RESPONSES OF A RAT RHABDOMYOSARCOMA AND RAT SKIN TO IRRADIATION WITH GAMMA RAYS AND 15 MeV NEUTRONS AT LOW DOSE RATES

H.B. KAL

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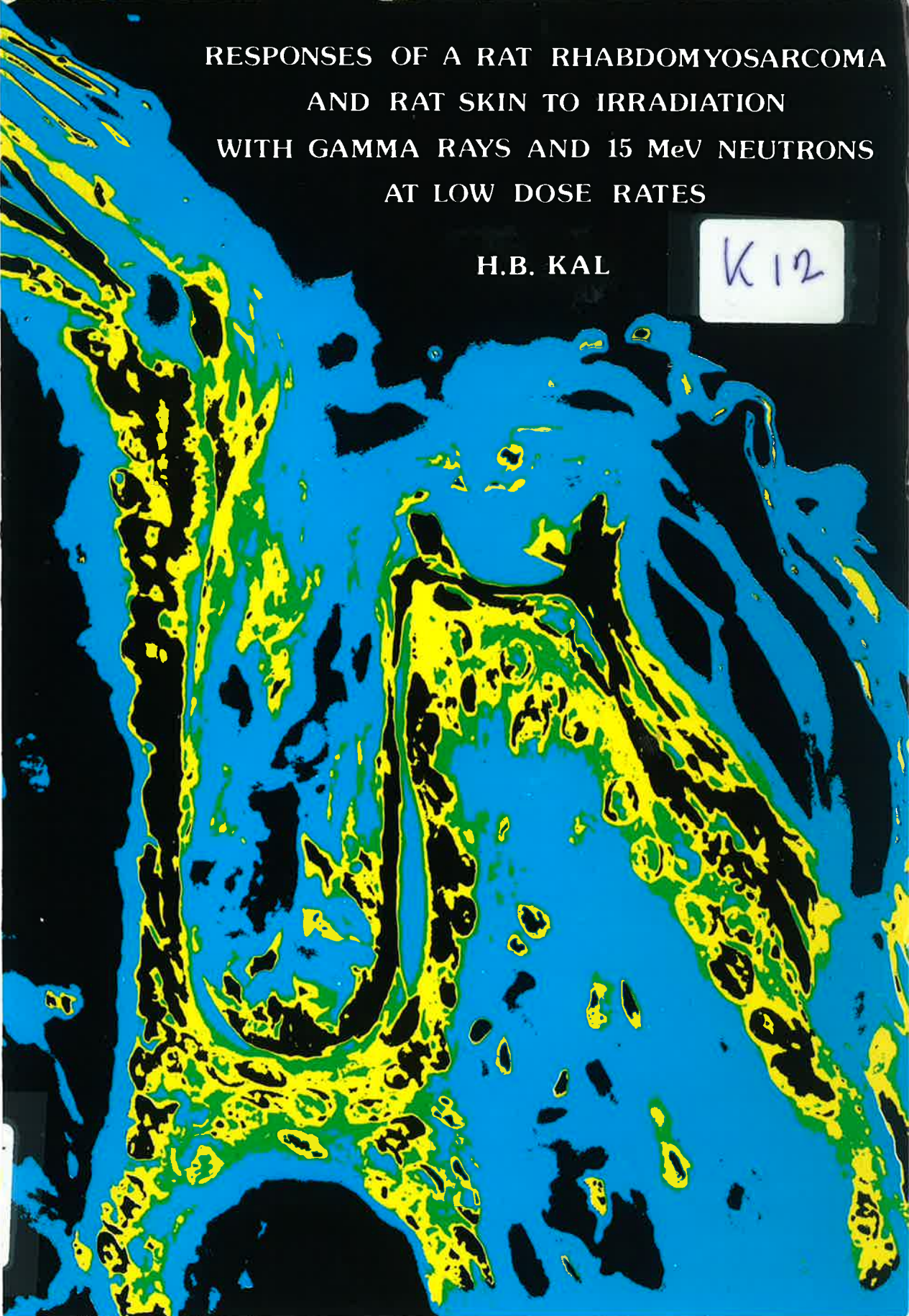

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The work described in this thesis has been performed at the Radiobiological Institute TNO, Rijswijk (ZH), The Netherlands.

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STELLINGEN

I

De waarde van de geautomatiseerde cytofotometrie voor het oncologisch onderzoek zal belangrijk worden verhoogd door het beschikbaar komen van eenvoudige technieken voor het isoleren van tumorcellen uit gemengde populaties.

II

De suggestie dat een langdurige gammabestraling een goedkoop alternatief zou zijn voor neutronenbestraling wordt niet door experimentele gegevens onderschreven.

H. R. Withers and K. Y. Chen, Brit. J. Radiol., 44, 818 (1971).

III

Exogene chemische carcinogenen kunnen borstkanker induceren in proefdieren. Uit de literatuur kan de conclusie worden getrokken dat deze chemische carcinogenen in het algemeen niet beschouwd worden als een factor van belang voor de inductie van borstkanker bij de mens.
Bij ioniserende straling wordt aangenomen dat voor lage stralingsdoses een lineaire dosis-effect relatie bestaat voor de inductie van tumoren. Als dit ook het geval is voor chemische carcinogenen die bij hoge doses in proefdieren tumoren induceren, dan kan niet uitgesloten worden dat lage doses van deze chemische carcinogenen van belang kunnen zijn bij de inductie van tumoren bij de mens.

IV

Het is gewenst de eenheden rad, curie en röntgen als speciale eenheden op te nemen in het Internationale Eenheden Stelsel.

V

De verbreiding van de „R-factor" in ziekenhuizen kan worden bestudeerd uit de gegevens van biotype en antibiogram van gram negatieve staven, alsmede van de localisatie van de patiënt van wie deze bacteriën werden geïsoleerd. Dagelijkse bewerking van deze gegevens met behulp van snelle informatieverwerkende apparatuur kan in de toekomst tot preventieve maatregelen leiden welke in ziekenhuizen het gevoeligheidspatroon van gram negatieve staven voor antibiotica kunnen verbeteren.

VI

Het is mogelijk uit PLM curven die percentages gelabelde mitoses geven als functie van de tijd na labelling, de spreiding in de duur van de fasen van de celcyclus te bepalen met behulp van een computeranalyse. Een nauwkeurige bepaling is echter alleen mogelijk als de vorm van deze curven slechts weinig varieert met de belichtingstijd die nodig is om gelabelde en ongelabelde cellen in autoradiogrammen te onderscheiden.

VII

De motivering dat het regelmatig röntgenonderzoek van bepaalde organen met moderne apparatuur niet bezwaarlijk is omdat de gonadendosis zeer gering zou zijn, houdt geen rekening met het toegenomen inzicht dat bij een zelfde dosis het risico van tumorinductie wellicht groter kan zijn dan de kans op genetische effecten.

S. Prêtre, Hlth. Phys., 25, 210 (1973)
Ned. T. v. Geneesk., 117, 1321 (1973)

VIII

Een besparing van de oliestookkosten op schepen kan worden verkregen door alle zeilen bij te zetten.

Amsterdam, 28 maart 1974 H. B. Kal

RESPONSES OF A RAT RHABDOMYOSARCOMA AND RAT SKIN TO IRRADIATION WITH GAMMA RAYS AND 15 MeV NEUTRONS AT LOW DOSE RATES

ACADEMISCH PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE UNIVERSITEIT VAN AMSTERDAM OP GEZAG VAN DE RECTOR MAGNIFICUS DR. A. DE FROE, HOOGLERAAR IN DE FACULTEIT DER GENEESKUNDE, IN HET OPENBAAR TE VERDEDIGEN IN DE AULA DER UNIVERSITEIT (TIJDELIJK IN DE LUTHERSE KERK, INGANG SINGEL 411, HOEK SPUI) OP 28 MAART 1974, DES NAMIDDAGS TE 5.00 UUR

DOOR

HENDRIK BASTIAAN KAL
geboren te Vlaardingen

UITGEVERIJ WALTMAN - DELFT - 1974

PROMOTOR: PROF. DR. G. W. BARENDSEN
CO-REFERENT: PROF. DR. K. BREUR

Aan Klaske,

Marleen, Arnoud en Majanka

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CHAPTER I

INTRODUCTION

Following the early application of X-rays and radioactive isotopes in radiotherapy, a large amount of clinical data has been accumulated for several regimens of tumour treatments with ionizing radiations. The effectiveness of these regimens has been analyzed with respect to the probability of cure or recurrence of tumours and the probability of severe damage to normal tissues. In the course of more than 60 years of application, results of administration of the dose in single treatments and in various fractionated and low dose rate regimens have been evaluated. A number of these schemes have been selected on the basis of critical assessments of normal tissue reactions in comparison with tumour eradication. Fractionated application of doses of X-rays or gamma rays at daily intervals and low dose rate treatments have been shown to cause relatively more damage to tumours than to normal tissues when compared with single dose treatments. Differences in responses of normal tissues and tumours to ionizing radiation, which are a necessary requirement in the treatment of malignant diseases, have thus been optimized as much as possible from available data. The schemes presently used in radiotherapy offer no guarantee, however, that they are truly optimal for each individual type of tumour with respect to maximizing differences between responses of normal tissues and tumours to ionizing radiation. This is due to the fact that the mechanisms determining the probability of success or failure of a given treatment are not yet fully known. In addition, not all patients suffering from a malignant disease can be cured with radiotherapy or chemotherapy, surgery, or combinations of these treatments. It cannot be ruled out that, through a better understanding of cell proliferation parameters, improvements can be made in the treatment of certain types of tumours. This might be done by taking advantage of the differences in responses to ionizing radiation when administered in specific regimens of fractionation or protraction which lead to more effective destruction of the tumour while maintaining the integrity and function of the irradiated surrounding normal tissues. In order to achieve this aim, more fundamental knowledge of the mechanisms involved in cell reproductive death and cell proliferation is required.

The fundamental knowledge of responses of cells to ionizing radiation has been increased considerably during the past two decades since the introduction of new assay systems into mammalian cell radiobiology to test whether single isolated cells have retained the capacity of unlimited proliferation. Among these are the *in vitro* clone technique of Puck and Marcus [1955] and the *in vivo* transplantation technique of Hewitt [1953]. With the *in vitro* clone technique, dose-survival relationships for different types of radiation can be obtained for various mammalian cell lines that can be cultured *in vitro*. Such a dose-survival relationship can be represented as a "sur-

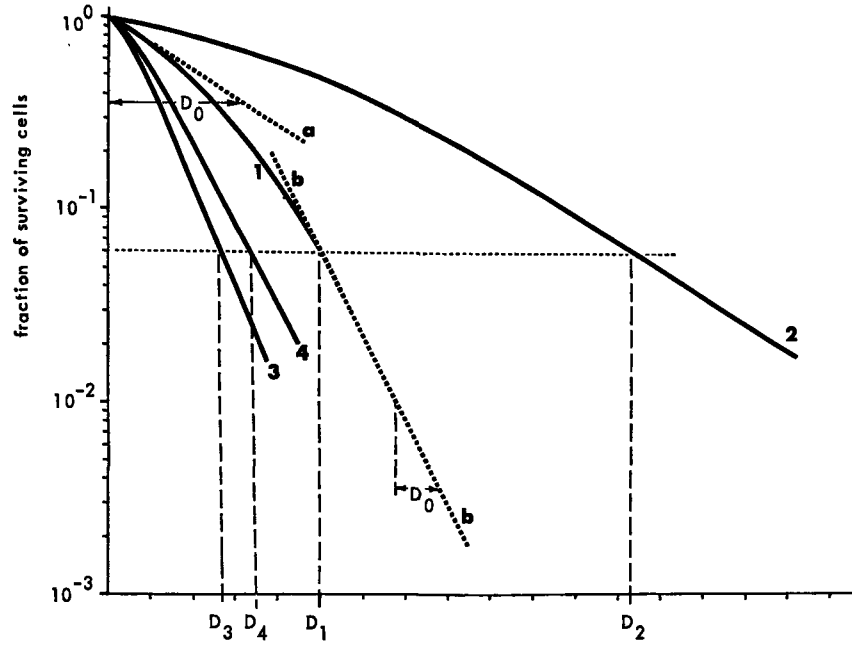


Fig. I-1 Theoretical dose-effect relationships, represented as "dose-survival curves" of well-oxygenated and anoxic mammalian cells. Curves 1 and 2 pertain to cells in equilibrium with air and nitrogen, respectively, as obtained for irradiation with X-rays. Curves 3 and 4 represent cells in equilibrium with air and nitrogen, respectively, as obtained for irradiation with 15 MeV neutrons.
The initial slope of the X-ray survival curve at low doses, represented by the dashed line a, can have D_0 values between 400 and 500 rad; the final slope (line b) can have D_0 values between 70 and 200 rad, depending on the cell line investigated.
The oxygen enhancement ratio, OER, at a given survival level, e.g. 6%, is defined by the ratio D_2/D_1. It has values between 2.5 and 3.0 for X-rays or gamma rays. The OER of fast neutrons derived as D_4/D_3, is in the order of 1.5 – 1.6.
The relative biological effectiveness, RBE, at a given survival level is defined by the ratio D_1/D_3 for oxygenated cells and D_2/D_4 for anoxic cells.
The RBE for fast neutrons of 15 MeV energy relative to X-rays in the high dose region is in the range of 1.6 – 2.0 for most oxygenated mammalian cells. For anoxic cells, the RBE is in the range of 2.5 – 4.0.

vival curve" when the fraction of surviving cells is plotted on a logarithmic scale as a function of the dose on a linear scale (figure I-1). In general, survival curves obtained with X-rays or gamma rays exhibit a shoulder at low doses which is followed by a more or less straight part in the high dose region. A detailed analysis [Barendsen et al., 1960] has revealed that these curves generally have an initial slope at low doses which can be described by a D_0 * of 400–500 rad and that the final slope at large doses can generally be described by a D_0 of 70–200 rad (figure I-1, curve 1, lines a

* The D_0 is the dose in rad required to produce a decrease in the surviving fraction of cells in the exponential region of the survival curve by a factor of e^{-1}.

and b). It has been suggested that, for relatively small doses of up to about 100 rad, the damage observed is almost entirely due to single event * actions. This single event action results from the fraction of the total energy deposited by the relatively small "high-LET" ** component of the X or gamma radiation – 5 to 10% of the energy dissipated. The steeper final slope in the high dose region indicates that accumulation of sublethal damage is the main cause of cell reproductive death at high doses. It is caused by "multiple event" action that results from the fraction of the total energy deposited by the relatively large "low-LET" component of the X or gamma radiation – 90 to 95% of the energy dissipated.

Elkind and Sutton [1960] have shown by split-dose experiments that repair of sublethal damage can be completed within 6 to 8 hours after the administration of a dose. This implies that two successive doses separated by a time interval of several hours are less efficient for cell inactivation than the same total dose given as a single short exposure. The fact that accumulation of sublethal damage can cause cell inactivation, and the occurrence of the Elkind repair of sublethal damage within 8 hours, determines the effectiveness of the administration of doses in radiotherapy in several ways. As a result of dose fractionation, and the application of a dose by a low dose rate regimen, accumulation of sublethal damage can be prevented and the possibility is provided that normal tissues can recover from radiation damage in the interval between two successive dose fractions or during a continuous low dose rate irradiation. It should be noted, however, that similar phenomena occur in tumour cells. Consequently, a better insight into the relative importance of these mechanisms will be essential for the selection of the optimum time schedule for the administration of doses in radiotherapy.

In the past two decades, it has been well documented that oxygen is a dose modifying factor for mammalian cells [Gray et al., 1953]; i.e., well-oxygenated cells are more radiosensitive than are cells that were anoxic at the time of irradiation. The oxygen enhancement ratio, OER, is defined as the ratio of two doses required for a specific effect when administered to cells in anoxic and in well-oxygenated conditions, respectively. This ratio is in the order of 2.5–3 for X-rays and gamma rays. An example is provided by curves 1 and 2 of figure I-1.

The extent of repair of sublethal damage as well as the magnitude of the oxygen enhancement ratio have been shown to depend on the type of radiation used [Barendsen et al., 1960; 1963; Barendsen and Walter, 1964]. A survival curve obtained for cells irradiated with a "high-LET" radiation (e.g., alpha particles or fast neutrons) exhibits an almost exponential shape, i.e., it has a small shoulder (figure I-1, curves 3 and 4). The fraction of the total energy deposited by the low-LET component, e.g.,

* An "event" can be defined as the passage of a particle through the sensitive structure of a cell.

** The linear energy transfer (LET) is a measure of the spatial distribution of the energy deposition and is defined by the quotient of dE/dL, where dL is the distance traversed by the ionizing particle, and dE is the mean energy loss due to collisions. For a discussion of LET, see chapter II.

delta rays *, depends on Z^2/β^2, where Z is the effective charge of the particle and β its velocity relative to the velocity of light [Curtis, 1970]. If $\beta < 0.1$, this fraction is small as compared to the fraction of the total energy of X-rays or gamma rays deposited by particles of low LET. As a consequence, repair of sublethal damage between dose fractions or during continuous exposure at low dose rate will have a smaller influence. The OER for "high-LET" radiation depends on the LET. It is 1.1 for alpha particles of 2–3 MeV and 1.5–1.6 for fast neutrons; see figure I-1, curves 3 and 4. This means that oxygen as a dose modifying factor has less influence with respect to cell survival for treatments with high-LET radiation as compared to low-LET radiation.

Not only have repair of sublethal damage and anoxia been found to be important factors in radiobiology, but also the specific phase of the cell cycle ** has been shown to be a factor that can influence radiation responses of mammalian cells. It has been shown that the radiosensitivity of the cell varies with the phase of the cell cycle. In general, the pattern of responses to X-irradiation shows an increase in radioresistance up to a maximum in late S. This is followed by a sensitive period in the late G_2 and M phases of the cycle. For high-LET radiation such as fast neutrons and alpha radiation, the response pattern is qualitatively very similar; the variation between the most sensitive and most resistant phases of the cell cycle is less than in the case of X-rays but it is still appreciable [Hall et al., 1972].

The factors determining the probability of survival for single cells *in vitro* are also found to play a role when intact structures such as organs or whole animals are subjected to an irradiation treatment [Barendsen, 1968; Broerse and Barendsen, 1973]. However, in intact structures, other factors will be important with respect to the response of these structures to ionizing radiation. In intact structures, especially in tumours, it is not unlikely that a fraction of the cells will suffer from malnutrition or will be in a hypoxic condition due to an insufficient blood supply. Furthermore, repopulation of cells during fractionated treatments or during continuous irradiation applied at a low dose rate can occur. Reoxygenation of severely hypoxic cells can also play a role during fractionated radiation treatments [Thomlinson, 1969; Howes, 1969; van Putten and Kallman, 1966; Barendsen and Broerse, 1970] and during continuous irradiation at low dose rate [Kal and Barendsen, 1972; Frindel et al., 1972; Hill and Bush, 1973]. Finally, it should be emphasized that two fractions of cells can frequently be distinguished in tumours and normal tissues: a fraction of proliferating cells (P cells) and a fraction of nonproliferating resting or quiescent cells (Q cells). It is possible that nonproliferating cells in tumours are relatively radioresistant as compared with proliferating cells, and cells from the fraction of nonproliferating cells which survive a treatment might repopulate the tumour by entering the proliferation cycle

* If the primary energy transfer due to a collision of a particle is larger than a few hundred eV, the electron liberated can ionize other atoms or molecules at considerable distances from the track of the particle. These electrons are called "secondary electrons" or delta rays.

** For a discussion of the cell cycle phases, see section III-5.

after treatment. It is likely, therefore, that each tumour, tissue, or organ will respond in a different manner to a particular radiation treatment and that studies on multicellular tissues are required in order to elucidate the influence of various types of mechanisms.

The aim of this study was to determine various factors that play a role at the cellular level in two different tissues, a rat rhabdomyosarcoma and the skin of the same rat, by the use of two types of radiation administered in several schemes. X-rays and gamma rays were used as low-LET radiation, and 15 MeV neutrons produced by the $^{3}_{1}H(d,n)^{4}_{2}He$ reaction were employed as radiation having a considerably higher mean LET. A comparison of the effects of radiation treatments on rhabdomyosarcoma cells growing *in vivo* and *in vitro* was made. As previously mentioned, the effects of radiation treatment on cells *in vitro* can be studied in more detail because complicating factors in tissues such as severe hypoxia and the existence of nonproliferating cells do not play a role.

In this study, the effects of acute versus protracted irradiation on tumour cells and on normal cells were compared. The continuous irradiations were performed at dose rates between 40 and 150 rad/h. These dose rates are frequently used in clinical conditions in the treatment of certain types of tumours. The results of this study may contribute to a better understanding of the various factors which are involved in treatments where continuous irradiation is applied at low dose rates and may be used to explain the satisfactory results frequently observed by the use of radium and ^{192}Ir implants in the treatment of certain human tumours [Pierquin, 1970; Pierquin and Baillet, 1971b]. They may also explain why continuous irradiation at low dose rate is more advantageous than acute irradiation or fractionated treatments in specific conditions and may provide an insight into whether this would also be the case for fast neutron irradiation – especially since the radioactive nuclide ^{252}Cf which emits fast neutrons at low intensities has become commercially available and may be used for intracavitary treatments of cancer.

The basic aspects of energy deposition by X-rays and fast neutrons and their relationship to the effectiveness of inducing cell reproductive death will be discussed in some detail in chapter II.

The materials and methods used for the biological experiments are described in chapter III. The biological materials were: 1. tumour cells of a rat rhabdomyosarcoma which can be grown *in vitro* as well as *in vivo* and which can be considered as a model for malignant tissue, and 2. the rat skin which can be considered as a model for a dose limiting normal tissue. The clonogenic capacity of cells surviving a radiation treatment and the remaining area of transplanted pieces of skin were the biological end-points considered. The cloning procedure, the skin transplantation method, and the method used to obtain and analyze cell size and DNA content distributions will be discussed.

Chapter IV is concerned with the dose-effect relationship for tumour cells growing *in vitro*. The effectiveness of acute and protracted irradiations and the factors which

play a role during and after irradiation such as repair of sublethal damage and redistribution of cell ages will be discussed.

In chapter V, dose-effect relationship for tumour cells irradiated in the animal will be described. The relative biological effectiveness, RBE, of 15 MeV neutrons will be discussed and the factors which determine the survival of tumour cells such as repair of sublethal damage, reoxygenation, and repopulation will be analyzed.

Results of irradiations of rat skin will be described in chapter VI. RBE values of 15 MeV neutrons for shrinkage of rat skin area will be reported and a possible gain in therapeutic margin will be discussed.

In chapter VII, a discussion is presented concerning the application of continuous irradiation at low dose rates in clinical radiotherapy. An alternative for protracted irradiation will be discussed and it will be shown that the quantitative relationships derived from experiments on the rat skin are quite similar to those observed for human skin tolerance.

CHAPTER II

BASIC ASPECTS OF ENERGY DEPOSITION AND THEIR RELATIONSHIP TO THE EFFECTIVENESS OF INDUCING CELL REPRODUCTIVE DEATH

1 Mechanisms of energy deposition by various radiations in biological material

Directly ionizing particles dissipate energy in tissue at a rate which is usually represented by the linear energy transfer, LET. This LET depends on the charge and velocity of the particles and it determines the relative biological effectiveness, RBE.

In an analysis of experiments in which cultured cells of human kidney origin were irradiated with electrons, deuterons, and alpha particles of different energies, Barendsen [1968] has distinguished three broad regions of LET; namely, the low-LET range below 10 keV/μm of tissue, the intermediate-LET range of 20–80 keV/μm of tissue and the high-LET range in excess of 200 keV/μm. The regions with LET values ranging from 10 to 20 and 80 to 200 keV/μm can be considered as transition regions in which some of the characteristics that will be discussed later change rapidly.

Three types of ionizing radiation will be discussed in this chapter: 1. X-rays and gamma rays, which dissipate energy through fast electrons and are generally designated as low-LET radiation; 2. fast neutrons which dissipate energy through low-LET as well as intermediate- and high-LET secondary particles and is generally designated as a radiation of intermediate-LET; and 3. protons, alpha particles, and heavy charged particles such as carbon and oxygen ions. These are of interest here because fast neutrons give rise to these particles in tissue. Alpha particles and heavy charged nuclei are commonly designated as radiation of high LET. X-rays, gamma rays, and 15 MeV neutrons have been employed in the experiments to be described in later chapters.

Electromagnetic radiations such as X-rays and gamma rays dissipate energy in biological material by the liberation of electrons which lose their energy by collisions with molecules or atoms. The secondary electrons liberated in these collisions produce a very large number of ionizations and excitations along their tracks as they are gradually brought to rest in the material. The energetic electrons among those liberated will deposit their energy over a relatively long track length. Therefore, the number of ionizations per unit length is relatively small; i.e., the linear energy transfer is low. These energetic electrons give rise to slower electrons and become slow electrons at the end of their tracks. The slow electrons will deposit their energy in a relatively small volume. As a consequence, the number of ionizations per unit path length is relatively large and corresponds to relatively high LET values. The maximum LET value, LET_{max}, is about 30 keV/μm for electrons of very low energy. The fraction of the total energy deposition from X-rays or gamma rays through electrons with a low

LET, i.e., with LET values below 10 keV/μm, is relatively high; about 90–95% for X-rays and gamma rays of energies in excess of 100 keV. Only a small part of the energy is deposited by electrons with an LET in excess of 10 keV/μm.

Fast neutrons dissipate their energy mainly by elastic collision processes which impart energy to the hydrogen nuclei. Especially at energies in excess of about 10 MeV, interactions with other nuclei in tissue such as carbon, nitrogen, and oxygen nuclei take place. Products of nuclear reactions with these elements, such as alpha particles, contribute significantly to the energy deposition [Randolph, 1964]. For the distribution of the dose of fast neutrons in LET, two important regions can be distinguished corresponding to the energy dissipated through protons and the energy dissipated through interactions with C, O, and N nuclei and through nuclear reactions producing alpha particles. Protons set in motion by neutrons through elastic collisions have ranges which for the greater part are large in comparison to the diameter of the cells. A proton of 1 MeV energy has a range of about 25 μm in tissue. This is approximately equal to the diameter of some mammalian cells. A proton of 10 MeV energy, how-

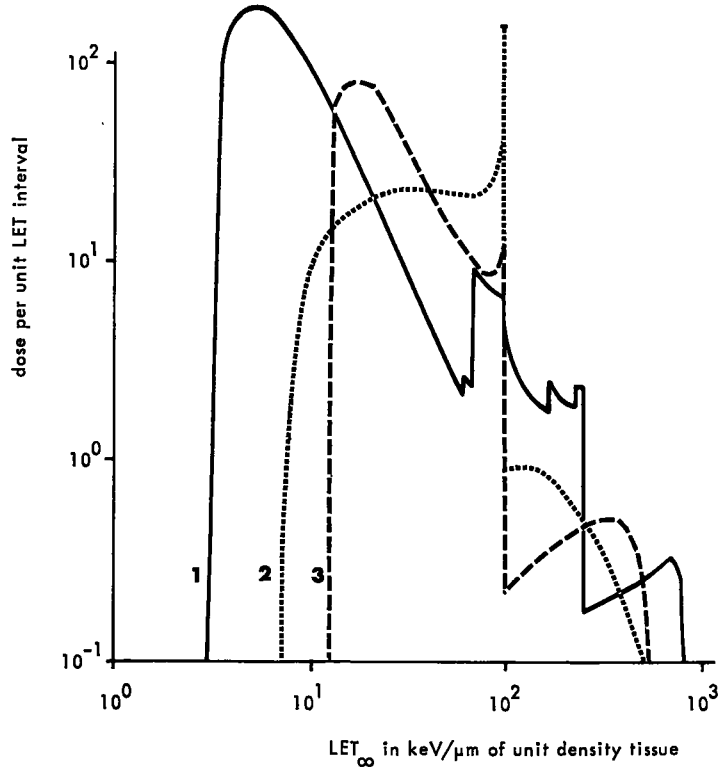


Fig. II-1 Distributions of LET_∞. The absorbed dose per unit LET interval for unit density tissue is plotted on a logarithmic scale as a function of the log LET for 14.6 MeV, fission, and 3 MeV neutrons curves 1, 2, and 3 respectively [Bewley, 1968].

ever, has a range of about 1200 μm in tissue. Therefore, protons dissipate energy with a distribution of the dose in LET which extends from low values of a few keV/μm to a maximum of about 96 keV/μm at the Bragg peak. Energy dissipated by fast neutrons through interactions with C, O, and N nuclei and nuclear reactions producing alpha particles is deposited mainly in the LET region between 100 and 1000 keV/μm. The LET spectra for fission neutrons and neutrons of 3 MeV and 14.6 MeV energy, as calculated by Bewley [1968], are presented in figure II-1.

As indicated in chapter I, the damage to cells caused by radiation consists of: 1. lethal damage which is related to the intermediate- and high-LET component of the radiation; and 2. accumulation of sublethal damage which is related to the low-LET component. Electrons liberated by low-LET radiation have a low probability of dissipating enough energy locally to inflict heavy damage on sensitive cell structures; consequently, the effectiveness for producing lethal damage will be relatively low. Therefore, most damage caused by low-LET electrons must be cumulative in order to produce cell reproductive death. If, however, the ionization density is extremely high, as in the case of high-LET radiations, more ionizations may be produced in sensitive biological structures than are required to produce the biological end-point. As a consequence, part of the energy deposited will be wasted and the effectiveness for producing cell reproductive death will decrease with increasing LET at these very high ion densities. Due to the difference in the LET distribution, two types of radiation can cause quantitatively different biological effects. The ratio of two doses of ionizing radiation causing the same damage to a biological system is the relative biological effectiveness, RBE, which is defined as:

$$\text{RBE (A)} = \frac{\text{dose of standard radiation required for a specific effect}}{\text{dose of radiation A causing the same effect}}.$$

Usually 250 kVp X-rays are taken as the standard radiation. An example is provided by curves 1 and 3 of figure I-1. The variation in the RBE as a function of the LET for damage to the clone forming capacity of mammalian cells in tissue culture will be discussed in more detail in II-3.2 .

Because of the small sizes of those structures assumed to be critical for normal cellular function and because of the distance between them, the number of interactions due to the traversal of a single charged particle within the critical structure is relatively small [Dewey et al., 1972]. As discussed in chapter I, energy deposition of these charged particles can be characterized by Z^2/β^2. A small change in the number of primary interactions per unit distance can result in a large change in the probability of inducing cell death; e.g., a mammalian cell will generally not be inactivated by a single electron ($Z = 1$) with an energy below about 300 eV [Barendsen, 1968]. This is due to the fact that the energy that can be deposited is too small to create the minimum number of ionizations required for cell inactivation. However, cell reproductive death can be induced through accumulation of damage caused by passages of several electrons each causing sublethal damage.

Electrons with energies in excess of about 1 keV will deposit their energy over a relatively long distance. As a consequence, the ionization density is too small to inactivate a cell as the result of the passage of a single particle. Electrons with energies in the region of 0.3–1 keV are considered to deposit their energy in a small volume with a sufficient number of ionizations to inactivate the cell by the traversal of a single particle.

Hogeweg and Barendsen [1972] concluded that the critical structure in the cell in which a given minimum damage must be produced in order to cause impairment of the capacity for unlimited proliferation has dimensions of less than 70 nm and that the minimum energy which must be deposited in this structure is of the order of 700 eV. This conclusion was reached by measuring event size distributions for alpha particles with a tissue-equivalent proportional counter and by comparison of the results with the data obtained by Barendsen [1964] for human kidney cells. The data of Hogeweg and Barendsen indicate that the relative effectiveness per particle when expressed as a cross section closely approaches a value of about 40 μm^2 at LET values in excess of 160 keV/μm. It is about 0.5 of this value at 80 keV/μm. This implies that, at 80 keV/μm, one-half of the particles passing through the critical structure is ineffective for producing impairment of the capacity for unlimited proliferation through single traversals. At an LET of 110 keV/μm, about 15% of the passages of alpha particles are still ineffective for producing the biological effect. From these results, it is clear that the dimensions of the critical structures and the minimum energy deposition required to inactivate the cell can only be derived as probability values. These results do not indicate the nature of the biological structures or the mechanism of damage. Dewey et al. [1972] proposed a model in which the decrease in radiosensitivity correlated well with the dispersion of chromatin as the cells moved from mitosis into S phase. In this model, lethal lesions result from the interaction of single lesions in the chromatin fibers. Interacting lesions may be produced in close proximity to one another as a result of ionizations caused by a traversal of a single particle, or they may result from single sublethal lesions as a result of ionizations caused by a traversal of a single particle interacting with other single sublethal lesions. Thus, the mitotic cells should be the most radiosensitive cells because their tightly packed chromatin fibers should provide the highest probability for interactions between lesions.

2 LET distribution

It is clear that the energy deposition by a given type of radiation cannot be described by a single LET value [Broerse, 1966]. The LET of a charged particle changes along its track, especially at the end of the range where a rapid rise in energy release per unit path length is found. This region is known as the Bragg peak. Therefore, energy deposition by a given radiation can be approximately described by some kind of an average LET value. Detailed data on the LET distributions of ionizing radiations are

very important, since the RBE and OER of various radiations for different levels of damage are related to these distributions [Barendsen et al., 1963].

Two different types of average LET have been commonly used. The track average LET, $\bar{L}_T$, is the mean LET associated with the track distribution. The absorbed dose average LET, $\bar{L}_D$, is the mean LET associated with the dose distribution.

The mean LET associated with the track distribution is $\bar{L}_T = \int_0^\infty t(L)L\,dL$; where t(L)dL represents the fraction of the total track length, T, having values of LET between L and L + dL. Thus, if T(L) is defined as the track length associated with LET up to L and divided by total track length, T, t(L) = dT(L)/dL.

The mean LET associated with the absorbed dose distribution is $\bar{L}_D = \int_0^\infty d(L)L\,dL$, where d(L)dL represents the fraction of the absorbed dose, D, delivered between L and L + dL; thus, if D(L) is that part of the dose with LET up to L and divided by the total absorbed dose, D, d(L) = dD(L)/dL. Although the energy deposition of a given type of radiation can be described by these two average LET values, it has been concluded from a comparison of the biological effects produced by different types of radiation that the concept LET, especially the concept average LET, has serious shortcomings [Broerse, 1966]. The LET is an approximation which is necessary because of the complicated nature of the mechanism of energy deposition. An ideal characterization of each type of radiation would require exact knowledge of the spatial distribution of the energy deposition and of the ionizations produced. As discussed in II-1, low-LET radiations as well as intermediate- and high-LET radiations dissipate energy through various interactions resulting in a complex LET spectrum. Examples of such complex spectra are shown in figure II-1.

These distributions of dose in LET were calculated by Bewley [1968] for monoenergetic neutrons of 14.6 MeV energy generated by the reaction $^2_1D + ^3_1T \rightarrow ^4_2He + ^1_0n$, for 3 MeV neutrons produced through the reaction $^2_1D + ^2_1D \rightarrow ^3_2He + ^1_0n$, and for fission neutrons produced by bombarding a ^{235}U converter plate with thermal neutrons.

For low-LET radiation such as X-rays and gamma rays, the distribution of dose in LET is less complex as compared with the distribution of dose in LET for intermediate and high energy neutrons. Dose distributions in LET can be calculated by means of the continuous slowing down approximation (csda) in which charged particles are assumed to lose energy continuously along their paths according to the linear energy transfer, L. A distribution of dose as a function of LET of ^{60}Co gamma rays is shown in figure II-2. The csda, however, ignores the discrete nature of energy increments removed from the particle along its track due to electrons liberated with sufficient energy to produce further ionizing events. The energy transferred may be so low that only an ion cluster of 2, 3, 4, etc. ion pairs are formed, or it may be large enough to produce a separate track known as a delta ray. Therefore, a better approximation of the real physical situation is obtained by applying a "two group model" in which the collisions are divided into two groups by an energy cut-off, Δ. The LET distribution obtained with the csda can be described by the LET (with $\Delta = \infty$) which

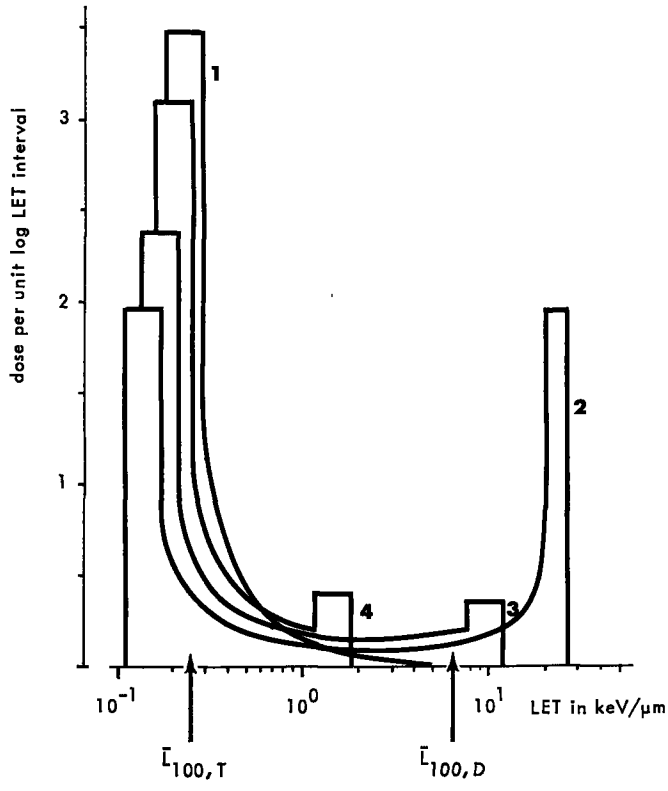


Fig. II-2 Distributions of absorbed dose in LET for water obtained with the electrons set in motion by ^{60}Co gamma rays as calculated by the continuous slowing down approximation (csda), curve 1, and with the "two group model" with cut-off values, Δ, of 100, 1000, and 10000 eV, for curves 2, 3 and 4, respectively [ICRU, 1970].

is defined as the energy loss per unit distance of the charged particles originally set in motion by electromagnetic radiation. LET_∞ is the same as the stopping power. An LET_{100} with cut-off energy of $\Delta = 100$ eV would be an LET obtained when tracks due to secondary particles with an energy of 100 eV or more are considered as separate tracks. The dose distribution in LET of ^{60}Co gamma rays for different cut-off values, Δ, is shown in figure II-2. From this figure, it is clear that, for cut-off values in the range of 0.1–1 keV, the dose distribution in LET for LET values larger than 10 keV/μm (i.e., for relatively high LET values) cannot be neglected. It is clear that, for high LET values such as for particles produced by fast neutron radiation, the importance of the choice of a cut-off value is less critical than for low-LET radiation.

In order to provide a more direct specification of energy distributions, Rossi [1959] has defined the quantity, Y, which is the energy deposited in a single event in a small sphere divided by its diameter; Y is called the event size. Another essential parameter, Z, is the local energy density which is defined as the quotient of the energy delivered to

a microscopic sphere divided by its mass. A number of Y and Z spectra have been measured by use of a spherical proportional counter. A more detailed description of the microscopic distribution of energy deposition is obtained in this way, but it remains to be seen whether they will be found generally useful in the specification of radiation quality. Their greatest value may be in the fact that they make possible predictions concerning the size of critical structures when experimental biological data for different radiations are taken into account and, thereby, help in the identification of mechanisms of radiobiological action.

3 Biological effects as a function of LET

3.1 *Survival curves*

It has been shown for many mammalian cell survival curves that the high dose regions, below 10^{-1} survival level, can frequently be described to a good approximation by a formula of the form $S = 1-\{1-\exp(-D/D_0)\}^N$, where D is the dose in rad; N, the extrapolation number representing the number obtained by extrapolating the exponential part of the survival curve to a dose of 0 rad; and D_0 is the dose increment in rad which is necessary to reduce the survival of the cell population by a factor e^{-1} for the exponential region of the survival curve. For an exponential survival curve, $N = 1$. The formula is then reduced to $S = \exp(-D/D_0)$. A complete survival curve can frequently be described to a first approximation by the analytic expression $S = \exp(-D/D_1)\,[1-\{1-\exp(-D/D_2)\}^N]$, where D_1 characterizes the slope of the initial part of the curve. For $N = 1$, the relationship between this expression and the above formula is given by $1/D_1 + 1/D_2 = 1/D_0$; for $N > 1$ this formula is correct to within a few per cent. N has the same meaning in both expressions.

In general, survival curves obtained with X-rays or gamma rays can be described by an initial slope, D_1, of 400–500 rad and a final slope at large doses by a D_0 of 70–200 rad. The extrapolation numbers range from 1 to 50, depending on the type of cells and on irradiation conditions.

For 250 kVp X-rays and 3.4 MeV alpha particles with a mean LET of 140 keV/μm, Barendsen [1962] measured survival curves of kidney cells of human origin, T-1 cells [van der Veen et al., 1958], employing both single and fractionated exposures. A few results are shown in figure II-3. The survival curve for 250 kVp X-rays (low-LET radiation) has a shoulder in the low dose region followed by a more or less linear part in the high dose region (curve 1). The occurrence of a shoulder followed by an exponential part indicates that the damage is due to two different phenomena as indicated earlier. The first is the lethal damage caused by the fraction of the total energy deposited by the high-LET component of the X-radiation and is represented by the initial part of the curve. The second phenomenon involved is the sublethal damage resulting from the fraction of the total energy deposited by the low-LET component of the radiation dose. Accumulation of this sublethal damage occurs with high doses and also causes loss of reproductive capacity. The survival curve for alpha

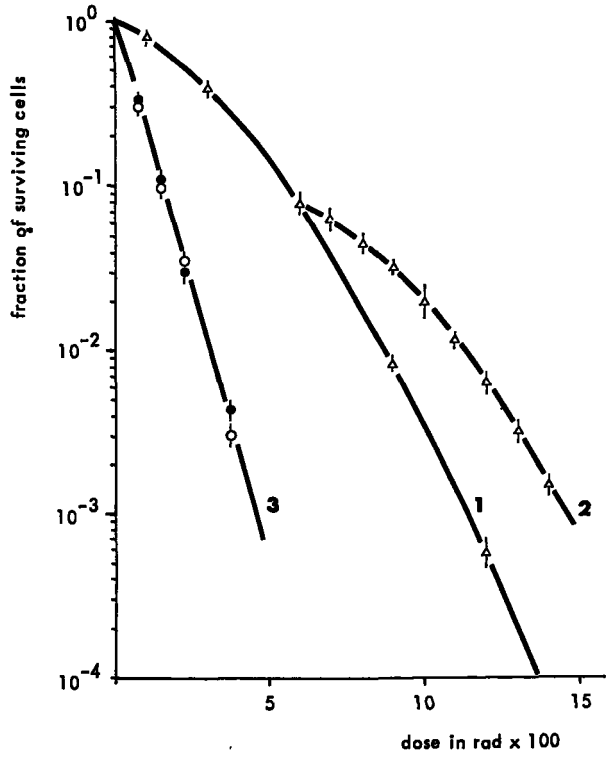


Fig. II-3 Effect of dose fractionation on survival curves obtained for T-1 cells with 3.4 MeV alpha particles and 250 kVp X-rays. All cells were in equilibrium with air [Barendsen, 1962].

Curve 1: single exposures to 250 kVp X-rays.
Curve 2: survival curve obtained with a dose of 600 rad of X-rays, followed 8 hours later by different doses of X-rays.
Curve 3: open circles, single exposures to alpha radiation; closed circles, total doses of alpha radiation fractionated in 2 equal parts administered at intervals of 8 hours.

particles shows an exponential shape (curve 3). This indicates that there is little or no sublethal damage with this high-LET radiation; all damage observed is due to lethal damage.

As noted earlier, Elkind and Sutton [1960] had demonstrated that mammalian cells repair sublethal damage within 6 to 8 hours. The cells that survive respond to subsequent irradiations as if they had not been previously irradiated. Because of this capacity of mammalian cells to recover from sublethal damage, fractionated doses are less effective than single doses, as shown for X-rays by curve 2 in figure II-3. In the case of alpha particles, no sublethal damage is present; hence, dose fractionation has no effect and fractionated doses are just as effective as one single dose (open and closed circles on curve 3 of figure II-3).

3.2 *Relative biological effectiveness (RBE)*

Because of the differences in shapes of survival curves as described in the previous section, the RBE of high-LET radiations varies depending on the survival level at which it is calculated. The RBE increases with increasing surviving fractions and thus the RBE values e.g. at the 80% survival level are larger than the RBE values determined at the 1% survival level. The variation in the RBE calculated at the 80% and 1% survival level as a function of the LET for damage to the clone-forming capacity of kidney cells of human origin in tissue culture is illustrated in figure II-4 [Barendsen, 1968].

Three broad areas in LET values can be distinguished in this figure: area a gives the RBE as a function of the LET for LET values up to 10 keV/μm. The RBE is only slightly dependent of the LET. The energy deposited by particles with LET values of less than 10 keV/μm can cause cell reproductive death in mammalian cells only through accumulation of sublethal damage. Because of the Elkind repair of sublethal damage, this damage would be dependent on dose fractionation and on dose rate. The energy deposited by particles with LET values in the range of 20–80 keV/μm (area b) would be sufficient to inactivate the cell by a single event which is independent of dose fractionation or dose rate. The RBE increases with increasing LET and reaches a maximum at about 100–150 keV/μm. It should be pointed out, however, that the damage caused by particles of these LET values can still be modified by oxygen, as will be discussed in the next paragraph. The decrease in RBE for LET values in excess of 200 keV/μm (area c) is due to the saturation effect mentioned earlier. The dose

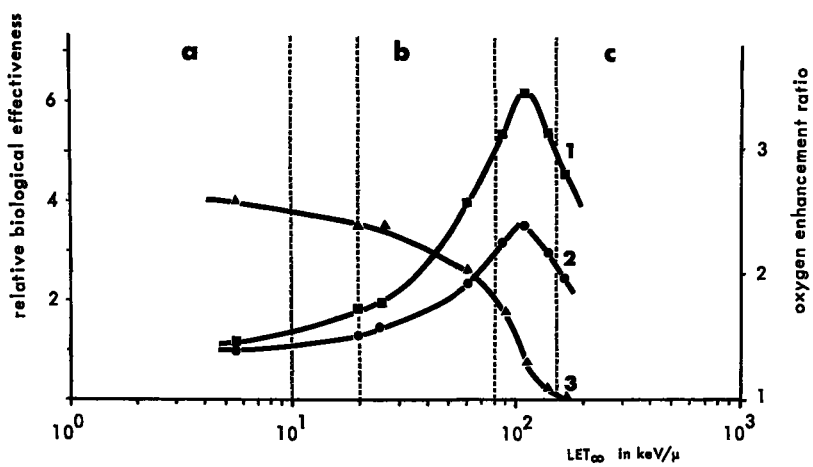


Fig. II-4 The relative biological effectiveness (RBE) and the oxygen enhancement ratio (OER) for inhibition of colony formation by T-1g cells as a function of LET [Barendsen, 1968].

Curves 1 and 2: RBE as a function of LET corresponding to 80% and 1% survival, respectively.

Curve 3 : OER as a function of LET.

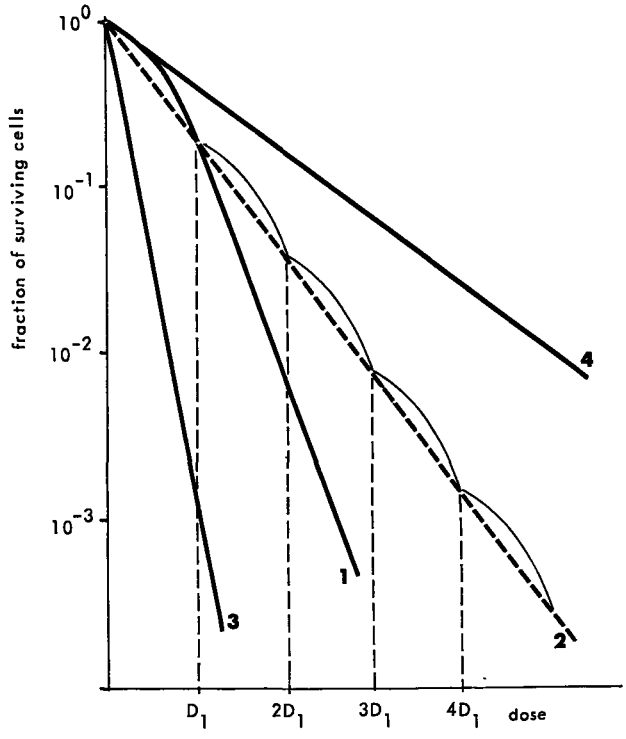


Fig. II-5 Idealized experiment. Curve 1 is the survival curve for single acute exposures of X-rays. Curve 2 is obtained if each dose is given as a series of fractions of size D_1 with a time interval between the fractions sufficient for full recovery to take place. Multiple small fractions approximates to a continuous low dose rate exposure, curve 4. Curve 3 is the survival curve for multiple small dose fractions of alpha rays, the curve is not significantly different from the curve obtained for single acute exposures to alpha rays.

deposited locally is in excess of the dose necessary to kill the cells and the excess is wasted. The effect of oxygen is less pronounced in this area. The regions with LET values ranging from 10–20 and 80–200 keV/μm can be considered as transition regions in which some of the characteristics mentioned change rapidly.

In fractionated exposures, the slope of the resulting survival curve for low-LET radiation is reduced (see figure II-5). If the size of the fractions is reduced, the slope of the resulting survival curve would also be reduced, see figure II-5, curve 2. The slope of the low-LET radiation survival curve becomes exponential when there is continuous exposure at a low dose rate, see figure II-5, curve 4. Under these conditions, the RBE of intermediate- and high-LET radiations is at a maximum level and is independent of the total dose administered.

3.3 *The oxygen enhancement ratio (OER)*

It has been found that the efficiency of low-LET radiation in producing a specific

effect may be influenced to a greater extent than the efficiency of high-LET radiation [Barendsen and Walter, 1964). As mentioned earlier, the presence of oxygen leads to a considerable change in radiosensitivity. For low-LET radiation, the dose required to produce the same biological effect in the absence of oxygen is about 2.5 to 3 times that needed in the presence of oxygen. This ratio is known as the oxygen enhancement ratio, OER. The OER decreases with increasing LET and reaches unity at about 160 keV/μm (figure II-4). There is some indication that irradiation with gamma rays at low dose rates gives a very low OER which is comparable to that obtained with californium [Withers and Chen, 1971]. However, Drew et al. [1974] demonstrated an OER of 2.50 for gamma irradiation at 37.3 rad/h with HeLa cells. Atkins et al. [1973] found an OER of 2.5 for Chinese hamster cells at the same dose rate. These values are in agreement with other data [Anderson et al., 1972; Berry, 1971, Djordjevic et al., 1973] and are not significantly different from OER's observed with acute X-ray irradiation.

In conclusion, these data show that the OER is dependent on the LET; however, a dose-rate dependency was not found.

3.4 *Protracted versus acute irradiation*

Low-LET radiations cause mainly sublethal damage and most of the lethal damage observed after large doses of acute irradiation is due to accumulation of sublethal damage. Cells have the capacity to recover from sublethal damage during protracted irradiation. Thus, protracted irradiation will be inefficient for cell killing as compared with acute irradiation during which repair of sublethal damage occurs only to a small extent; i.e., the slope of the survival curve obtained after protracted irradiation is decreased as compared with acute irradiation (figure II-5, curve 4). High-LET radiation causes mainly lethal damage which is independent of dose rate and it is more effective than low-LET radiation for a given dose. The effect of lowering the dose rate of high- and low-LET radiation is to further increase the RBE. When all sublethal damage is repaired during protracted exposures, the ratio of the slopes of the survival curves obtained after acute and protracted irradiation may give information on the events directly causing lethal damage. During protracted irradiation of mammalian cells cultured *in vitro*, however, not only repair of sublethal damage, but also the redistribution of cells in phases of the cell cycle plays a role. In addition, if the irradiation time is longer than the cell cycle time and the dose rate is sufficiently low, progression through the cell cycle and cell division can occur. This can result in the formation of a clone of cells and lead to a greater probability for survival. In tumours, reoxygenation can occur, cell loss during protracted irradiation can cause changes in the tumour volume, and resting cells in the tumour may start proliferating. It is clear that these phenomena may affect the results of treatments with low dose rate irradiations in a similar way as observed for fractionated treatments by Young and Fowler [1969]. The influence of these parameters will be discussed in more detail in chapter IV.

4 Further rationale for this study

In the next chapters, the various factors that influence radiation responses at the cellular level in a malignant and normal tissue will be discussed. These tissues are a rat rhabdomyosarcoma and the skin of the rat. The responses of these tissues were studied by the use of two types of radiation; firstly, 300 kV X-rays were used for acute irradiations and ^{137}Cs gamma rays for protracted irradiations. These X-rays and gamma rays can be considered as low-LET radiations with a dose average LET of about 1 keV/μm. Secondly, 15 MeV neutrons generated by the D-T reaction with a dose average LET of 92 keV/μm [Bewley, 1968] and a track average LET of 12 keV/μm, can be considered as an intermediate-LET radiation and were used in acute and protracted exposures.

Alpha particles or other heavy charged particles, which can be considered as high-LET radiations, would have been preferable for fundamental investigations of the mechanisms by which effects of ionizing radiation on living cells are initiated. However, these particles are not suitable to expose uniformly relatively large objects. Therefore, 15 MeV neutrons were used in the present investigations. These fast neutrons are of great interest for applied radiobiology, since they offer the only practical possibility of uniformly exposing relatively large objects to intermediate-LET radiation. The relatively low OER of 1.5 to 1.6 for 15 MeV neutrons in combination with the favourable depth dose characteristics [Barendsen and Broerse, 1968] indicates that these neutrons are the most promising for the clinical treatment of tumours containing a fraction of anoxic cells [Greene and Wood, 1967].

For the evaluation of the possible use of fast neutrons in radiotherapy, the responses of both normal and tumour tissue must be investigated. A therapeutic gain can be expected only if the RBE for damage to the tumour is larger than the RBE for damage to the dose-limiting normal tissues [Barendsen, 1971; Field and Hornsey, 1971]. For this reason, the evaluation of the effects of acute and protracted exposures must be made on both a tumour and a normal tissue. The skin of the rat appeared to offer a suitable model as a dose-limiting normal tissue for the evaluation of the effects af acute and protracted exposures, since a method for quantitative scoring of radiation damage had been developed [Barendsen, 1969].

A study of the effects on both normal and tumour tissue irradiated at low dose rates is of special importance since the radioactive isotope, ^{252}Cf, emitting neutrons at low intensities has become available and may be used in the clinic for intracavitary treatments.

CHAPTER III

MATERIALS AND METHODS

1 Introduction

In the experiments with high and low dose rate irradiations, dose-effect relationships have been determined for responses of a rhabdomyosarcoma and the skin of rats of the inbred WAG/Rij strain. This tumour was chosen because a variety of responses to single and fractionated acute doses was known and assay methods had been developed by Reinhold [1965] and Barendsen and Broerse [1969]. A method to assay radiation effects on the skin of this rat strain had been developed by Barendsen [1969] and data on responses to single and fractionated acute doses had been obtained [Barendsen, 1969; Broerse et al., 1971].

2 Properties and origin of the rhabdomyosarcoma

2.1 *Characteristics of the tumour*

The transplantable tumour line was derived from a rhabdomyosarcoma which originated in December 1962 in the mandibula of a rat of the inbred WAG/Rij strain. Eight months previously, this rat had received a total-body irradiation of 864 rad of X-rays followed by transplantation of bone marrow from BROFO rats. The tumour was first propagated for nine passages by subcutaneous injection of cell suspensions which were prepared from it by the method of Reinhold [1966].

Subsequently, a selection procedure consisting of several steps was applied. First, a suspension of tumour cells was cultured *in vitro*. A fraction of the cultured cells attached to the bottom of a Petri dish and developed into a monolayer. Then, a single clone of cells was isolated from this cell line and these cells were inoculated into the flank of a WAG/Rij rat where a tumour developed. A cell suspension was prepared from this tumour and the cells were cultured *in vitro*. This transfer from tumour to the *in vitro* culture system and the inoculation of a single clone back into the animal was repeated several times and finally resulted in a tumour line designated as R-1 in December 1966. These tumours are transplantable in the flanks of WAG/Rij rats by implantation of small pieces of tumour material or by subcutaneous inoculation of a cell suspension obtained either from a cell culture or from a tumour in a rat.

The tumours originating from inoculated R-1 cells or implanted pieces of tumour grow as solid masses having shapes which closely approximate a sphere. The cells in the tumour are arranged in intertwining strands and are spindle-shaped with an elongated nucleus. The cytoplasm is usually scanty. The stroma is not abundant and consists of tiny bundles of collagenous and reticular fibres which form a small-meshed network in close contact with the cells [Reinhold, 1965; 1967]. Tumours with volumes

between 500 and 1000 mm^3 have a volume doubling time, T_d, of 4 ± 1 days. Those of about 1500 mm^3 have a T_d of 6.0 days. Determinations of the cell cycle time of the proliferating cells in these tumours yielded a value of about 20 hours. A growth fraction of proliferating cells of about 0.4 could be derived. The R-1 cells growing under cell culture conditions have a doubling time of the mean number of cells per clone of 17 ± 2 hours. This value is not significantly different from the cell generation cycle of 16 hours as determined from autoradiographic analysis of pulse-labelled cultures [Hermens and Barendsen, 1967, 1969]. This indicates that the growth fraction is approximately 1.0 and that cell loss is negligible in small clones.

2.2 *Assay for fractions of clonogenic cells in irradiated tumours*

The tumours used in the present study had volumes between 0.7 and 1.0 cm^3 with a doubling time of about 4 days.

The cells of the R-1 tumour can be directly cultured *in vitro* [Barendsen and Broerse, 1969] after application of a cell dispersion technique [Reinhold, 1965; 1966]. Cells derived from unirradiated tumours give rise to clones with a plating efficiency of 35 ± 10%. This technique offers the possibility of assaying changes in the fractions of clonogenic cells in tumours after a given treatment.

Clones which contain a few hundred to a few thousand cells develop in 10 to 11 days after plating. The number of clones can be scored after fixation with Bouin's solution and staining with haematoxylin. If single cells from irradiated tumours are cultured *in vitro*, the spread in clone size is considerably greater than for cells derived from unirradiated controls. Clones containing 50 or more cells at 10 or 11 days after plating are considered to have originated from a clonogenic tumour cell that retained the capacity for unlimited proliferation. The reproducibility of the plating efficiency has been found to be better if conditioned medium * and 10,000 feeder cells ** are used per dish.

2.3 *Cell culture and clone technique*

The medium used for culturing R-1 cells consisted of Hanks' salt solution and Eagle's amino acid and vitamin mixture. Phenol red was added as a pH indicator. This solution was filtered through a porcelain bacterial filter (G5) or a millipore membrane filter and kept in stock at 4° C. Ten per cent newborn calf serum and 100 IU of penicillin per ml was added before use.

Cells were cultured in glass bottles at a temperature of 37° C. A cell suspension

* Conditioned medium was prepared by adding 150 ml Hanks-Eagle medium containing 20% newborn calf serum to a culture flask together with 2×10^6 R-1 cells. These cells were allowed to grow for 72 hours. During this period the medium was "conditioned". After centrifugation and removal of cells and cell debris, the conditioned medium was stored at −20° C until used.

** Feeder cells were prepared by irradiating R-1 cells *in vitro* with a dose of 4000 rad of 300 kV X-rays.

was obtained by gentle trypsinization and a portion of the cells (300,000) was transferred to another bottle twice a week for propagation of the culture.

The clone technique of Puck and Marcus [1955] as modified by Barendsen for these R-1 cells was applied to determine the capacity for unlimited proliferation. Cells obtained by trypsinization of the stock culture or from the tumour by the dispersion technique were counted and plated in plastic Falcon flasks or in specially designed culture dishes [Barendsen and Beusker, 1960]. These consisted of a glass ring and a 6 μm thick Melinex* bottom which were covered by a glass top. These dishes or flasks had been provided with 3 ml Hanks-Eagle medium (HE) + 20% calf serum 24 hours earlier. After 4 hours, at least 95% of the cells became attached to the bottom of the dish or flask. During that period, they had the opportunity to repair the damage caused by the trypsinization procedure. Cells in dishes or in flasks can be used for different experiments. Cells that retain the capacity for unlimited proliferation will grow into a colony of cells, a clone, if the culture conditions are adequate. In order to provide the proper conditions, the temperature was kept at 37° C and the air was saturated with water vapor containing 5% CO_2 so as to maintain the required pH of 7.2.

3 Skin transplantation system

To investigate a possible differential effect of protracted irradiation on malignant and normal tissues, the effects of protracted neutron and gamma irradiation on the experimental rhabdomyosarcoma were compared with responses of the skin of the WAG/Rij rat.

A skin transplantation technique for the assessment of radiation-induced damage to the skin had been developed [Barendsen, 1969]. Albino female WAG/Rij rats of 3-month age were used as skin donors. Following total-body irradiation, two circular white pieces of dorsal skin from one animal were transplanted onto the backs of 3–6 month old unirradiated brown (WAG/Rij × BN/Bi)F_1 hybrid rats in which two graft beds per animal had been prepared by excision of similar pieces of skin [Balner, 1964] (figure III-1). Pieces of skin from an unirradiated animal (2 pieces per animal) were similarly transplanted to serve as controls. A piece of skin from a control animal, or a piece of skin irradiated with a relatively small dose, was transplanted onto the back of a recipient together with a piece of skin from an animal which had received a rather high dose of radiation.

The choice of the combination of donor and host eliminates the possibility of the rejection of the grafts due to immunological differences. Furthermore, the influence of damage produced in subcutaneous tissue which otherwise cannot be completely avoided, even with beta irradiation of low penetrating power [Hulse, 1962], is equal for all grafts. The difference in colour easily distinguished the grafted skin from the host.

* polyethylene terephthalate ICI.

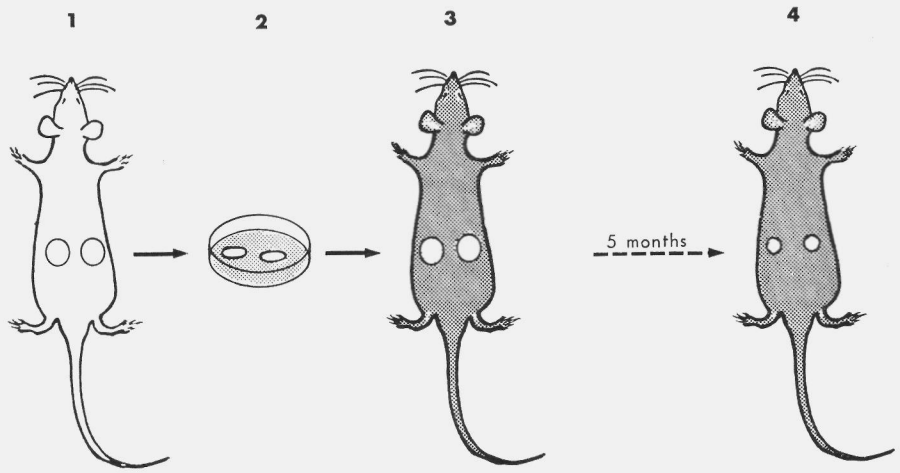


Fig. III-1. Skin transplantation method.

1. An albino WAG/Rij rat is used as skin donor.
2. Following total-body irradiation two circular pieces of dorsal skin are excised from the donor.
3. These white pieces of skin are transplanted onto the back of an unirradiated brown (WAG/Rij × BN/Bi)F_1 hybrid rat in which two graft beds were prepared.
4. Shrinkage of transplanted skin area is determined 5 months following irradiation.

At least 4 control animals (8 pieces of skin) and at least 2 animals per dose were used (4 pieces of skin) for each experiment. Unirradiated as well as irradiated grafts healed within 10 days. Little difference was observed between irradiated and unirradiated skin grafts either macroscopically or microscopically with respect to healing of the graft. The irradiated skin grafts start to decrease in size after about two weeks. After 3–5 months, the ratios of the remaining areas of grafted skin relative to controls approach constant values which depend on the dose (figure III-2). The areas of the transplanted pieces of skin could be readily measured with calipers in two perpendicular directions. The remaining areas of grafted skin relative to controls were measured 5 months after transplantation to obtain dose-effect relationships.

4 Irradiation techniques and dosimetry

4.1 *Tumours*

Acute irradiations of tumours with 300 kV X-rays were carried out with a Philips-Müller X-ray generator operated at 10 mA. Rats were anaesthetized by injection of 0.45 mg of Veterinary Nembutal per gram body weight and were placed on a horizontal 4 mm thick lead platform with a centre hole of 25 mm diameter. The tumours in the flanks of the rats were placed over the hole through which the X-ray beam passed vertically. The dose rate was 340 rad/min; the focus-skin distance, FSD, was 20 cm;

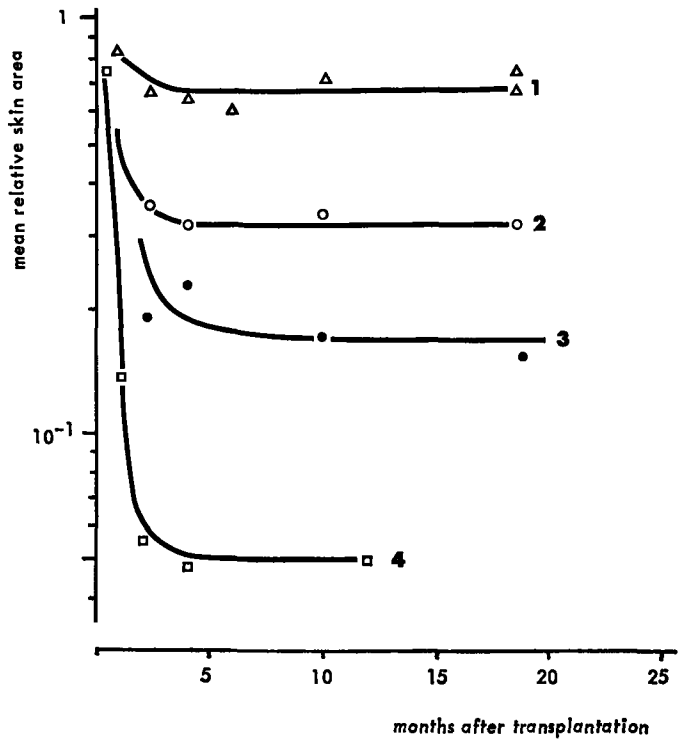


Fig. III-2. Area changes as a function of the time interval after irradiation for transplanted areas of skin. Curves 1 to 4 show relationships for single acute doses of 800, 1200, 1600, and 2000 rad of 300 kV X-rays, respectively.

the half-value layer, HVL, was 2.0 mm Cu. Absorbed doses were determined by measurements with a Baldwin Ionex ionization chamber.

Protracted irradiations of tumours with gamma rays were carried out with a 1200 Ci ^{137}Cs gamma source. Nonanaesthetized rats were given a total-body irradiation and water and food were provided *ad libitum*. Rats were housed in small cages at different distances from the gamma source which were selected on the basis of the dose rate to be given. For a dose rate of 75 rad/h, the distance, source-rat, was 130 cm. Absorbed doses were determined by measurements with a Philips ionization chamber type 37486/10.

Acute and protracted neutron irradiations were carried out with a Van de Graaff accelerator.* Monoenergetic neutrons of 15 MeV were produced through the reaction $^{3}_{1}H+^{2}_{1}D \rightarrow {}^{4}_{2}He+{}^{1}_{0}n$ by bombarding a fixed tritium target with 400 keV deuterons at a current of 250 μA. The yield of this generator was equal to 2×10^{10} neutrons/sec. For acute irradiations, the tumours had to be irradiated at a rather short distance of

* Manufactured by High Voltage Engineering (Europa) N.V., Amersfoort, The Netherlands.

about 6 cm from the target [Barendsen and Broerse, 1969] in order to obtain a dose rate of 20 rad/min. Animals were anaesthetized.

During protracted irradiations with 15 MeV neutrons, the rats were housed in small Macrolon cages. They were placed at a distance of 30 cm from the target in order to irradiate them with a dose rate of 48 rad/h. These rats were given a total-body irradiation and were not anaesthetized. Food and water were provided *ad libitum*. The absorbed doses were determined by measurements with a B15 ionization chamber filled with tissue-equivalent gas [Broerse and van Ammers, 1966].

4.2 *Cells*

Acute irradiation of cells plated in dishes or in Falcon plastic flasks were performed with 300 kV X-rays with a HVL of 2.1 mm Cu. Dishes or flasks were placed on a 8 cm thick hardboard layer to provide maximum backscatter. Cells were irradiated through a layer of 4 mm medium in the dishes or flasks.

Continuous irradiation of cells plated in Falcon plastic flasks with ^{137}Cs gamma rays at low dose rates were carried out at different distances from the 1200 Ci gamma source depending on the dose rate required. The flasks were irradiated on a platform which was placed in a waterbath at a temperature of $37 \pm 0.5°$ C. A stirrer was employed to maintain a homogeneous temperature in the bath. Cells were irradiated from below through a water layer of approximately 5 cm thickness.

Cells plated in Falcon plastic flasks received continuous irradiation with 250 kVp X-rays at low dose rates which were obtained by varying the current and/or the distance from tube focus to cells. Flasks were placed in a waterbath and irradiated from above through 4 mm of medium.

4.3 *Skin*

For acute X irradiation, the animals were placed in a circular perspex cage with a diameter of 30 cm [Barendsen, 1969]. The distance from the focus of the X-ray tube to the bottom of the cage was 70 cm; the dose rate was 40 rad/min. The animals were irradiated dorsoventrally and the cage was placed on an 8 cm thick layer of masonite to provide maximum backscatter. Dose variations over the animal did not exceed 4% of the midline dose.

For acute neutron irradiations, the rats were irradiated individually in small nylon cylinders at a distance of 7 cm from the target. The dose rate was 20 rad/min [Barendsen, 1969].

The irradiation conditions mentioned in section 4.1 were used for protracted neutron and gamma irradiations.

5 Cell volume and DNA content distributions

5.1 *Measurements of cell volume distributions*

Electronic particle counters are widely applied as a standard tool for counting a

variety of microscopic particles such as blood cells, bacteria, etc. It is also possible to obtain size distributions of the counted particles by use of an electronic counter with a pulse height analyzer. Cell size distributions can be used to determine the unknown volume of cells by comparing their volume with volumes of well-known calibration particles such as polystyrene latex particles and pollen grains.

Continuous irradiation at a low dose rate may result in a radiation-induced inhibition of division leading to an accumulation of cells in the G_2 phase of the cell cycle [Hermens and Barendsen, 1969]. As a result, the mean cell volume will shift to larger values as compared to unirradiated controls. This aspect of cell volume change can also be studied by using an electronic particle counter with a pulse height analyzer. The Coulter Counter model B interphased with a 128 channel pulse height analyzer was used for counting and sizing rhabdomyosarcoma cells. The counter automatically determines the number and volumes of particles suspended in a diluent containing an electrolyte. A flow of particles in this diluent is directed through a small constricting aperture of 100 μm into a glass tube. Two immersed platinum electrodes at opposite ends of the aperture cause an electric current to flow through it. As the particle passes through the orifice, it displaces its own volume of electrolyte solution. This increases the resistance between the electrodes and results in a pulse-shaped electrical signal whose amplitude is related to the particle volume. This pulse is electronically amplified and transmitted to a scaling circuit. The amplified pulse is also fed into a pulse shaper and multichannel pulse height analyzer with teletype output. The pulse shaper accepts negative input pulses having an amplitude of between 0.2 and 10 volt and a duration of maximal 40 μsec. It provides a positive output pulse with an amplitude proportional to the input pulse and a duration of 3 μsec which is optimal for the pulse height analyzer.

The aperture which was used has a diameter of 100 μm. The coincidence correction with this aperture is less than 3% for suspensions with 20,000 cells/ml. Suspensions were diluted to about 20,000 cells/ml in order to eliminate the need for coincidence correction. Cell volume distributions could usually be obtained for several tens of thousands of cells. After sizing, the teletype prints out the numbers of counted cells per channel and also punches the data on paper tape. This paper tape can be used as input for a small computer interphased with an incremental plotter. Several parameters such as mean channel number, skewness, and kurtosis (see paragraph III-6) can be calculated, and a size distribution can be plotted.

5.2 *Measurements of DNA content distributions*

The cell cycle stages which were first recognized by Howard and Pelc [1953], consist of pre- and post-synthetic gaps (G_1 and G_2), the DNA synthesis period (S), and the mitotic period (M). This terminology forms the basis of modern cell cycle analysis and provides the temporal frame work upon which biochemical events can be arranged. A number of published techniques based on unique biochemical or physical proper-

ties of cells in specific phases of the cell cycle have been employed for cell life cycle analyses. These methods are based either on the appearance of mitotic figures or on the occurrence of specific metabolic processes. Scoring of labelled cells, which is tedious, must be performed visually under the microscope. A compensation, however, is that the cells are evaluated individually in terms of their uptake of a specific precursor.

An "impulse cytophotometer", the ICP11, as described by Dittrich and Göhde [1969] has provided a new approach to the study of various cell proliferation parameters. In order to determine the DNA content of cells, cells from a monocellular suspension are fixed in pure alcohol at −30° C. After centrifugation, washing with physiological saline, and incubation with RNAse at 37° C for 30 minutes, cells are treated with the fluorescent dye ethidium bromide which stains the double-stranded DNA. In the impulse cytophotometer cells are drawn through an orifice of 100 µm and are intensely illuminated by light from a high pressure mercury tube. An illuminated cell which is stained with ethidium bromide will show fluorescence and the fluorescent light is then electronically amplified by a photomultiplier. The amplitude of the pulse is proportional to the intensity of the fluorescent light and, therefore, proportional

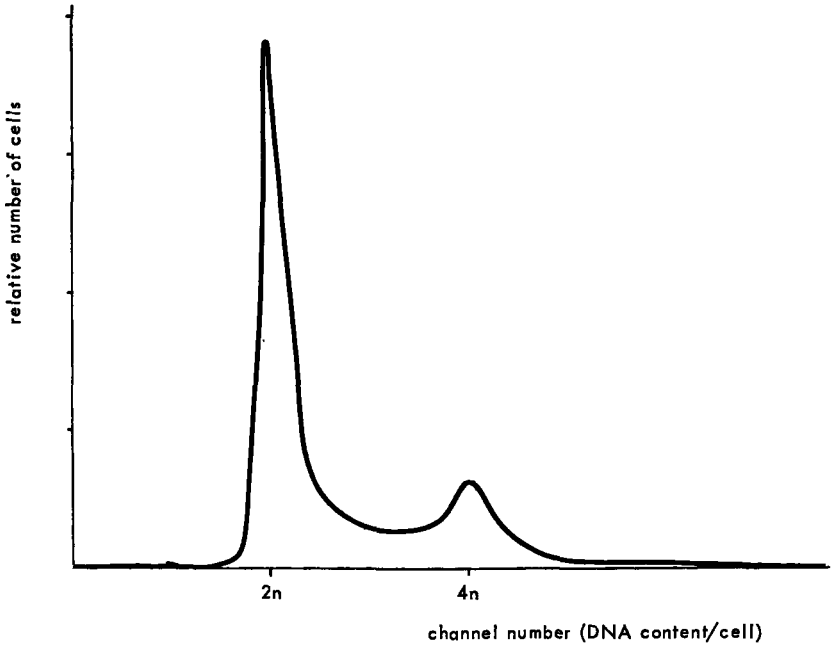


Fig. III-3. Distribution of DNA content per cell of R-1 cells. Numbers of cells counted per channel are plotted on an arbitrary scale as a function of the channel number, which is proportional to the DNA content per cell. The first peak represents cells in G_1 phase with a DNA content of 2n; the second peak represents cells in G_2+M phase with a DNA content of 4n. Cells in S phase are represented between these two peaks.

to the DNA content of the cell. The pulses are stored in a multichannel analyzer and displayed on an oscilloscope. After measurement of a sufficient number of cells, the content of the multichannel analyzer is read out and a hard copy is obtained by an X-Y plotter. The impulse cytophotometer requires only 50 µsec to measure a cell. Up to one thousand cells can be processed per second. At this high speed of determination, it is possible to count a sufficient number of cells to obtain adequate statistical accuracy of measurements.

A typical distribution of DNA content per cell is shown in figure III-3, where a DNA content distribution of cultured R-1 cells is plotted. The first peak represents cells in G_1 phase with a DNA content of 2n; the second represents cells in G_2+M phase with a DNA content of 4n. Cells in S phase are represented between these two peaks.

6 Analysis of cell volume and DNA content distributions

6.1 *Analysis of volume distributions*

In order to facilitate the analysis of changes in shapes of size distributions, a mathe-

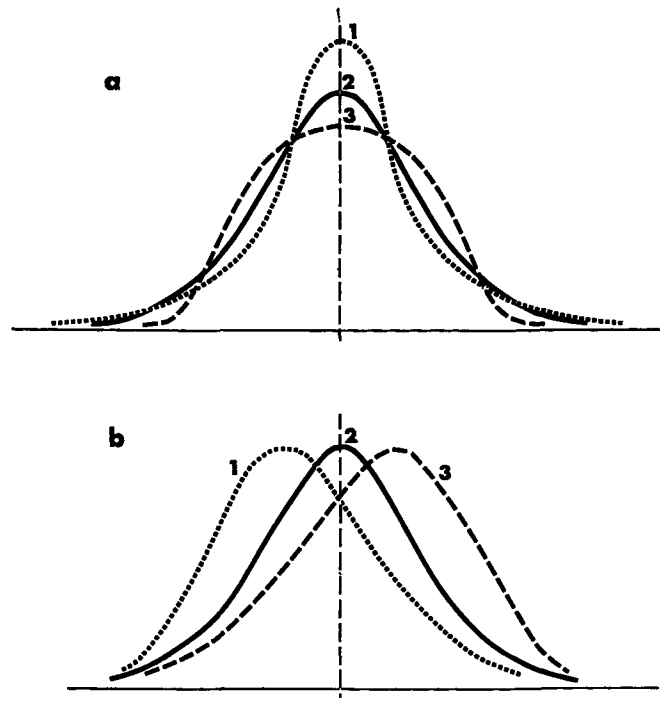


Fig. III-4. a. kurtosis: 1: lepto curve (g_2 positive), 2: normal curve ($g_2 = 0$), and 3: flattened curve (g_2 negative).
b. skewness: 1: skewness to the left (g_1 negative), 2: normal curve ($g_1 = 0$), and 3: skewness to the right (g_1 positive).

matical description for the volume distribution was sought. It was found that the volume spectrum can be represented by a log normal distribution:

$$N(V) = C/V \exp \{(\log V - \mu)^2/2\sigma^2\}$$

where N(V) is the number of cells in channel V; C is a normalization constant such that the area under the distribution of log V is unity; μ is the mean log volume; and σ^2 is the variance in log volume about μ [Rosenberg and Gregg, 1969]. In other words, the logarithms of the cellular volumes are described by a Gaussian relationship. Using the Fisher normality tests [de Jonge, 1960], one can calculate the skewness and kurtosis of the distribution. If the distribution is standard normal (i.e., a real Gaussian curve), the skewness, g_1, and the kurtosis, g_2, are equal to zero; if the distribution has a skewness to the left, the g_1 value will be negative. The g_1 value will be positive if the distribution has a skewness to the right. The g_2 value of the kurtosis test will be positive if the distribution is a lepto curve (i.e., has a pointed shape) and it will be negative if the distribution is a flattened curve (see figure III-4).

In order to calculate several parameters, the output of the multichannel analyzer

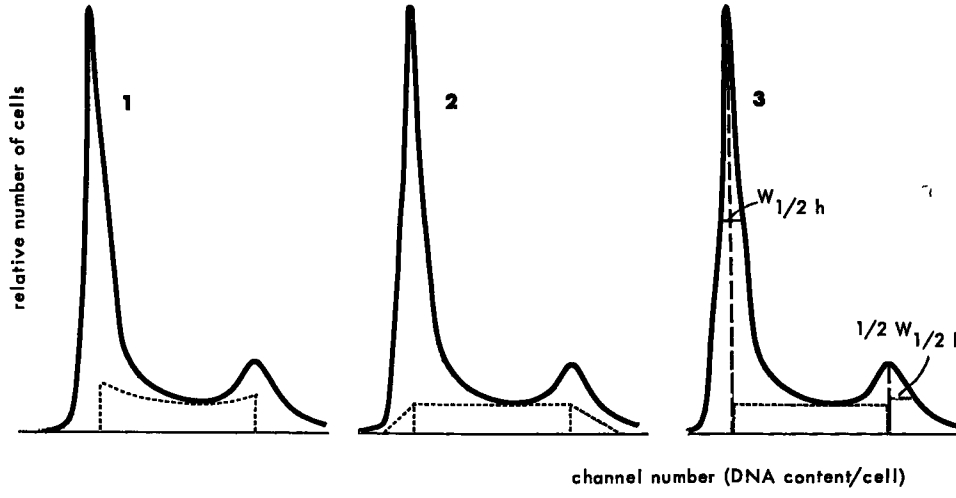


Fig. III-5. Analysis of a DNA content distribution.

1: least-squares best-fit of a normal distribution function to the G_1 and G_2+M peaks and a second-degree polynomial to the S distribution [Cram et al., 1971].

2: least-squares best-fit of a normal distribution function to the G_1 and G_2+M peaks; the S distribution is approximated by a rectangle and triangles [Zywietz, 1973].

3: areas representing the fractions of G_1 cells and G_2+M cells are approximated by the product of the height and the halve width at half the height of the G_1 peak and twice the product of the height and the half of the width at half the height of the G_2+M peak, respectively; the area representing the fraction of the cells in S phase is assumed to be rectangular.

teletype has been used as input for a small digital computer, the PDP-12. After taking the logarithms of the volumes, the mean channel number, the skewness, kurtosis, and the variances of these parameters are calculated.

6.2 *Analysis of DNA content distributions*

In order to determine the fraction of cells in G_1, S and G_2+M phases several methods can be used. Among these are: 1. the method of Cram et al. [1971] who used a computer program that makes a least-squares best-fit of a normal distribution function to the G_1 peak, a second-degree polynomial to the S distribution, and another normal distribution function to the G_2+M peak (curve 1 of figure III-5); 2. the method of Zywietz [1973] who used a computer program that makes a least-squares best-fit of a normal distribution function both to G_1 and G_2+M peaks; the S distribution is approximated by a rectangle and two triangles (curve 2 of figure III-5); 3. the method of Smets [1973]. In this method the area of the G_1 peak, representing the total number of cells in G_1, is obtained assuming that it can be approximated by a triangle whose area is calculated from the product of the number of cells in channel number 2n and the width determined at half the height ($W_{\frac{1}{2}h}$) as shown in figure III-5, curve 3. The area of the G_2+M peak is obtained by calculating the area of a triangle with a top at channel number 4n. The area is derived as twice the product of the number of cells in channel number 4n and the half of the width at half the height of this peak ($\frac{1}{2}W_{\frac{1}{2}h}$) as indicated in figure III-5, curve 3. The area of the S distribution is obtained by calculating the area of the rectangle as shown in figure III-5, curve 3.

Because the hard copy obtained by the impulse cytophotometer is not convenient for a computer analysis, the estimation of the areas representing the fractions of cells in G_1, S and G_2+M phases was done by the method of Smets. A comparison of the fractions determined in this way with the results obtained by Hermens [1973] show a reasonably good fit.

CHAPTER IV

SURVIVAL OF CULTURED RHABDOMYOSARCOMA CELLS AFTER ACUTE AND PROTRACTED IRRADIATION

1 Introduction

In this chapter, the extent of repair of sublethal damage after acute and during protracted gamma irradiation will be discussed and a comparison between experimental results and predictions based on the theory of Lajtha and Oliver [1961] concerning the dose equivalent of sublethal damage will be made. Results on cell survival obtained by Barendsen and Broerse [1969] after acute X-ray and fast neutron irradiations will be reviewed and compared with results of continuous low dose rate irradiations. In addition, cell volume distributions and DNA content distributions of cells have been obtained with the Coulter Counter and the impulse cytophotometer, ICP11. From the results obtained by these techniques, a better insight could be obtained into the extent to which ionizing radiation affects the progression of cells through the cell cycle.

2 Survival of R-1 cells in culture after acute and protracted gamma irradiation

In order to determine their radiosensitivity and to investigate the capacity of cultured R-1 cells to repair sublethal damage during protracted irradiation, survival curves were obtained for R-1 cells irradiated with ^{137}Cs gamma rays at dose rates of 122 rad/min and of 75 and 40 rad/h. Cells were kept at room temperature during the acute irradiation (dose rate 122 rad/min) and at 37° C in a waterbath as described in section III-3.2 during the irradiations at dose rates of 75 and 40 rad/h. Immediately after irradiation, cells were returned to the incubator at 37°C. Control cells were treated in the same way, except for the irradiation procedure.

The fractions of surviving cells after a radiation treatment were determined by the clone technique of Puck and Marcus [1955] as described in section III-1. The points through which the survival curves were drawn are mean values derived from the number of clones obtained ten days after irradiation on at least four Melinex dishes or Falcon flasks that had been irradiated with the same dose. The standard errors (standard deviation of the mean) represented by vertical bars in figure IV-1 were calculated by the formula

$$SE = \sqrt{\sum_{i=1}^{n} (x_i - \bar{x})^2 / n(n-1)},$$

where x_i represents the number of clones per dish, $\bar{x}$ the mean number of clones, and

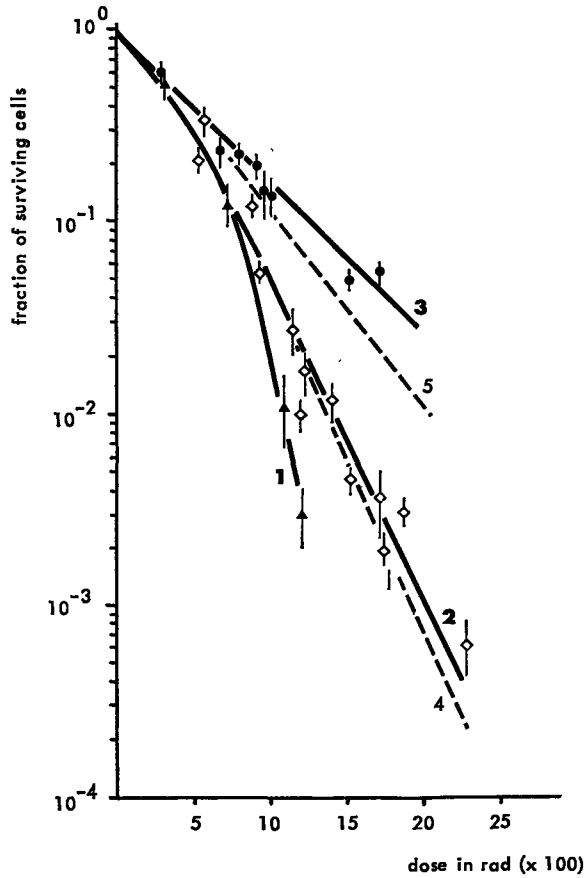


Fig. IV-1. Effect of lowering the dose rate on survival curves of R-1 cells treated with ^{137}Cs gamma rays.

Curve 1: dose rate, 122 rad/min;
Curves 2 and 3: dose rate, 75 and 40 rad/h, respectively;
Curves 4 and 5: curves 2 and 3, corrected for cell multiplicity, respectively.

n the number of dishes. However, when the value of the SE was found to be smaller than the standard deviation from the Poisson distribution, $SP = 1/n \sqrt{\Sigma x_i}$, the latter value was used as standard error. Obviously, the standard error for the control value must be added to the standard error for the points representing fractions of surviving cells after a certain dose of irradiation in order to derive the correct standard error of the surviving fractions given by the points and vertical bars in the figures.

In figure IV-1, the fractions of cultured R-1 cells which have retained the capacity for unlimited proliferation *in vitro* after irradiation are represented as a function of the dose administered at dose rates of 122 rad/min and of 75 and 40 rad/h. As discussed in section II-3, survival curves for single cell systems can be characterized by an

Table IV-1. The extrapolation numbers and D_0 values of the survival curves as represented in figure IV-1 for different dose rates. (The numbers in parentheses represent 95% probability values).

dose rate in rad/h	extrapolation number N	D_0 in rad
1210	10 (3.0–36)	153 (145–165)
75	2.2 (1.9–4.1)	262 (248–277)
75	2.6 (2.3–2.9)*	240 (225–265)*
40	0.9 (0.6–1.3)	563 (520–616)
40	1.2 (0.8–2.0)*	422 (395–455)*

* corrected for cell multiplicity

extrapolation number, N and a D_0 value. In table IV-1, the N and D_0 values for the survival curves represented in figure IV-1 are shown. It is clear that, with decreasing dose rate, the extrapolation numbers decrease and the D_0 values increase. The D_0 and N values derived from the survival curve obtained after acute gamma radiation are in the same range of values as those found for other mammalian cell lines, i.e., D_0 values in the range of 70–200 rad and N values between 1 and 50.

3 Multiplicity

Cells will continue to proliferate during continuous irradiation if the radiation is administered at relatively low dose rates. In order to account for this effect, the

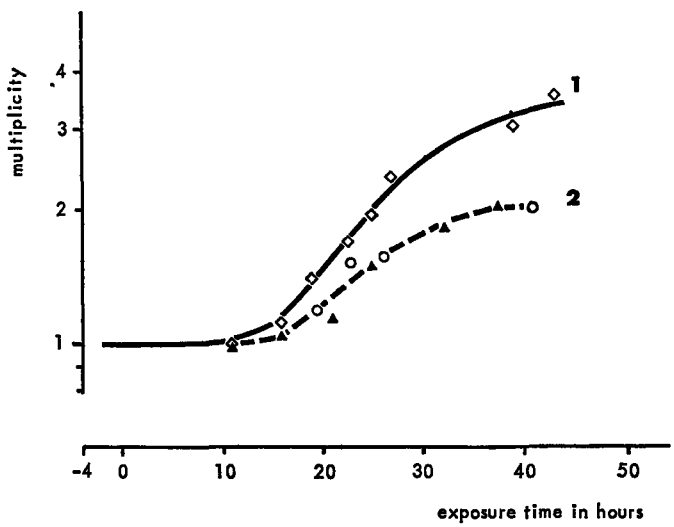


Fig. IV-2. The multiplicity, i.e., the number of R-1 cells relative to the number of R-1 cells at t = 0 (i.e., the start of the continuous exposure), as a function of time for control cells (curve 1) and cells treated with ^{137}Cs gamma rays administered at dose rates of 40 (▲) and 75 (○) rad/h as a function of exposure time, t (curve 2). Cells were plated on Falcon flasks four hours before the continuous irradiation was started (t = −4).

multiplicity (i.e., the mean number of cells per clone) has been derived as the ratio of the number of cells at exposure time, t, relative to the number of cells at $t = 0$. Cell countings were performed with the Coulter Counter. The ratio of the number of exposed cells at time, t, and control cells at the start of exposure 4 hours after plating of the cells is given in figure IV-2 as a function of exposure time, t, for dose rates of 40 and 75 rad/h of gamma radiation. From these results, it can be concluded that, 1. after plating cells do not enter mitosis for a period of about 15 hours, 2. low dose rate irradiation leads to an increase in the cell cycle time.

It is clear that the survival curves obtained after the 40 and 75 rad/h gamma irradiation must be corrected for cell multiplicity for exposure times in excess of 15 hours. The multiplicity correction has been carried out by the method of Sinclair and Morton [1965]. These authors obtained single-cell-response curves by correcting the surviving fraction for the average number of cells per colony as follows: the fractional survival, f, for colonies of average multiplicity, $\overline{N}$, is related to the average single cell survival, S, by $f = 1-(1-S)^{\overline{N}}$; therefore, $S = 1-(1-f)^{1/\overline{N}}$. Values for $\overline{N}$ are obtained from data shown in figure IV-2, although, strictly speaking, $\overline{N}$ should be valid for viable cells only. The error in using the observed value for $\overline{N}$ is not large, however, and is in the order of a few per cent as discussed by Sinclair and Morton [1965]. The N and D_0 values obtained with the multiplicity correction are included in table IV-1.

4 Repair of sublethal damage

4.1 *Introduction*

The factors which influence the shape of cell survival curves were discussed by Fowler [1964] who also dealt with the choice between a multi-target (single-hit) or a single-target (multi-hit) model. A "hit" means a physical event such as an excitation, ionization, or ion cluster. If energy can diffuse appreciable distances, the "target volume" may be larger than the sensitive site in the cell which is essential to its continued function. The multi-target, one-hit-on-each-target, model gives rise to survival curves which are exponential at high doses and which have an extrapolation number that is equal to the assumed number of targets. The single-target (multi-hit) model gives rise to survival curves with no finite extrapolation number, since the curves continue to bend with increasing dose. The general conclusion of Fowler was that the cell survival data are often insufficiently precise to make a choice of a specific model. Both models are compatible with a mechanism of sublethal damage when either the number of targets hit or the number of hits per target is below that required for lethal damage.

An important factor that can affect the shape of a survival curve is the possible heterogeneity of a cell population. This may influence the size of the shoulder and may even result in the shoulder being lost altogether. It is possible, therefore, that sublethal damage may exist independently of whether or not a shoulder is present in the

survival curve. Even when the data can be fitted to a shouldered curve, this does not provide direct evidence of the capacity of a cell to repair sublethal damage. Therefore, only survival curves obtained with split-dose experiments can demonstrate whether or not cells have the capacity to recover from this damage.

No attempts were made in the present study to synchronize R-1 cells for the determination of the radiosensitivity of cells in a particular phase of the cell cycle by obtaining dose-survival curves. Earlier attempts to synchronize cells by removal of mitotic cells by shaking were unsuccessful [Barendsen, personal communication]. Therefore, in order to demonstrate the existence of sublethal damage caused by gamma rays in R-1 cells, two types of split-dose experiments were performed. In one experiment, the first dose administered to the cells was 600 rad of ^{137}Cs gamma rays given at a high dose rate (122 rad/min) to inactivate the most sensitive cells. The cells surviving this dose must carry sublethal damage which can be revealed by analyzing the effectiveness of a second dose of 600 rad of ^{137}Cs gamma rays given at various time intervals after the first dose. In a second experiment, a first dose of 850 rad of ^{137}Cs gamma rays was administered to the cells given at a low dose rate in order to allow cells to repair sublethal damage during protracted irradiation. A second acute dose of 600 rad of ^{137}Cs gamma rays was given at different time intervals to test

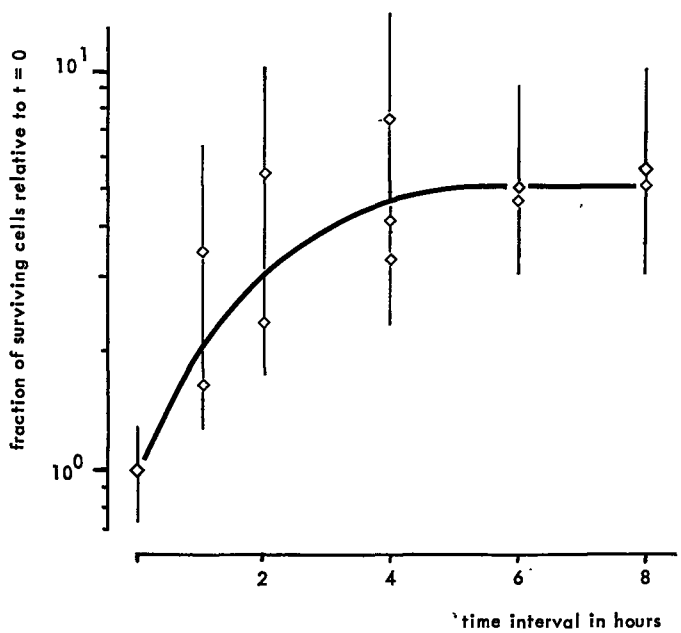


Fig. IV-3. Repair following a split-dose treatment. The first dose of 600 rad of ^{137}Cs gamma rays was administered to R-1 cells at a dose rate of 122 rad/min. The second dose of 600 rad was given at different time intervals, t, after the first dose. The fractions surviving cells are related to the fraction surviving cells at t = 0 (i.e., after a total dose of 1200 rad of gamma rays).

whether this repair was optimal. These experiments will be described in the next paragraph.

4.2 *Repair of sublethal damage after acute and during protracted irradiation*

R-1 cells plated on Melinex dishes or in plastic Falcon flasks received a single dose of 600 rad of gamma rays at a dose rate of 122 rad/min. Immediately and after different time intervals of incubation of the irradiated cells at 37 °C, a second dose of 600 rad of gamma rays was given. A single dose of 1200 rad of gamma rays reduces the fraction of surviving cells to 0.3%. If a second dose of 600 rad is given 1 hour after the first dose of 600 rad, then the surviving fraction is higher by a factor of about 2 as compared to the single dose of 1200 rad. This is due to repair of sublethal damage (Elkind repair). If the time interval is prolonged, repair becomes more clearly evident. The curve representing the fractions surviving cells after the split-dose treatment relative to the fraction surviving cells at t = 0, is shown in figure IV-3. The indication is that repair of sublethal damage is completed within 6–8 hours.

In figure IV-4, results of split-dose experiments with a first dose of 850 rad of gamma rays administered at a dose rate of 75 rad/h and a second acute dose of 600 rad of gamma rays given at different time intervals after the first dose are shown. The points show that no repair of sublethal damage could be demonstrated in the interval between the two doses. This must be due to the fact that repair of sublethal damage was optimal during the continuous irradiation at 75 rad/h.

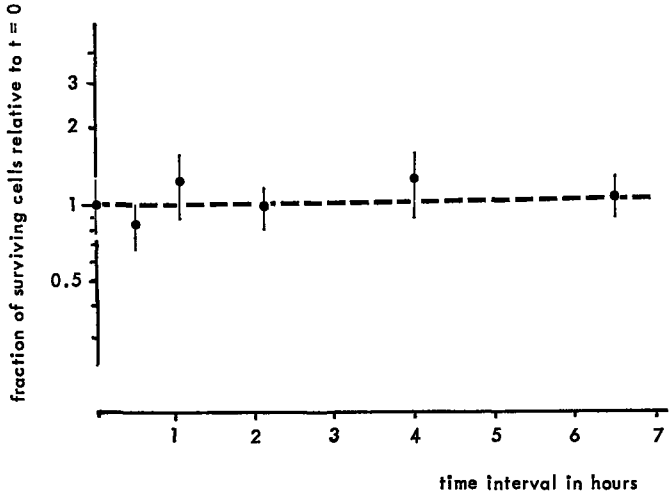


Fig. IV-4. Split-dose treatment of R-1 cells. The first dose was 850 rad of ^{137}Cs gamma rays administered at a dose rate of 75 rad/h. The second dose was 600 rad of ^{137}Cs gamma rays, dose rate 122 rad/min, given at different time intervals, t, after the first dose. Fractions surviving cells are related to the fraction surviving cells at t = 0 (i.e., after a dose of 850 rad at 75 rad/h which was immediately followed by a dose of 600 rad at 122 rad/min).

If repair of sublethal damage during continuous irradiation is complete then the fraction of surviving cells that would be expected after the acute dose of 600 rad of gamma rays is

$$S = S_{850} \times S_{600} = 0.085 \times 0.21 = 0.017.$$

However, the surviving fraction determined when the additional acute dose of 600 rad of gamma rays was given immediately after the first dose of 850 rad of gamma rays at 75 rad/h is 0.0065. This fraction is lower by a factor of 2.6 than the fraction expected on the basis of the assumption mentioned. It indicates that other phenomena must play a role and cause an increased radiosensitivity. The fact that such a small surviving fraction was observed after the additional acute dose of 600 rad can be explained by the assumption that cells are redistributed over the cell cycle during continuous irradiation and that a relatively larger number of cells is present in a more radiosensitive phase of the cell cycle than was the case at the start of the irradiation. This aspect of redistribution of cells will be discussed in the next paragraph.

5 Cell volume and DNA content distributions

A number of Falcon flasks each containing medium supplemented with 10% calf serum and 1,000,000 cells at the start of the exposure 24 hours after plating were irradiated at dose rates of 40 and 75 rad/h with ^{137}Cs gamma rays. Flasks were removed from the gamma source at regular time intervals and cells were trypsinized and counted with the Coulter Counter. A cell size distribution was made following dilution to a density of about 20,000 cells/ml. This size distribution was analyzed by the PDP-12 computer with respect to the parameters mean channel number, kurtosis, and skewness.

In figure IV-5 volume distributions as a function of exposure time, t, for cells irradiated at a dose rate of 40 rad/h are shown. From these figures, it is clear that the cells in the irradiated cell population have different mean cell volumes as compared with the mean volume of cells in the untreated population. This indicates that cells are redistributed in the cell cycle phases.

In figure IV-6, the mean cell volume, kurtosis, and skewness as derived from volume distributions of figure IV-5 and distributions obtained at other time intervals are presented as a function of time. The shifts in mean cell volumes and the variations in skewness and kurtosis also indicate that changes in the age distributions of cells must have taken place. However, quantitative relationships concerning the changes in fractions of cells in G_1, $G_2 + M$, and S phases as a function of exposure time cannot be derived from these data.

In figure IV-7, histograms of DNA content per cell of cells exposed to continuous irradiation at 40 and 75 rad/h are shown. From these data it is possible to derive quantitative relationships with respect to changes in the age distribution of cells. The

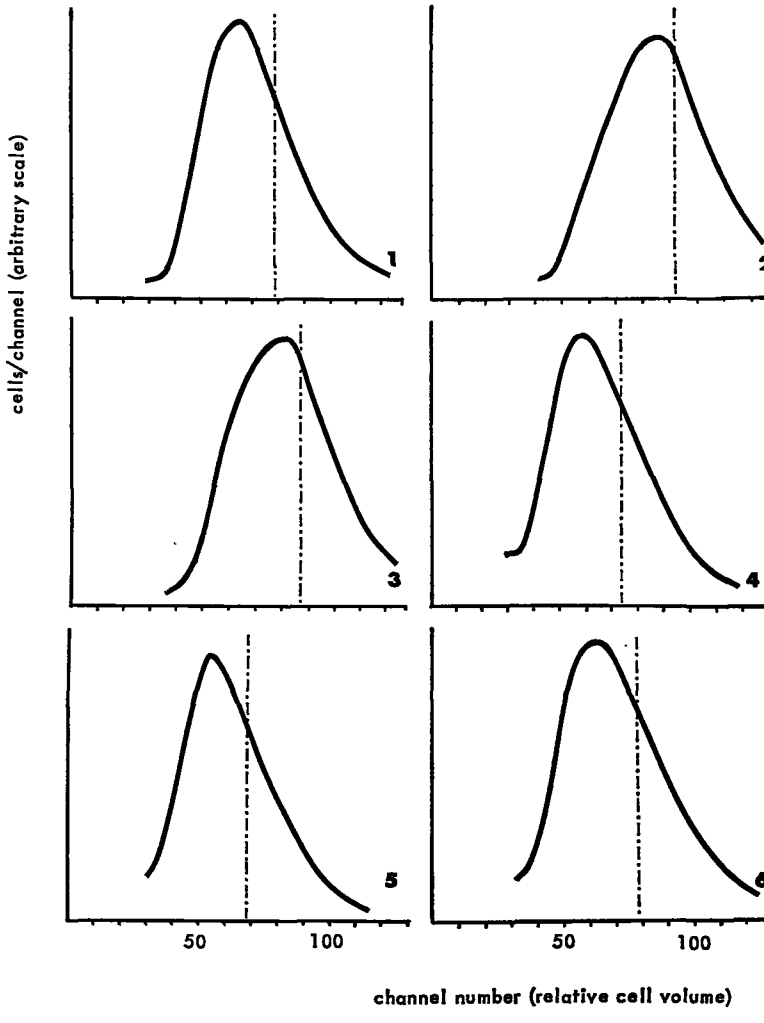


Fig. IV-5. Volume distributions of R-1 cells irradiated at a dose rate of 40 rad/h as a function of exposure time, t. Number of cells counted per channel are plotted on an arbitrary scale as a function of channel number, which is proportional to the relative cell volume. The vertical lines indicate the mean cell volume. Curve 1 shows the volume distribution of control cells, curves 2–6 show the volume distributions of cells at t = 4, 8, 12, 16 and 20 hours, respectively.

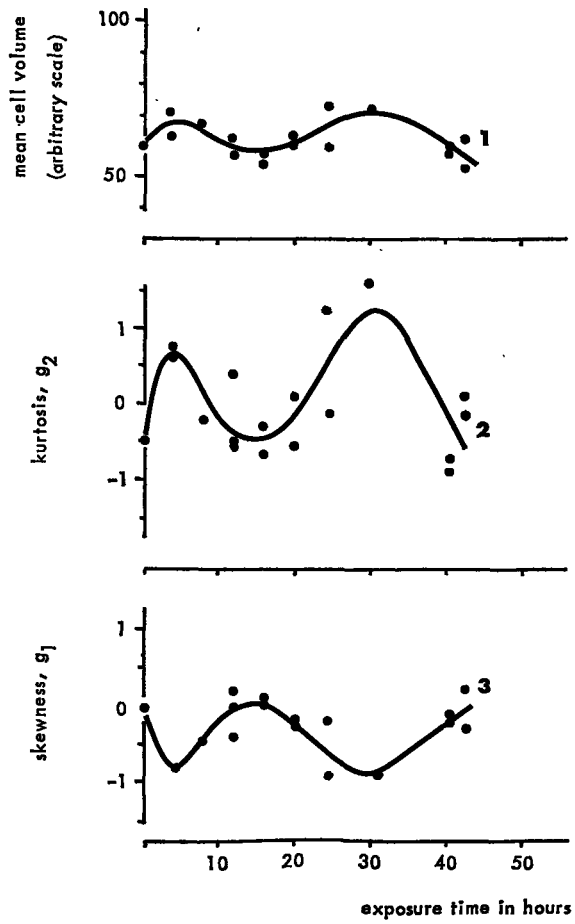


Fig. IV-6. Mean cell volume (curve 1), kurtosis, g_2, (curve 2) and skewness, g_1, (curve 3) of volume distributions of R-1 cells irradiated at 40 rad/h as a function of exposure time, t.

percentages of cells in G_1, S, and $G_2 + M$ phases as derived from these and other DNA histograms obtained at different time intervals are presented in figures IV-8 and IV-9 as a function of exposure time. It is clear that during the first 8 to 10 hours of the continuous irradiation at 40 rad/h (figure IV-8) the fraction of G_1 phase cells decreases. This is followed by an increase to about 80% of its value at $t = 0$ after an exposure time of 16–20 hours. The fractions levels off to a value which is about 75% of that at $t = 0$ for exposure times in excess of 24 hours. The fraction of $G_2 + M$ phase cells reaches a maximum value at an exposure time of about 20 hours and decreases to a value which is somewhat higher than it is at $t = 0$. The fraction of S phase cells increases during the first 4–6 hours of the irradiation; it decreases until a minimum value has been reached at $t = 16$–18 hours. The fraction of S phase cells increases again for

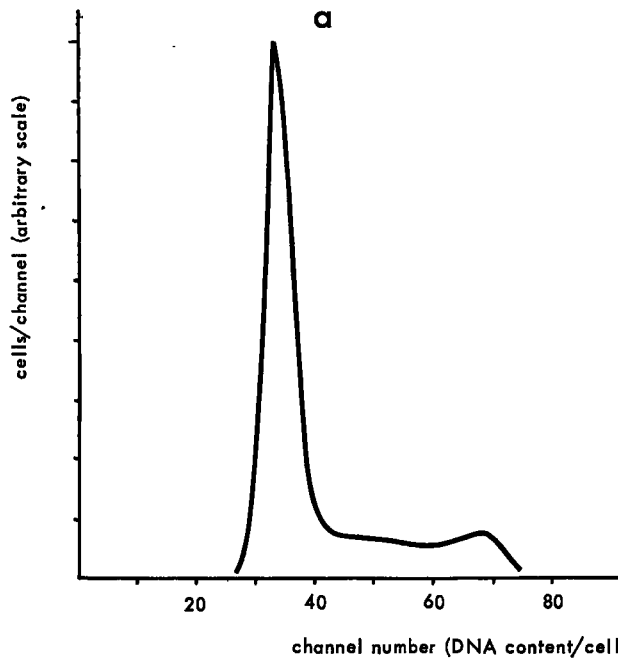
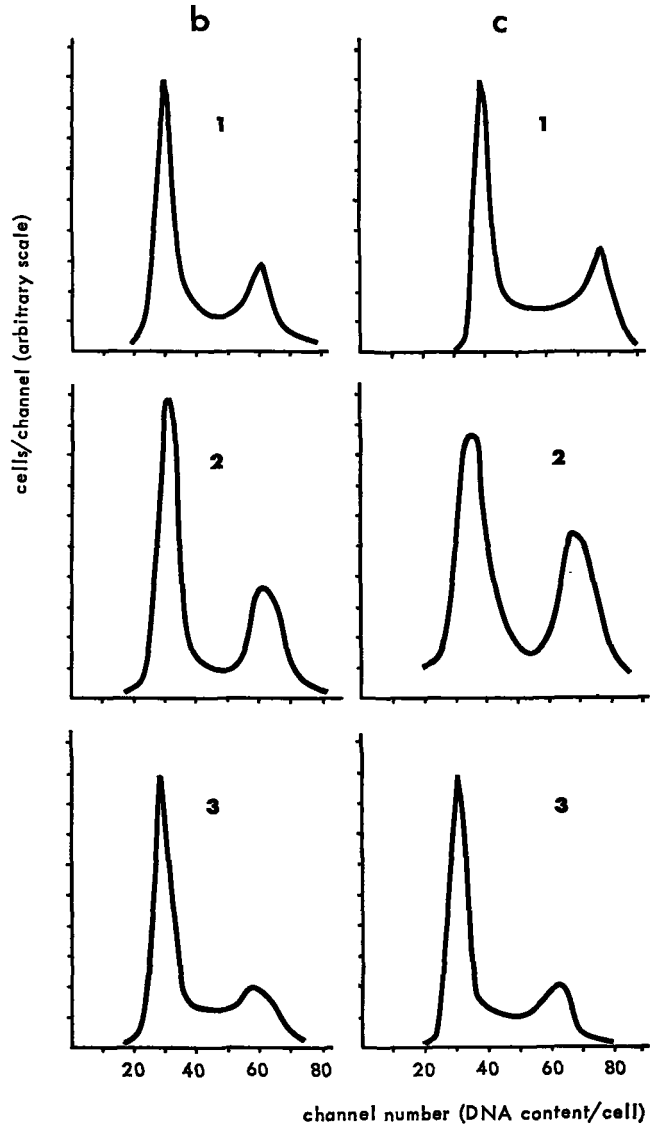


Fig. IV-7. Distributions of DNA content per cell for R-1 cells as a function of exposure time, t. panel a: control R-1 cells, t = 0 hours

exposure times in excess of 20 hours up to about 30 hours and does not change significantly during the time from 30 hours to t = 40 hours. For the continuous irradiation at 75 rad/h, the differences are more pronounced than with irradiation at 40 rad/h as shown in figure IV-9. The fraction of G_2+M phase cells increases to a value of about 37% at t = 22 hours. This maximum value is higher by a factor of about 2 than that of an untreated population of cells. During continuous exposure at 40 rad/h, the fraction of G_2+M cells increases to a value of about 30% at t = 20 hours. This value is higher by a factor of 1.5 as compared with that at t = 0.

It can be concluded that continuous irradiation has little effect on the progression of cells through the G_1 phase, but produces a delay in the S and G_2+M phases. This is demonstrated by the increase in the fraction of cells in S phase during the first 4–6 hours which is followed by an increase in the fraction of cells in the G_2+M phase (figures IV-8 and IV-9). This delay leads to an increase in cell cycle time. This observation is in agreement with the values of the multiplicity factor presented as a function of exposure time in paragraph IV-3 and with data of Bedford and Mitchell [1973] for V79 Chinese hamster cells irradiated at 90 rad/h. The fact that more cells are found in the G_2+M phase, which is known as a very radiosensitive phase (see paragraph



Cont. fig. IV-7.

panel b: R-1 cells irradiated at a dose rate of 40 rad/h. Curve 1: t = 4 hours; curve 2: t = 16 hours; curve 3: t = 41 hours.

panel c: R-1 cells irradiated at a dose rate of 75 rad/h. Curve 1: t = 7.5 hours; curve 2: t = 16 hours; curve 3: t = 41 hours.

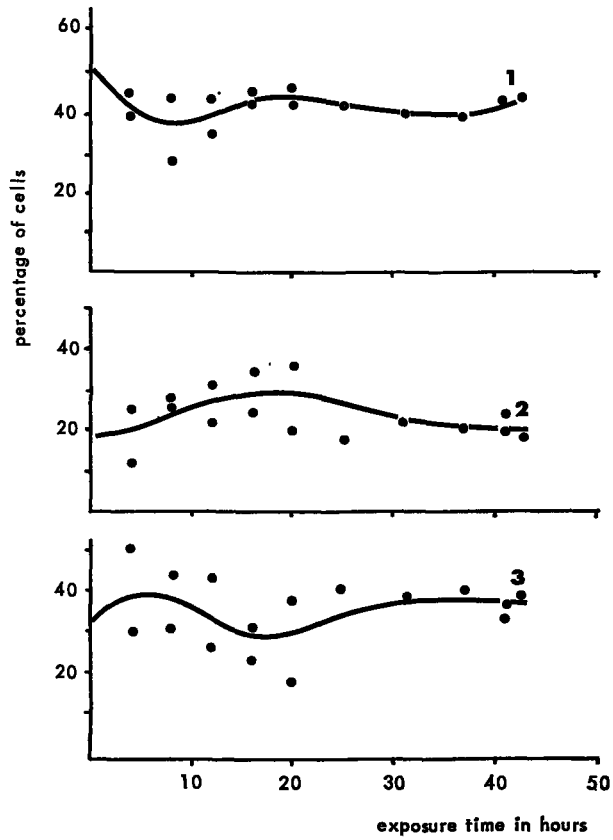


Fig. IV-8. Percentages of R-1 cells irradiated at 40 rad/h in G_1 phase (curve 1), G_2+M phase (curve 2), and S phase (curve 3) of the cell cycle as a function of exposure time, t.

IV-7.3), could well explain the observed increase in radiosensitivity after treatment with a dose of 850 rad of ^{137}Cs gamma rays administered at a dose rate of 75 rad/h as discussed in the previous paragraph.

The fraction of cells in G_2+M phase is larger after a dose of 1500 rad administered in 20 hours at a rate of 75 rad/h as compared with a dose of 850 rad administered in 11.3 hours. It might be expected that an additional acute dose of 600 rad of ^{137}Cs gamma rays given after 20 hours would be even more effective than if administered at $t \approx 11.3$ hours. With the cloning technique, however, it is not possible to observe a sufficiently larger number of survivors after a dose of 1500 rad of gamma rays given at a dose rate of 75 rad/h followed by an acute dose of 600 rad of ^{137}Cs gamma rays, because the fraction would be too low to yield sufficiently a large number of clones.

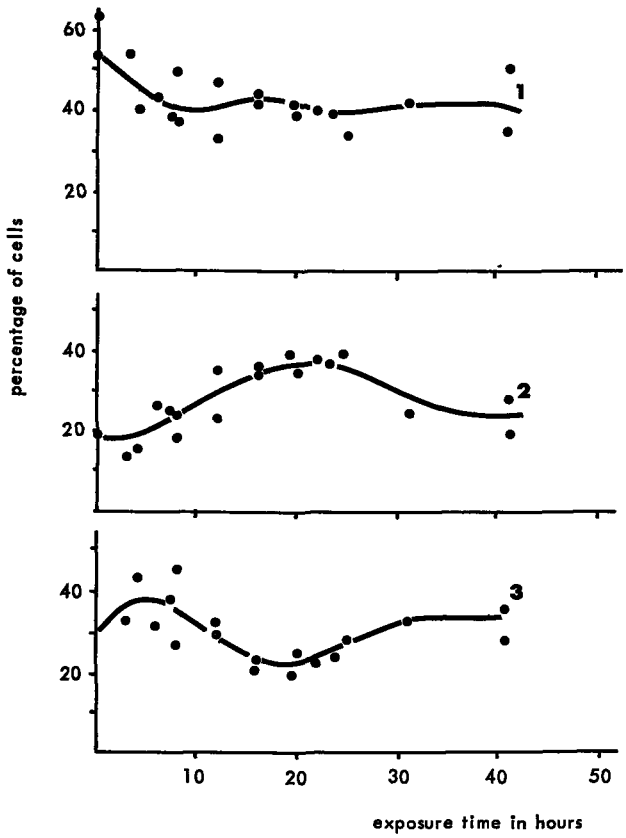


Fig. IV-9. Percentages of R-1 cells irradiated at 75 rad/h in G_1 phase (curve 1), G_2+M phase (curve 2), and S phase (curve 3) of the cell cycle as a function of exposure time, t.

6 Survival after acute irradiation with 15 MeV neutrons

Although no experiments were performed with protracted fast neutron irradiation on single cultured R-1 cells, it is necessary to discuss the available data on acute fast neutron irradiation of such cells in order to obtain RBE values for fast neutron irradiation relative to X-rays. Experiments in which acute fast neutron irradiation was given to rhabdomyosarcoma cells, were performed by Barendsen and Broerse [1969] and their results will be reviewed. Monoenergetic neutrons of 15 MeV energy were produced by bombarding tritium with 400 keV deuterons at a current of 250 μA, accelerated with a Van de Graaff accelerator. Due to the limited output of this generator, the dose rate obtained was about 25 rad/min. In figure IV-10, curve 1 represents the dose-survival curve for rhabdomyosarcoma cells after acute fast neutron irradiation. The extrapolation number, N, is 3 and the D_0 value is 85 rad. For comparison, curve 2 represents the dose-survival curve after acute X-ray exposure.

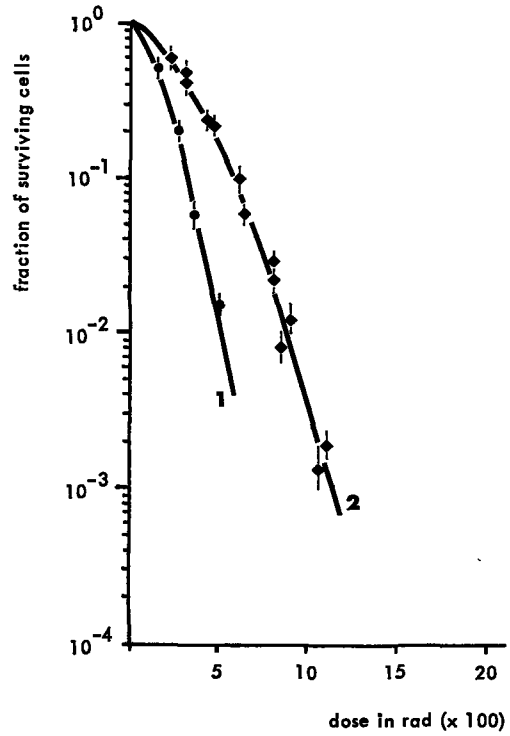


Fig. IV-10. Survival curves for R-1 cells exposed to fast neutrons and X-rays at 20 rad/min and 150 rad/min, curves 1 and 2, respectively [Barendsen and Broerse, 1969].

Characteristics of the irradiation conditions were: 250 kVp X-rays, 30 mA, 2.1 mm Cu, dose rate 150 rad/min. Survival curve 2 can be characterized by $N = 10$ and $D_0 = 120$ rad. The RBE of 15 MeV neutrons relative to X-rays will depend on the level of damage considered. The doses required to obtain 50% survival are equal to 255 rad of X-rays and 120 rad of 15 MeV neutrons. From these values, an RBE for 15 MeV neutrons equal to 2.1 can be calculated. The RBE values for the 10 and 1% survival level are 1.8 and 1.7, respectively.

7 Discussion

7.1 *Survival of R-1 cells*

Reduction in the rate at which a given dose of gamma radiation is administered to rhabdomyosarcoma cells cultured *in vitro*, results in a reduction in cell killing in relation to the known dose-response curves for mammalian cells *in vitro*, which show that part of the cell killing is due to accumulation of sublethal damage. This can be interpreted by the assumption that sublethal damage is repaired during protracted

irradiation. This means that the shoulder of the dose-response curve is reconstructed continuously during irradiation at low dose rates. The limiting form of the dose-response curve will, therefore, be an exponential; i.e., the extrapolation number will be unity. That is clearly indicated in table IV-1 where the N value for the survival curve obtained at a dose rate of 40 rad/h approaches 1.

As discussed by Barendsen [1962], the limiting slope of the survival curve obtained after protracted irradiation will be equal to the initial slope in the low dose region of a survival curve obtained after acute irradiation. Such initial slopes corresponding to D_0 values of 450 $\pm$ 100 and about 500 rad for X-rays have been derived for T-1 cells [Barendsen, 1962] and HeLa cells [Hall et al., 1966] respectively. Irradiations applied at low dose rates have been carried out with several types of mammalian cells; namely, HeLa cells by Bedford and Hall [1963, 1966] and Chinese hamster cells by Fox and Nias [1970] and Hall [1972]. In table IV-2, the cell type and dose rate, the extrapolation number, N, the D_0 values, and the temperature at which cells were kept during continuous irradiation are summarized. From the experiments carried out at 37°C, a ratio of 1.8 for HeLa cells can be derived for the D_0 values at the lowest and at the high dose rate. The experiment of Fox and Nias was performed at room temperature in an attempt to obtain a noncycling system and to analyze the relative contributions to the reduced killing at low dose rates and at high dose rates. Observations on cellular multiplicity and pulse labelling confirmed that there was no progression during the 3-day exposure period at room temperature and no loss in plating efficiency was observed. Cells were able to repair sublethal damage to a normal extent during exposure. The experiment of Hall [1972] was also performed at room

Table IV-2. Extrapolation numbers and D_0 values of survival curves.

cell type	type of radiation	dose rate	temperature in °C	D_0 in rad	N	Reference
Chinese hamster cells	X-rays	100 rad/min		200	3	Fox and Nias, 1970
	^{60}Co γ-rays	25 rad/h	20	625	1	
S_3 HeLa cells	^{60}Co γ-rays	44.9 rad/min		181	1.6	Bedford and Hall, 1963
	Ra γ-rays	19 rad/h	37	282	1	
	Ra γ-rays	9.5 rad/h	37	320	1	
S_3 Hela cells	^{60}Co γ-rays	103 rad/min		180	1.5	Bedford and Hall, 1966
	^{60}Co γ-rays	30 rad/h	37	260	1	
Chinese hamster cells	X-rays	70 rad/min		200	3	Hall, 1972
	Ra γ-rays	52.5 rad/h	20–24	359	1	
	Ra γ-rays	36 rad/h	20–24	398	1	
	Ra γ-rays	26 rad/h	20–24	421	1	
	Ra γ-rays	19.4–11.7 rad/h	20–24	479	1	
R-1 cells	^{137}Cs γ-rays	122 rad/min		153	10	present study
	^{137}Cs γ-rays	75 rad/h	37	240*	2.6*	
	^{137}Cs γ-rays	40 rad/h	37	422*	1.2*	

* corrected for cell multiplicity

temperature. Storage of cells at room temperature effectively prevented cell division and did not change the radiation response to an acute exposure throughout the storage period.

From the experiments of Fox and Nias [1970] and of Hall [1972], it is clear that repair of sublethal damage during protracted exposure results in a survival curve with a D_0 value which is about 2 to 2.7 times the D_0 value for a survival curve obtained after acute exposure assuming an RBE for gamma rays of 0.85. As indicated in figure IV-2, an increase in cellular multiplicity of R-1 cells becomes manifest about 20 hours after plating of the cells. With the correction of Sinclair and Morton [1965] for cellular multiplicity, the D_0 value for the survival curve obtained after irradiation at a dose rate of 40 rad/h changes from 563 to 422 rad. Comparison of this value with the value of 153 rad for acute exposure yields a ratio of D_0 values at low and high dose rates of 2.7. This value is close to the ratios derived from the experiments of Hall [1972], and Fox and Nias [1970]. It can be concluded that the D_0 values of survival curves obtained at low dose rate, when corrected for cell multiplicity are of similar magnitude as those for the initial slopes of the survival curves obtained at high dose rates. This conclusion is in agreement with the hypothesis that at low dose rates only single particle traversals produce cell reproductive death [Barendsen, 1962].

7.2 *Changes in cell volume and DNA content distributions*

The variation observed in the parameters of the cell volume distributions as a function of irradiation time obtained from cells irradiated at 40 rad/h are evident (figure IV-6). The increase in mean cell volume during the first period of the continuous irradiation indicates that a redistribution of cells in the cell cycle must have taken place; the decrease in skewness and the increase in kurtosis of the volume distributions are also reflections of this redistribution.

The measurements of DNA content distributions with the ICP11 have shown more definitely quantitative data concerning the redistribution of cells. The data indicate, that a steady-state is obtained for exposure times in excess of 26 hours during irradiation of 40 rad/h; i.e., no further changes in the fractions of cells in G_1, S, and G_2+M phases could be observed. At a dose rate of 75 rad/h, further changes in cell ages are not observed for exposure times in excess of about 30 hours. It must be noted that the cell volume and DNA content distributions were obtained from irradiated cells of which the majority were not capable of further proliferation because of the radiation dose administered. These cells, however, have not lost the capacity to adhere to the bottom of the Falcon flask, in contrast to a fraction of cells which looses this capacity for exposure times in excess of about 25 hours.

It is quite clear that more quantitative information can be obtained from DNA content distributions than from cell volume distributions. Therefore, rapid cytophotometers are useful tools for determining changes in fractions of cells in the phases of the cell cycle influenced by various types of agents.

7.3 *Variation in radiosensitivity over the cell cycle*

The existence of variation in the radiosensitivity of cells as a function of cell age is a well-known phenomenon. Sinclair [1968] considered three different cell types: Chinese hamster lung cells of the V79 line, HeLa cells, and mouse L cells. He summarized the principal features of the X-ray survival age-response as follows: 1. cells are generally most sensitive at mitosis (M phase): 2. if G_1 is of appreciable length, a resistant period is usually evident early in G_1 which is followed by a decline in survival towards S, cells at the end of G_1 may be as sensitive as those in mitosis; 3. in most cell lines, resistance increases during S to a maximum in the latter part of S. This is usually the most resistant part of the cycle; 4. in most cell lines, the G_2 period is sensitive – perhaps as sensitive as in mitosis.

DNA content histograms of rhabdomyosarcoma cells cultured *in vitro* and subjected to protracted irradiation clearly show that cells are blocked in G_2+M phase of the cell cycle (figure IV-8) during continuous irradiation for exposure times up to about 20 hours. According to Sinclair, the G_2+M phase was found to be the most radiosensitive in several cell lines. It is likely, therefore, that the increased radiosensitivity of protracted irradiated cells as described in paragraph IV-2, can be explained by a redistribution of the cells into the relatively more radiosensitive G_2+M phase of the cell cycle.

7.4 *Dose equivalent of sublethal damage*

Lajtha and Oliver [1961] and Oliver [1964] introduced the concept of "sublethal damage dose equivalent". This may be defined as that dose of radiation, D_E, which when given instantaneously to the cells would bring them to the same state of sublethal damage, and hence the same state of sensitivity, as that in which the cells are found at the moment considered. Lajtha and Oliver deduced from the *in vitro* data of Elkind and Sutton [1959] that D_E decreased exponentially with a half-life, τ, of 1.5 hours; i.e., they assumed that t hours after an acute dose, D_1, the dose equivalent of sublethal damage, D_E, is represented by $D_E = D_1 e^{-\mu t}$ where $\mu = 0.693/\tau$.

The surviving fraction of cells, S, after receiving a dose of D_1 rad which is followed t hours later by a dose of D_2 rad is thus given by the following equation [Oliver, 1964]: $S = S_{D_1} \times S_{(D_E+D_2)}/S_{D_E}$, where S_{D_1}, $S_{(D_E+D_2)}$, and S_{D_E} are the surviving fractions of cells after single doses of D_1, (D_E+D_2), and D_E rad, respectively.

On this basis, Lajtha and Oliver showed that the slope of the protracted cell survival curve after irradiating cells for t hours at d rad/h should be the same as the slope of the corresponding cell survival curve for acute irradiation when a dose of D_E rad has been delivered, where $D_E = d(1-e^{-\mu t})/\mu$. This equation has been used to relate the effects of protracted and acute regimens of radiation given in clinical practice [Lajtha and Oliver, 1961; Liversage, 1966; 1969a, b], see also chapter VII-5. A value of τ equal to 1.5 hours was assumed to be applicable to different mammalian tissues irradiated at normal body temperatures. Liversage [1969a] has discussed published evidence that

recovery appears to be complete within 7–16 hours in most mammalian systems. As τ is approximately one seventh of the time required for full recovery, this suggests that τ is in the order of 1 or 2 hours for most mammalian systems. The average errors in theoretical split doses calculated by substituting a value of τ equal to 1.5 hours have been shown to be very small for Chinese hamster lung cells, ovarian cells, and HeLa cells [Liversage, 1969a]. In the present split-dose experiment using fractional doses of 600 rad of gamma rays, τ was found to be 1.6 ± 0.2 hours. Repair of sublethal damage was almost completed within 8 hours. This result is in good agreement with the τ values found for the other cell systems mentioned above.

In the split-dose experiment where the first dose of 850 rad of gamma rays was given at a dose rate of 75 rad/h, no repair of sublethal damage could be demonstrated in the interval between the successive doses. This could be explained by the fact that repair of sublethal damage is sufficiently rapid during the protracted irradiation to prevent accumulation of sublethal damage. The dose equivalent of sublethal damage at the end of the protracted irradiation as predicted by the formula of Lajtha and Oliver is $D_E = d(1-e^{-\mu t})/\mu = 173$ rad, in which $d = 75$ rad/h and $\mu = 0.693/\tau$ with $\tau = 1.6$ hours. The additional dose of 600 rad at $t = 0$ would reduce the fraction of surviving cells according to

$$S = S_{850} \times S_{(173+600)}/S_{173} = 0.085 \times 0.11/0.68 = 0.014.$$

Although this fraction is slightly lower than the fraction of surviving cells expected if repair of sublethal damage was optimal during continuous irradiation at 75 rad/h, i.e., $S = 0.017$ (see IV-4), the dose equivalent of sublethal damage of 173 rad for the continuous exposure at 75 rad/h is not sufficient to account for the increased radiosensitivity, as determined after the additional acute dose of 600 rad of gamma rays which yielded a surviving fraction of 0.0065.

The observed increase in radiosensitivity is therefore most likely due to redistribution of cells during protracted irradiation. This increased radiosensitivity leads to a considerable reduction in survival as compared to the survival of cells in a noncycling system. Hence, the model of Lajtha and Oliver [1961], which does not take into account the increase in radiosensitivity related to redistribution and partial synchrony, requires further parameters to make it more generally applicable.

7.5 *RBE values*

When it became clear that the response of tumour cells *in situ* to protracted fast neutron irradiation was not statistically different from the response of these tumour cells to acute irradiation (see chapter V), no further experiments were performed with protracted fast neutron irradiation on rhabdomyosarcoma cells cultured *in vitro*.

Data from the literature indicated that variation in the radiosensitivity of cells related to cell age exists not only for low-LET radiation, but also for high-LET alpha radiation [Hall et al., 1972]. The same tendency in radiosensitivity as a function of the

cell cycle was found for alpha radiation, although the difference between the most resistant and most sensitive phases was considerably less as compared with low-LET radiation. From this observation, and the fact that some repair of sublethal damage may occur [Broerse and Barendsen, 1969] after acute fast neutron irradiation, it seems likely that little or no differences can be expected in the responses of cells to protracted neutron irradiation as compared to the responses of cells to acute irradiation. Thus approximate RBE values can be predicted for protracted fast neutron irradiation at different dose rates relative to ^{137}Cs gamma rays. For a gamma ray dose rate of 40 rad/h, an RBE value for 15 MeV neutrons of 3.2 relative to gamma rays can be calculated at the 10% survival level; for 75 rad/h, this value is 2.6. For acute fast neutron irradiation the RBE values relative to gamma rays were 2.0 and 2.1 for the 1 and 10% survival level, respectively, assuming an RBE of 0.85 of gamma rays relative to X-rays.

RBE values of ^{252}Cf neutrons administered at low dose rates are known from single cell survival curves of HeLa cells and Chinese hamster cells. The results published up to now indicate RBE values of 2.7 to 4.4 for to the clinically interesting dose rate of 30–50 rad/h using the standard gamma radiation [Atkins et al., 1973; Djordjevic et al., 1973; Fairchild et al., 1969; Hall et al., 1971]. An exception to this is the RBE of 6.7 obtained by Hall [1972] who circumvented the effects of cell progression and division during irradiation by carrying out the irradiation at room temperature. A comparison of the estimated RBE values for the R-1 cells with the published data cannot be made directly. The fact must be taken into account that the RBE as a function of dose or dose per fraction depends not only on the type of cells or tissues considered, but also on the neutron energy and the dose rate.

The dependence of the RBE on the mean neutron energy has been measured for cells of human kidney origin (T-1g cells) by Broerse et al. [1968]. In their experiments to obtain survival curves of T-1g cells, neutrons of several energies were obtained as follows: monoenergetic neutrons of 15 MeV and 3 MeV energy by bombarding ^{3}H and ^{2}H with 0.4 MeV deuterons, respectively; 8 MeV neutrons by bombarding Be with 16 MeV deuterons; 6 MeV neutrons by bombarding Be with 20 MeV ^{3}He; and 1 MeV neutrons by fission of ^{235}U induced by thermal neutrons from a reactor. In figure IV-11, the dependence of the RBE on the mean neutron energy at the 80, 20 and 1% survival level is shown (curves 1, 2 and 3, respectively). For an estimation of the RBE of ^{252}Cf neutrons from the data obtained in our experiments a factor must be considered, which takes into account the diffferences in mean neutron energy for irradiations performed at low dose rates. This factor is the ratio of RBE values derived from survival curves in the low dose region; i.e., at the initial part of the curves. As discussed in chapter II, the RBE varies as a function of the survival level and has its lowest value in the exponential region of the survival curve. A plateau value is reached in the initial part of the survival curve; i.e., in the low dose region. The RBE values at the 80% survival level obtained from the T-1g cell-survival curves as determined by Broerse et al. [1968] can be used as an approxi-

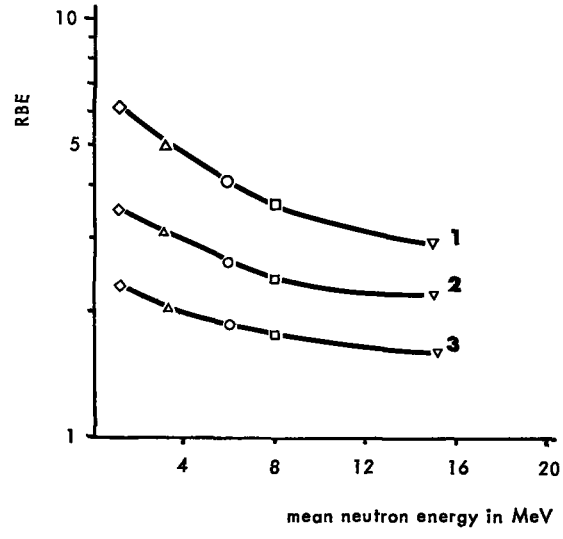


Fig. IV-11. Relative biological effectiveness, RBE, of fast neutrons as a function of the mean neutron energy for inhibition of clone formation by cultured human cells at the 80% survival level (curve 1) and at the 20 and 1% survival level (curves 2 and 3, respectively) [Broerse et al., 1968]. ◆: 1 MeV neutrons; Δ: 3 MeV neutrons; ○: 6 MeV neutrons; □: 8 MeV neutrons; and ∇: 15 MeV neutrons.

mate measure to arrive at a factor which allows an intercomparison between RBE values for low dose rate neutrons with different mean energies. These RBE values are 6.1 for 1 MeV fission neutrons; for 6 MeV neutrons, 4.2; for 8 MeV neutrons, 3.6; and for 15 MeV neutrons, 2.9. The mean neutron energy of californium neutrons has a value of about 2.3 MeV and a modal value of about 1 MeV [Stoddard, 1964]. Thus, at a certain dose rate, the RBE values obtained for 15 MeV neutrons can be compared with RBE values for ^{252}Cf neutrons, if the difference in mean neutron energy reflected by the ratio of 1.85 (be derived from figure IV-11) is considered.

The dependence of the RBE for ^{252}Cf neutrons on the ^{252}Cf dose rate (n+γ) derived in this way is shown in figure IV-12 where RBE data obtained from several experiments with californium neutrons on mammalian cells have been summarized. The RBE data of Hall et al. [1971] were derived from experiments where a known number of Chinese hamster cells were submitted to continuous irradiation lasting up to 10 days. The RBE values were obtained for depopulation of cells to a fraction of 10^{-1}. The data of Atkins et al. [1973], Fairchild et al. [1969] Djordjevic et al. [1973], and Hall [1972] were derived from single cell survival curves with either HeLa cells or Chinese hamster cells. The RBE values for ^{252}Cf neutrons lie approximately on a straight line when plotted as a function of the ^{252}Cf dose rate (n+γ) on a log-log scale. For ^{252}Cf dose rates (n+γ) of less than about 5 rad/h, it is assumed that the RBE will reach a plateau value. This can be deduced from survival curves of mamma-

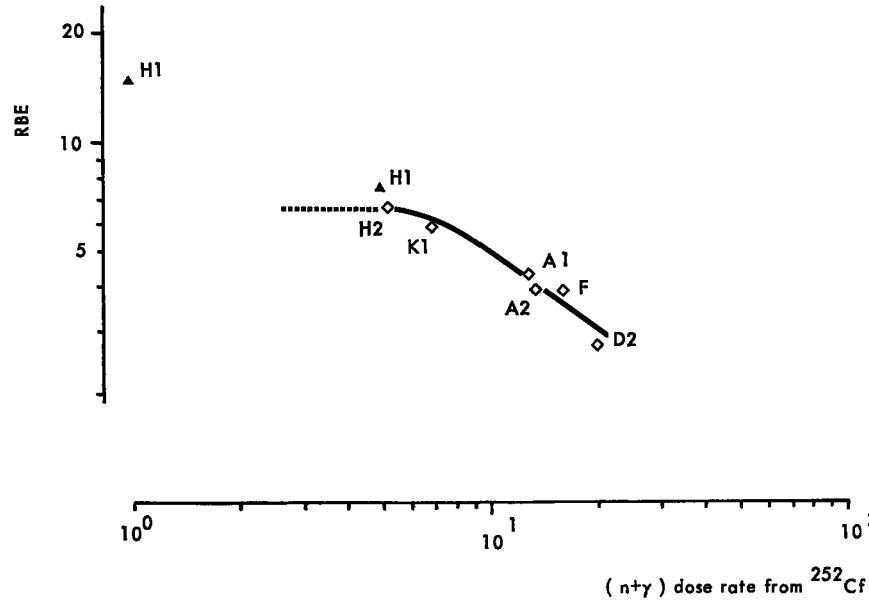


Fig. IV-12. Relative biological effectiveness, RBE, for ^{252}Cf neutrons as a function of ^{252}Cf dose rate (n+γ). The letters represent the following studies:

A1: Atkins et al. [1973], survival of HeLa cells;
A2: Atkins et al. [1972], survival of Chinese hamster cells;
D2: Djordjevic et al. [1973], survival of HeLa cells;
F: Fairchild et al. [1969], survival of HeLa cells;
H1: Hall et al. [1971], proliferation of Chinese hamster cells;
H2: Hall [1972], survival of Chinese hamster cells;
K1: present study, survival of R-1 cells (extrapolated value).

lian cells where a maximum in RBE values are reached in the low dose region. The high RBE value at about 1 rad/h reported by Hall et al. [1971] for depopulation of Chinese hamster cells does not conflict with this finding. The system used by Hall in these experiments is quite different because cell proliferation was allowed to occur during the irradiations and cannot be compared with the system in which survival of single cells as determined by the clone technique is used as the biological end-point.

The RBE equal to 3.2 for 15 MeV neutrons relative to ^{137}Cs gamma rays at a dose rate of 40 rad/h might therefore be multiplied by a factor of 1.85 when a comparison with the RBE for ^{252}Cf neutrons is made. The RBE obtained in this way is 5.9 and is similar to the RBE values found for ^{252}Cf neutrons for cultured mammalian cells; i.e., the calculated RBE value lies close to the line of figure IV-12 (point K1), on the assumption that a gamma dose rate of 40 rad/h corresponds to a ^{252}Cf dose rate (n+γ) of about 7 rad/h.

8 Conclusions

Two factors have been shown to play a major role in determining the reproductive death during protracted gamma irradiation: 1. repair of sublethal damage; and 2. redistribution of cell ages. These two factors have been shown to be adequate to explain 1. the reduction in effectiveness when a radiation dose is administered at a low dose rate as compared with an identical dose given at a high dose rate; and 2. the observed increase in radiosensitivity to an additional acute dose of 600 rad of ^{137}Cs gamma rays determined after a dose of 850 rad of ^{137}Cs gamma rays administered at 75 rad/h in comparison with unirradiated cells.

CHAPTER V

SURVIVAL OF TUMOUR CELLS IRRADIATED *IN VIVO* AND ASSAYED *IN VITRO*

1 Introduction

In the preceding chapter quantitative differences at the cellular level were discussed between dose-effect relationships obtained with radiations of different LET such as gamma rays and 15 MeV neutrons in relation to the dose rate employed. These differences may be expected to influence the biological effectiveness of different radiations in multicellular tissues such as skin and tumours. The dose-effect relationships of X-rays, gamma rays and 15 MeV neutron radiation on skin will be discussed in chapter VI. In this chapter, experiments on effects produced by doses of 300 kV X-rays, ^{137}Cs gamma rays, and 15 MeV neutrons applied at different dose rates to transplantable rhabdomyosarcomas growing in the flanks of WAG/Rij rats are described. Measurements were made of the loss of reproductive capacity as a function of dose. The results are represented as dose-survival curves. From these survival data, the influence of several phenomena could be deduced, namely reoxygenation of anoxic cells, repair of sublethal damage, and variation in radiosensitivity as a function of the phase in the cell cycle. With the results obtained, differences in RBE values derived for fast neutrons relative to X-rays for acute and protracted irradiations can be interpreted in terms of these phenomena.

The procedures were as follows: tumours were excised from the animal following irradiation, and, after application of the cell dispersion technique as described in chapter III, cells were counted and cultured directly *in vitro*. This adaptation of the clone technique of Puck and Marcus [1955] by Barendsen provides the possibility of assaying changes in the fractions of clonogenic cells in tumours after a given treatment. The Coulter Counter and the impulse cytophotometer, ICP11, offer the possibility of obtaining cell volume and DNA content distributions, which can be used to elucidate specific aspects of responses of tumours to irradiation.

2 Acute X-ray and fast neutron irradiation versus protracted gamma ray and fast neutron irradiation

2.1 *Survival after acute X-ray and protracted gamma ray irradiation*

For an evaluation of the processes which determine the total effect of a protracted exposure, it is not sufficient just to measure the fraction of clonogenic cells present in the tumour at the end of the treatment. It is also necessary to take into account changes in the absolute number of cells or in the cell density. Cell proliferation and cell loss during treatment can lead to changes in the total number of cells present during

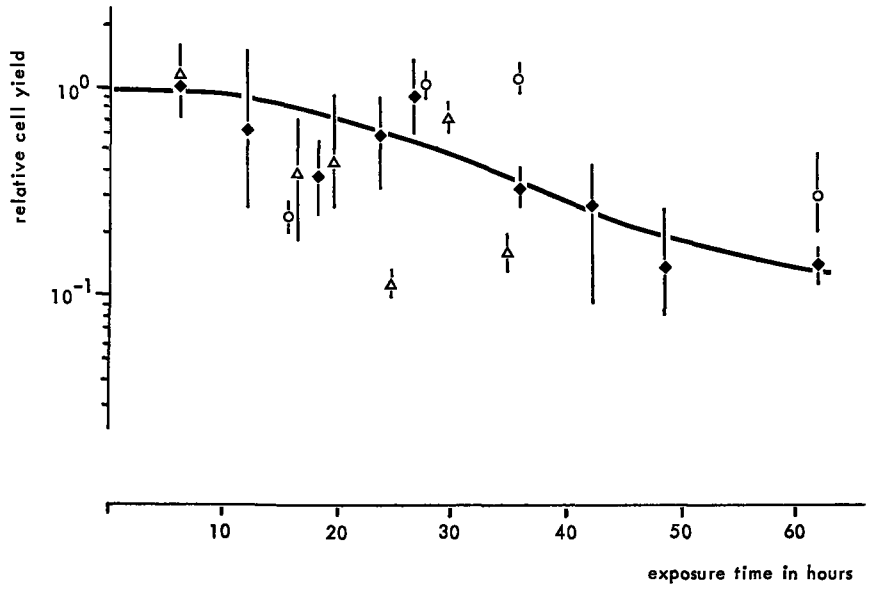


Fig. V-1. Cell yield of the cell dispersion technique as a function of exposure time of tumours irradiated with ^{137}Cs gamma rays applied at dose rates of 75(○), 110(◆), and 150 (Δ) rad/h, relative to the cell yield at t = 0. Bars represent the spread of the measured values for individual experiments.

protracted exposures. As a result, the tumour volume may increase during an initial period of irradiation and then decrease during the later part of the irradiation period. Furthermore, the mean cell volume, as measured with the Coulter Counter, has been found to increase during protracted irradiation. This was confirmed by DNA measurements to be discussed later, which clearly showed an accumulation of cells into G_2+M phase. Finally, irradiated cells may become more sensitive to the trypsinization treatment of the tumour which is required for preparation of the cell suspension to be analyzed for clonogenic capacity. It is not yet possible to make accurate corrections for all of these influences. Therefore, the yield from the cell dispersion technique, i.e., the number of cells per gram of tumour, has been used as an approximate measure to arrive at a correction factor for these effects as described by Barendsen and Broerse [1970] for treatments of the tumour with daily doses.

The relative cell yield per gram of tumour has been plotted as a function of exposure time in figure V-1. These data show that, for exposure times in excess of 12 hours, a correction factor must be applied; i.e., the fraction of surviving cells obtained after a protracted exposure of t hours will be multiplied by the cell yield factor at t hours. The points of figure V-1 for exposure times in excess of 10 hours might indicate that a minimum value exists at an exposure time of about 16–18 hours. In view of the large spread in the individual experimental points it cannot be ascertained whether a minimum in the relative cell yield is present.

Results of determinations after acute exposures and after exposures to protracted irradiation of fractions of surviving cells relative to the number of cells present at the start of the treatment are shown in figure V-2. Curve 2 of figure V-2 represents the fractions of surviving cells after single doses of 300 kV X-rays as determined by Barendsen and Broerse [1970]; the points represent mean values of at least three tumours assayed separately. The points of the low dose rate curve 1 represent results from assays of individual tumours which were exposed at dose rates of 75, 110, and 150 rad/h.

Survival curve 2, which was obtained with 300 kV X-rays for single doses administered at a dose rate of 340 rad/min, is characteristic for a population of cells consisting

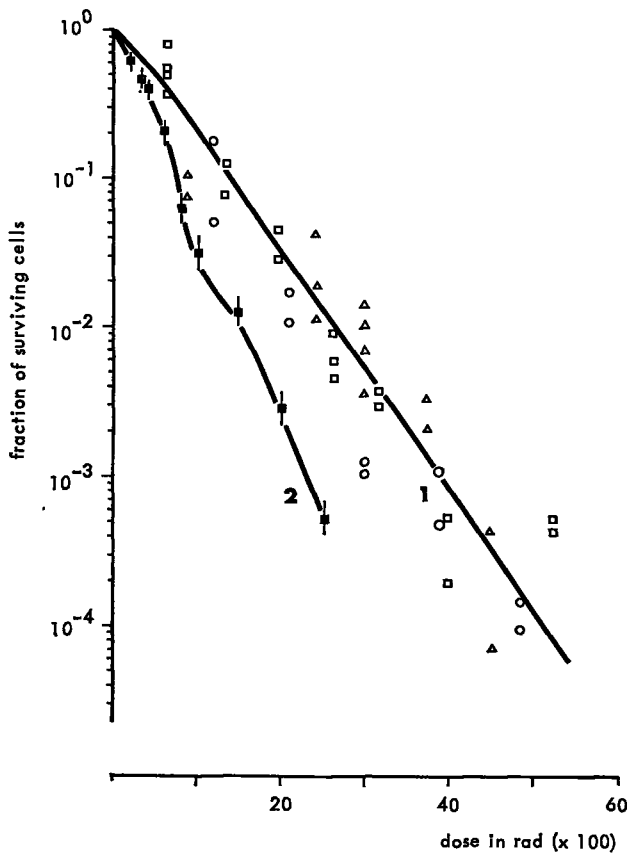


Fig. V-2. Survival curves obtained by irradiation of R-1 tumours. Curve 1: tumours irradiated with ^{137}Cs gamma rays in unanaesthetized rats receiving total-body irradiation at dose rates of 75(○), 110(□) and 150(Δ) rad/h. Symbols represent data obtained with individual tumours. Curve 2: tumours irradiated with 300 kV X-rays in anaesthetized rats at a dose rate of 340 rad/min. Symbols represent average values obtained with at least three tumours assayed separately. Bars represent standard errors.

of a large fraction of oxygenated cells (85%) and a smaller fraction (15%) of hypoxic cells [Barendsen and Broerse, 1969].

The fraction of hypoxic cells was determined as follows: 1. a dose-survival curve was obtained for cultured R-1 cells irradiated under well-oxygenated conditions (figure V-3, curve 1) and a dose-survival curve (figure V-3, curve 2) was obtained for R-1 cells after irradiation under anoxic conditions; 2. a dose-survival curve was obtained for tumour cells after irradiation of tumours in animals that were killed at least ten minutes earlier to allow cells to become anoxic (triangles in figure V-3, curve 2). In this way, survival curves were obtained for anoxic cells both *in vitro* and *in vivo*. The two survival curves were not significantly different from each other. The conclusion drawn was that rhabdomyosarcoma cells whether cultured *in vitro* or growing in the animal respond in a similar manner to radiation when irradiated under anoxic conditions. Therefore, Barendsen and Broerse [1970] assumed that well-

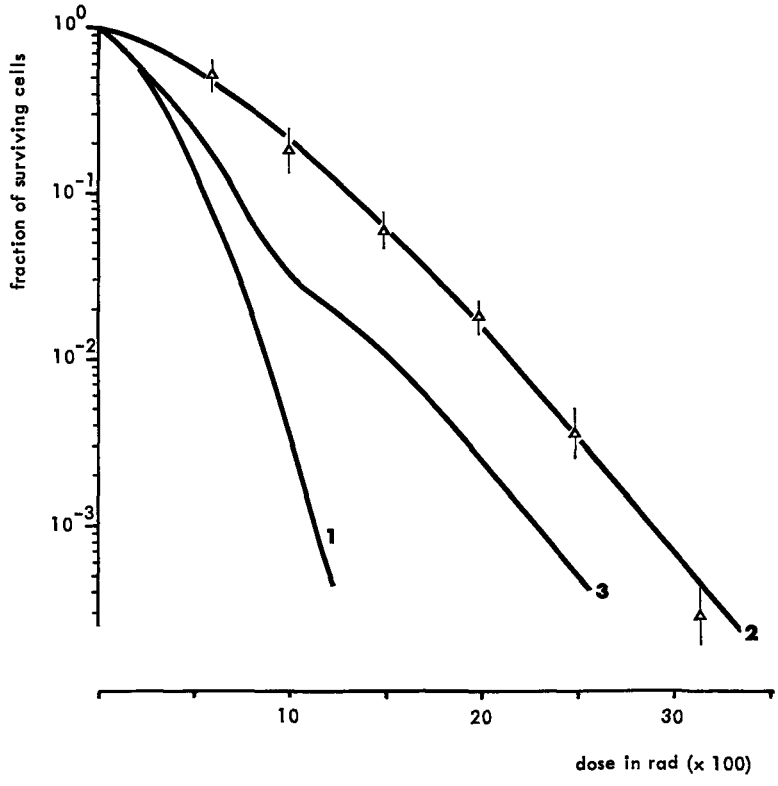


Fig. V-3. Survival curves obtained by irradiation of R-1 cells with 300 kV X-rays. Curves 1 and 2: survival curves for cultured R-1 cells in equilibrium with air and nitrogen, respectively. Curve 3: survival curve for R-1 tumour cells irradiated in the living anaesthetized animal. Triangles: experimental points of tumours irradiated in dead animals. From Barendsen and Broerse [1969].

oxygenated rhabdomyosarcoma cells cultured *in vitro* or growing in the animal respond to irradiation in an identical manner. This was confirmed by the finding that the D_0 value of the initial slope of curve 3 of figure V-3, which was obtained for the tumour in the low dose region and which represents mainly the survival of well-oxygenated tumour cells, is within experimental errors identical with the value of the initial slope of curve 1; i.e., the survival curve of well-oxygenated cultured R-1 cells.

The tumour survival curve 3 of figure V-3 was positioned between the dose-survival curves for cultured R-1 cells irradiated under oxygenated and anoxic conditions and could have been constructed from the data of these two curves with the assumption that the fraction of hypoxic cells in the tumour was 15%. Thus, the survival curve 2 of figure V-2 can be considered as consisting of two components each characterized by different D_0 and equal N values according to the above assumption that the cell population in the tumour consists of a fraction of well-oxygenated and a fraction of severely hypoxic cells. For the low dose region, the D_0 value is 120 rad and an extrapolation number, N, is equal to 10 [Barendsen and Broerse, 1969]. It represents mainly the slope of the survival curve for the well-oxygenated cells. For the region beyond 2000 rad, the D_0 value is 295 rad. This represents mainly the slope of the curve for severely hypoxic cells.

The three survival curves which could be obtained after exposures applied at dose rates of 75, 110, and 150 rad/h show no statistically significant differences due to the scatter of the experimental points. This scatter reflects not only the inaccuracy in the individual determinations but may also be due to true differences in responses between individual tumours. The survival curve obtained for the combined data has a small shoulder. Its extrapolation number is about 1.5 and the D_0 value of the slope is about 520 rad. The ratio of the doses of protracted gamma irradiation and acute X-ray irradiation required to reduce the fraction of surviving cells to 10^{-2} to 10^{-3} is 1.7. If an RBE of 0.85 for gamma rays with respect to X-rays is taken into account, this dose rate effectiveness ratio, DRER, is 1.45.

It cannot be concluded from the shape of this curve whether or not there is a fraction of hypoxic cells present at the end of the irradiation. The evaluation of the oxygenation status of the tumour cells at the end of the continuous treatment at low dose rate will be discussed in section V-3.2.

2.2 *Survival after acute and protracted fast neutron irradiation*

Data from the literature indicated that the relative biological effectiveness, RBE, of neutrons depends on: 1. the neutron energy; 2. the dose or dose fraction; and 3. the dose rate. It has been shown that the therapeutic gain factor, TGF, i.e., the ratio of RBE values of fast neutrons for some experimental tumours and of RBE values of fast neutrons for some normal tissues for doses of clinical interest, is in the order of 1.1–1.3 [Barendsen, 1971]. A TGF in excess of 1 suggests the possibility of an advantage in the use of fast neutrons for treatment of selected tumours in clinical trials.

A TGF of 1.2 means that the effective neutron dose can be increased by 20% without increasing the normal tissue response.

In order to analyze whether treatments with fast neutrons at low dose rates would also provide an advantage, it is important, therefore, to investigate the effects of protracted and acute fast neutron irradiation on both a tumour and a normal tissue in an animal model. This is of interest not only from a fundamental, but also from a practical point of view due to the fact that the nuclide ^{252}Cf has become available and can be used in radiotherapy. ^{252}Cf emits neutrons at low intensities with a neutron energy spectrum comparable to the spectrum of reactor fission neutrons.

The effects of protracted and acute neutron irradiation on a normal tissue, the rat skin, will be discussed in chapter VI. The effect of protracted and acute fast neutron irradiation on the rhabdomyosarcoma will be presented here.

As discussed in paragraph 2.1, the yield from the dispersion technique has been used to arrive at a correction factor for the calculation of surviving fractions after protracted neutron irradiations. The relative cell yield per gram of tumour as a function of exposure time has been plotted in figure V-4. This curve has a slightly different shape as compared to the curve of figure V-1 (dashed line in figure V-4), but,

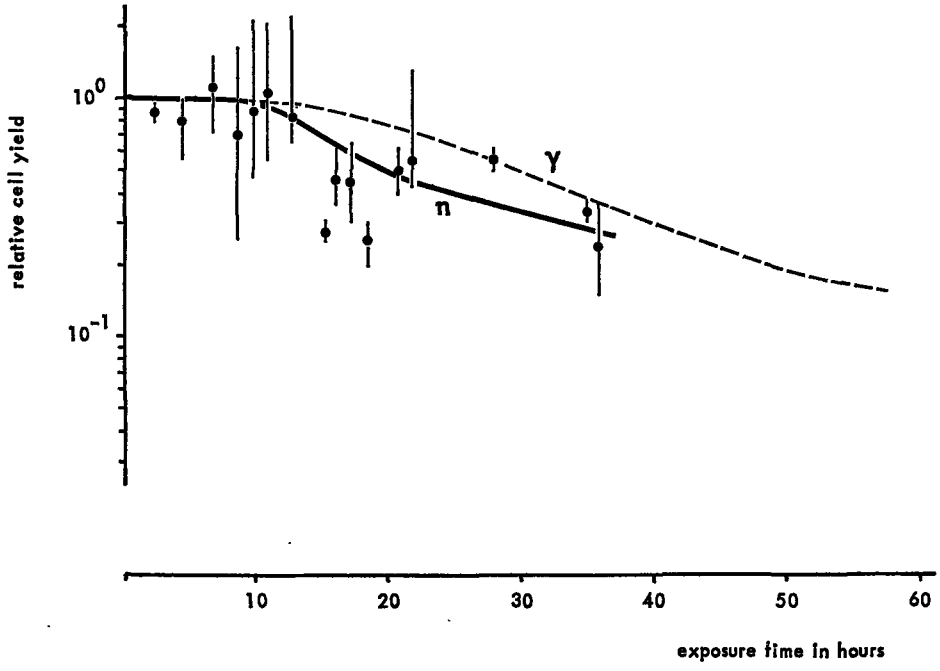


Fig. V-4. Changes in yield from the cell dispersion technique when applied to tumours irradiated with 15 MeV neutrons administered at a dose rate of 48 rad/h as a function of the treatment time relative to the yield at $t = 0$. Bars represent the spread in the measured values for individual tumours assayed separately. The dashed line representing the curve of figure V-1 is included for comparison.

in view of the large variations among the experimental points, it cannot be ascertained whether the differences are significant. The correction derived from this curve is very crude but these relatively large errors do not greatly influence the D_0 and N values of the survival curves (compare discussion in section 4.1).

In figure V-5, the results of determinations of fractions of surviving cells after exposures to 15 MeV fast neutrons administered at an acute dose rate of 1200 rad/h, curve 1, and at a rate of 48 rad/h, curve 2, are presented. If two or more points for one dose or for doses which differ less than 10% were obtained in different experiments, the mean values of these points are presented with bars representing the standard errors of the surviving fractions and the spread in doses. The data of curve 1 were obtained by Barendsen and Broerse [1969]. Curve 1 of figure V-5 can be described by a D_0 value of 110 rad and an extrapolation number N = 1.5; curve 2 can be described

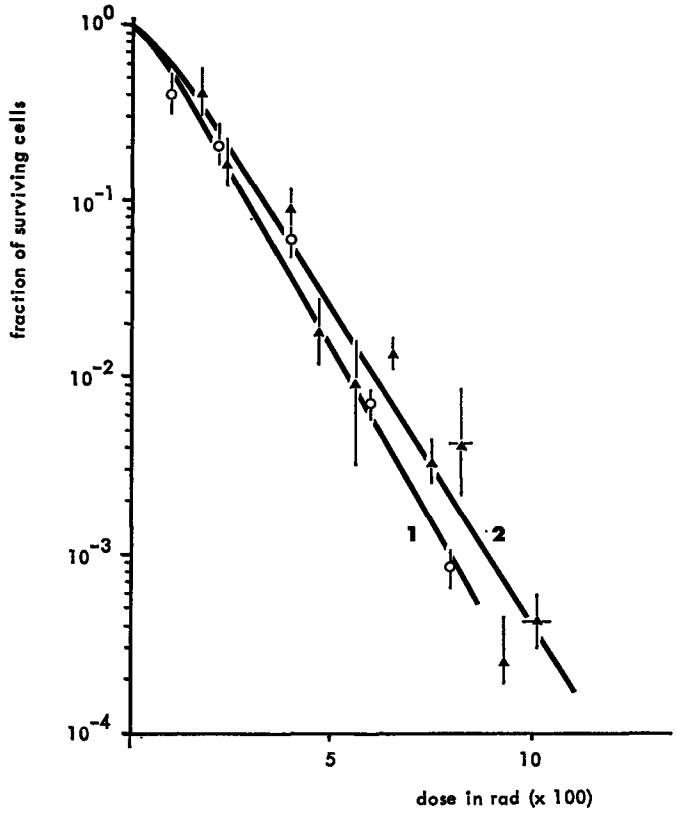


Fig. V-5. Survival curves of tumour cells obtained by irradiation of R-1 tumours with 15 MeV neutrons. Curves 1 and 2: tumours irradiated at dose rates of 1200 rad/h and 48 rad/h, respectively. A symbol represent the mean value of data obtained in different experiments for one dose or for doses which differ less than 10%. Bars represent standard errors of the experimental data and the spread in the doses.

by a D_0 value of 122 (106–138) rad and N = 1.5 (0.7–3.0). Due to the fact that both survival curves have the same extrapolation numbers, the dose rate effectiveness ratio, DRER, for survival levels below 10^{-1} is equal to 1.1. However, because of the scatter of the experimental points, the two survival curves are not significantly different.

2.3 *RBE values*

For the curves presented in figures V-2 and V-5, the effectiveness of 15 MeV neutrons applied at low dose rates relative to the effectiveness of ^{137}Cs gamma rays applied at low dose rates can be estimated. If an RBE factor of about 0.85 for the gamma radiation relative to 300 kV X-rays is taken into account, the RBE values for the 15 MeV neutrons applied at low dose rates can be calculated. Both curves of figures V-2 and V-5 have an extrapolation number not significantly different from 1. Therefore, the RBE can be considered as a constant factor and does not vary with the level of damage considered.

The doses required to obtain the 10% survival level are equal to 1360 rad for the gamma rays and 330 rad for the 15 MeV neutrons. From these values and the RBE of gamma rays relative to 300 kV X-rays, an RBE value of 3.5 ± 0.1 can be calculated for 15 MeV neutrons applied at low dose rates.

For acutely irradiated tumours, however, the RBE is not a constant value but varies with the level of damage considered. The RBE for 15 MeV neutrons for survival levels at 10, 1 and 0.1% are 2.3, 2.7, and 3.0, respectively. These values indicate that the RBE value for protracted neutron irradiation is larger by a factor of about 1.2 to 1.5 as compared with acute exposures.

3 Factors determining responses of tumours to protracted irradiation

3.1 *Introduction*

In chapter IV, it was shown that, during protracted irradiation of rhabdomyosarcoma cells cultured *in vitro*, cells were able to repair sublethal damage and that cells were slowed down in their progression through the cell cycle and spent more time in the G_2+M phase of the cell cycle. It may be expected that these phenomena also occur in the rhabdomyosarcoma growing as a solid tumour in the flank of the rat. Other factors may also play a role in the tumour. Among these, the most important is reoxygenation of severely hypoxic cells during protracted irradiation. Furthermore, little is known about the responses of resting cells to ionizing radiation. In the R-1 rhabdomyosarcoma approximately half of the cells are noncycling cells [Barendsen, 1973]. In the next paragraphs, the factors that determine the responses of the tumour to protracted gamma irradiation will be discussed in more detail.

3.2 *Oxygenation status of tumour cells*

In order to evaluate the oxygenation status of the tumour cells at the end of a pro-

tracted treatment of 20 hours at a dose rate of 100 rad/h of gamma radiation, an additional dose of 800 rad of 300 kV X-rays was given to tumours in living animals and to tumours in animals which were killed 10 minutes prior to this additional irradiation. In the latter case, all tumour cells are known to be severely hypoxic. This was verified by the fact that the dose-effect curves under this condition *in vivo* and under controlled hypoxia *in vitro* are similar (see figure V-3).

A first dose of 2000 rad was chosen because, if reoxygenation did not occur during protracted irradiation, the dose of 2000 rad would have reduced the fraction of severely hypoxic cells to about 0.075 and the fraction of well-oxygenated tumour cells to about 0.00085. These values are derived from idealized survival curves for well-oxygenated and anoxic R-1 cells (figure V-6). Curve 1 of figure V-6 for well-oxygenated cells calculated for a dose rate of 100 rad/h, is an approximation of survival curves obtained at a dose rate of 75 rad/h as shown in chapter IV and those obtained at a

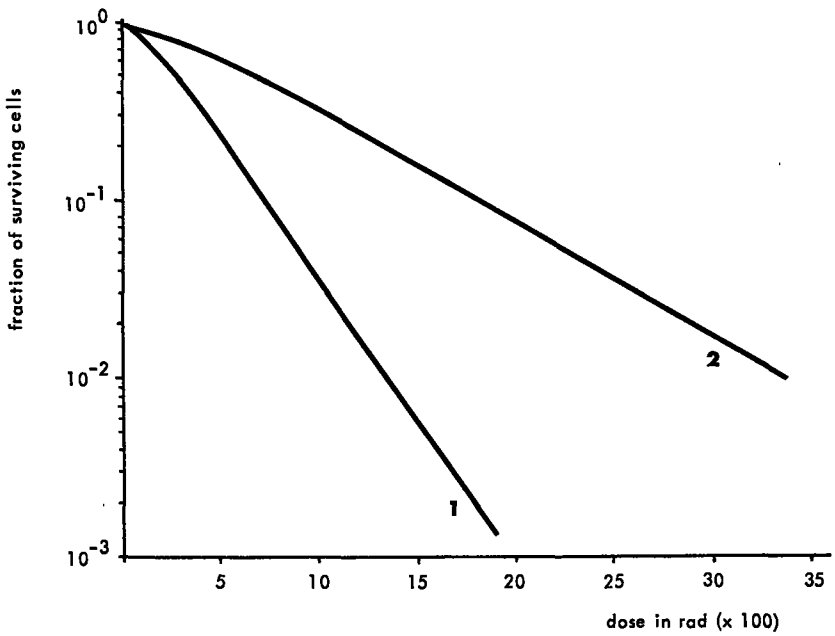


Fig. V-6. Idealized survival curves for well-oxygenated and anoxic R-1 cells. Curve 1: survival curve for cultured R-1 cells obtained with ^{137}Cs gamma rays at a dose rate of 100 rad/h. This curve is obtained as an approximation from the survival curves obtained at a dose rate of 75 rad/h (figure IV-1) and those obtained at a dose rate of 150 rad/h (unpublished results). Curve 2: calculated survival curve for R-1 cells under anoxic conditions during a continuous gamma irradiation as derived from curve 1 by applying an OER value of 2.5. A dose of 2000 rad reduces the well-oxygenated cells to a fraction of 0.0009 and the anoxic cells to a fraction of 0.075. In the tumour the ratio of well-oxygenated and severely hypoxic cells after 2000 rad of gamma rays, assuming no reoxygenation, is given by $(0.85 \times 0.0009)/(0.15 \times 0.075) = 0.068$ in which the fractions of well-oxygenated and severely hypoxic cells in unirradiated tumours are represented by the values 0.85 and 0.15, respectively.

dose rate of 150 rad/h (unpublished results). Curve 2 for anoxic R-1 cells at 100 rad/h is constructed from curve 1 of figure V-6 by applying an OER value of 2.5. As discussed in chapter II, in general, the OER did not vary significantly when the dose rate was changed from high to low values. The dose of 2000 rad would lead to a ratio between well-oxygenated and anoxic cells in the tumour of less than 0.07 when the

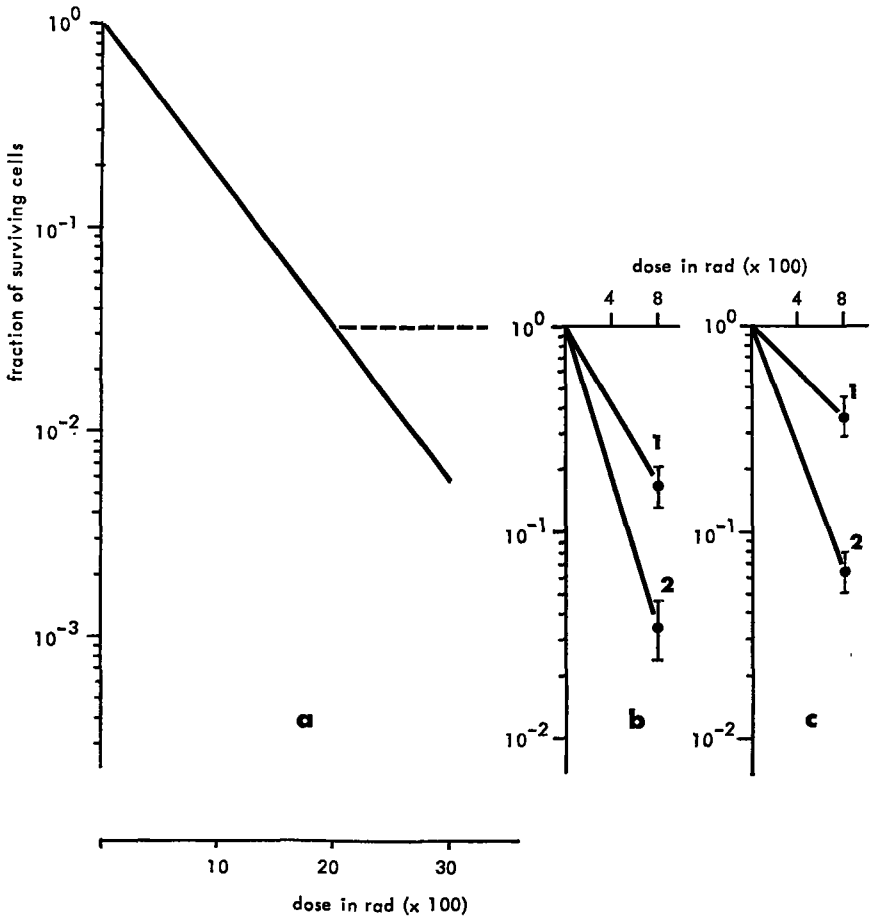


Fig. V-7. Fractions of surviving cells of irradiated R-1 tumours as a function of dose.

panel a: ^{137}Cs gamma rays applied at dose rates of 75, 110 and 150 rad/h. The line presents a least squares fit of the experimental points of figure V-2, curve 1, with the number of scored clones as a weighting factor.

panel b: 2000 rad of gamma rays applied at a dose rate of 100 rad/h followed by a single acute dose of 800 rad of 300 kV X-rays to tumours in the dead and living animal (points 1 and 2, respectively).

panel c: 800 rad of 300 kV X-rays, single acute dose only. Points 1 and 2 refer to tumours irradiated in the dead and living animal, respectively. Vertical bars represent the standard errors.

fractions of 0.85 and 0.15 for well-oxygenated and anoxic cells are taken into account. If reoxygenation occurs during protracted irradiation, this ratio would be larger. A larger initial dose would have been preferable to make the ratio of surviving oxygenated cells and surviving severely hypoxic cells as small as possible. It must be taken into account, however, that this initial dose must be followed by a second dose of 800 rad. The final fraction of surviving cells should not be less than 10^{-4} to 10^{-5}, since these are the smallest fractions detectable with the *in vitro* clone technique. On the other hand, the second dose must be as large as possible in order to obtain the maximum difference between the fractions of surviving cells from the oxygenated and the severely hypoxic compartment. If reoxygenation did not take place during irradiation, the fractions of surviving cells in the tumour irradiated with an additional acute dose of 800 rad of X-rays in the living and in the dead animal should be practically equal; namely, 0.29% and 0.31%, respectively.

Results which were obtained with this treatment are shown in figure V-7. The curve of panel a represents the low dose rate survival curve of figure V-2. The pretreatment with low dose rate gamma radiation to a total dose of 2000 rad reduces the fraction of clonogenic cells to 3.1%. The additional dose of 800 rad of X-rays reduces the fraction of clonogenic cells in tumours in living animals to 3.7% of the fraction present at the end of the protracted irradiation (point 2 in panel b of figure V-7); the fraction of surviving cells of the tumour irradiated in dead animals is reduced to 17% (point 1 in panel b of figure V-7). From the ratio of these two surviving fractions, 0.22, it can be concluded that reoxygenation must have taken place during the protracted irradiation.

Thus, for tumours exposed to gamma rays at 100 rad/h for 20 hours, the ratio of fractions of surviving cells in living and dead animals is 0.22 after a dose of 800 rad of X-rays; for tumours receiving a dose of 800 rad of X-rays without pretreatment as deduced from panel c of figure V-7, this fraction is 0.20. These values are not significantly different from each other. Therefore, it can be concluded that the oxygenation status of cells in the tumour has not changed during the low dose rate treatment of 20 hours and that reoxygenation must be a fairly rapid process.

3.2.1 *Rate of reoxygenation*

From irradiations of the rhabdomyosarcoma with 300 kV X-rays at daily doses of 300 rad, it had been concluded that reoxygenation must take place during the daily intervals; i.e., it is completed within 24 hours [Barendsen and Broerse, 1970]. From the experiment just described, it is clear that the rhabdomyosarcoma is a relatively rapidly reoxygenating tumour; i.e., reoxygenation is complete in even shorter time intervals than the 24 hours which can be derived from daily treatments. A rough estimate of the rate of oxygenation can be made as follows: if one assumes that no reoxygenation took place during protracted irradiation, then, at a dose of 1000 rad administered in 10 hours, about 96% of the fraction of well-oxygenated cells and about 70% of the fraction of anoxic cells are killed. Thus, about 60% of the survivors would

be severely hypoxic cells (figure V-6). As discussed in paragraph 3.2, it is clear that oxygenation took place; hence about 75% of these severely hypoxic cells were reoxygenated in 10 hours. If one assumes an exponential course, this means that 50% of the severely hypoxic cells are reoxygenated in less than 5 hours. Therefore, in the rhabdomyosarcoma transition from hypoxic to oxygenated conditions of half of the fraction of hypoxic cells takes place in less than 5 hours.

3.3 *Repair of sublethal damage*

As shown in chapter IV, cells can repair sublethal damage during protracted irradiation and it is fast enough to prevent accumulation of sublethal damage during irradiation at a dose rate of 75 rad/h. This was demonstrated by the observation that little or no repair of sublethal damage was observed in an interval of up to 6 hours if the protracted irradiation was followed by an additional single acute dose at subsequent time intervals. These findings were confirmed for the tumour in an experiment where, after a dose of 2000 rad of gamma rays administered in 20 hours, an additional dose of 800 rad of X-rays was given immediately afterwards and at 2 and 4 hours following the protracted irradiation. The surviving fractions measured in these experiments are shown in figure V-8 where point 1 represents the fraction of surviving cells after the dose of 2000 rad of gamma rays at a dose rate of 100 rad/h and points 2, 3 and 4 represent the fractions of surviving cells after an additional dose of 800 rad of X-rays at 0, 2, and 4 hours following the protracted irradiation. From these results, it is clear that these fractions are not significantly different from each other and, hence, no repair of sublethal damage could be demonstrated in the interval of up to 4 hours.

It can be concluded that repair of sublethal damage is sufficiently rapid to avoid accumulation of this type of damage during protracted irradiation applied at a dose rate of 100 rad/h. This finding agrees with the results obtained with R-1 cells cultured *in vitro* as discussed in chapter IV-4.2.

3.4 *Progression in the cell cycle*

It is well-known that an acute dose given to mammalian cells will cause a mitotic delay which may depend on the cell cycle time. Puck and Yamada [1961] have found that the mitotic delay is about 1 hour per 100 rad for HeLa cells. From the experiments described in chapter IV with cultured R-1 cells, it was also observed that during continuous irradiation at a dose rate of 75 rad/h cells were delayed in their progression and were partly accumulated in the G_2+M phase of the cell cycle. A dose of 800 rad of X-rays following the initial dose of 2000 rad of ^{137}Cs gamma rays administered in 20 hours reduces the fraction of clonogenic cells in tumours in living animals to 3.7% and the fraction of surviving cells in the tumour irradiated in the dead animal to 17%. A single dose of 800 rad of X-rays, however, given to tumours in living and dead animals without pretreatment with protracted irradiation results in

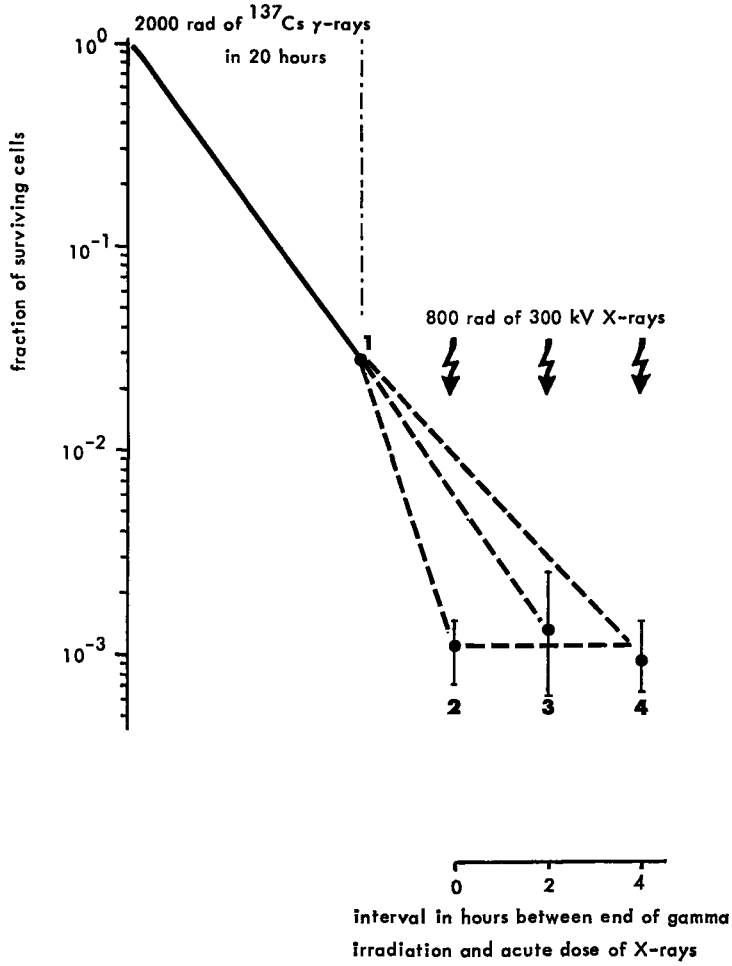


Fig. V-8. Fractions of surviving cells of irradiated R-1 tumours as a function of dose and time. Fractions of clonogenic cells are obtained after administration of 2000 rad of ^{137}Cs gamma rays at 100 rad/h, followed at different time intervals by acute doses of 800 rad of 300 kV X-rays. Point 1: surviving fraction after 2000 rad of low dose rate gamma radiation only. Points 2, 3 and 4: surviving fractions for intervals of 0, 2 and 4 hours, respectively.

fractions of clonogenic cells of 7 and 35%, respectively (panel c of figure V-7). This may be explained by assuming that cells at the end of the first dose when accumulated in a certain phase of the cell cycle are more radiosensitive than are cells in a non-synchronized tumour. This will be discussed in more detail in the next paragraph.

3.4.1 *Cell volume and DNA content distributions*

Cell volume distributions of tumour cell suspensions were obtained with the Coulter Counter interphased with a multichannel analyzer. In figure V-9, cell volume distribu-

tions of rhabdomyosarcoma cells from a control tumour and from tumours irradiated with gamma rays for 39 and 64 hours, respectively, at a dose rate of 110 rad/h are shown. The numbers of cells counted per channel are plotted on an arbitrary scale as a function of the channel number which is proportional to the relative cell volume. Significant differences are clearly observed, indicating that more large cells appear as the irradiation time increases.

The differences become even more evident when the DNA content per cell is considered. In figure V-10, histograms of DNA content per cell for an unirradiated tumour and for tumour cells irradiated at 100 rad/h for 20 hours are shown. The first peak represents the DNA content per cell of the normal diploid cells, such as cells of the connective tissues in the tumour which are not removed by the trypsinization technique [see also Raju et al., 1974]. The second peak represents tumour cells in the G_1 phase; the last peak, cells in $G_2 + M$ phase. Cells in S phase are represented between

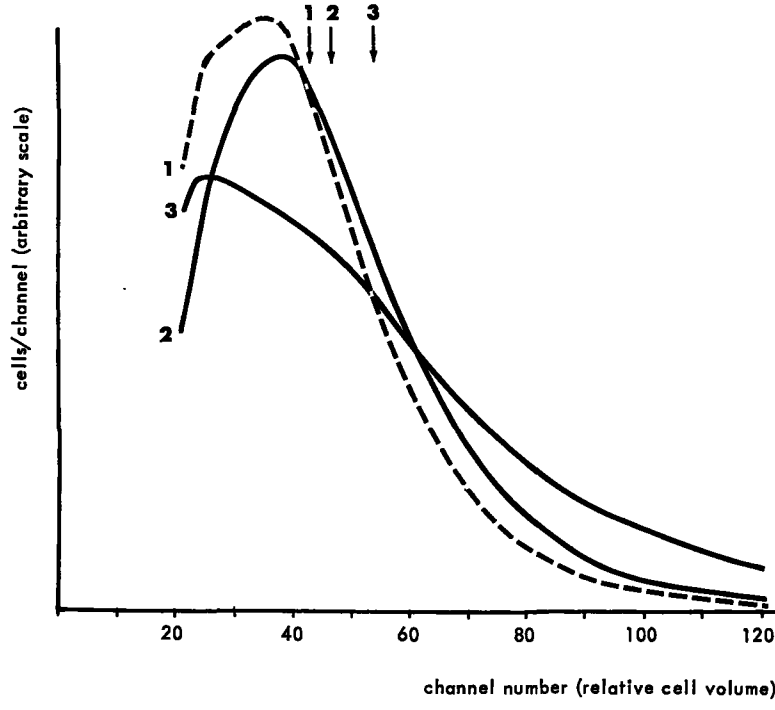


Fig. V-9. Cell volume distributions of R-1 tumour cells. Numbers of cells counted per channel are plotted on an arbitrary scale as a function of the channel number, which is proportional to the relative cell volume.

Curve 1: control tumour.
Curve 2: tumour irradiated 39 hours with a dose rate of 110 rad/h.
Curve 3: tumour irradiated 64 hours with a dose rate of 110 rad/h.
Arrows represent the mean cell volumes of the distributions.

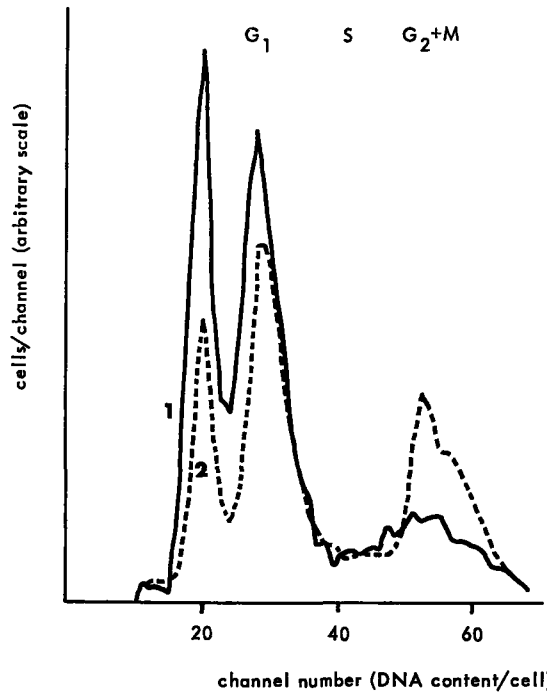


Fig. V-10. DNA content distributions of R-1 tumour cells. Numbers of cells counted per channel are plotted on an arbitrary scale as a function of the channel number, which is proportional to the relative DNA content per cell.

Curve 1: control tumour.
Curve 2: tumour irradiated for 20 hours at a dose rate of 100 rad/h.

the second and third peak. The percentages of cells in the G_1, S, and G_2+M phases are 60, 25, and 15, respectively. These percentages are 41, 10, and 49 respectively, in the tumour after protracted irradiation. These values indicate that, at the end of the protracted irradiation lasting for 20 hours, more cells are found in the G_2+M phase of the cell cycle.

The finding that more cells are present in the G_2+M phase which is known to be very radiosensitive, might well explain the increased radiosensitivity of protracted irradiated cells to a subsequent acute X-ray dose of 800 rad.

4 Discussion

4.1 *Correction for cell yield*

The cell dispersion method which was applied to obtain a suspension of cells from the solid rhabdomyosarcoma gives a yield of 5 to 10%. This implies that a possibility for selection exists; e.g., the possibility cannot be excluded that all cells in S phase or in

another cell cycle phase might be especially sensitive to the trypsinization procedure. Similarly, it could be hypothesized that all nonproliferating or quiescent cells (Q cells) are killed by the procedure and that, for the proliferating cells, the yield of the cell dispersion method is 10 to 20%. However, from the data of Barendsen et al. [1973] it can be concluded that a significant selection of either P cells in a particular phase of the cycle or of the Q cells due to the trypsinization technique is not observed. These conclusions are based on experiments with WAG/Rij rats bearing a rhabdomyosarcoma, in which repeated injections of ^{3}H-TdR at 4-hour intervals were used to label all P cells in order to distinguish these cells from Q cells. After the last injection, the tumours were excised and cell suspensions were prepared. From these suspensions, cells were cultured for different time intervals up to 120 hours. Counting of labelled cells and of clones in cultures of different ages by autoradiography revealed that Q cells have an approximately equal capability as P cells for producing clones of at least 16 cells within 4 days after plating. The labelling index of cells in 12-hour cultures was equal to that of cells in the tumours, indicating that the cell dispersion technique did not preferentially select P or Q cells.

As indicated in paragraph 3.4, most of the cycling cells are accumulated in the $G_2 + M$ phase of the cell cycle. As a result, the mean cell volume will increase; i.e., the mean cell density of the tumour will decrease. It will be clear that this variation in cell density must be reflected in the yield from the cell dispersion technique when applied to assess the number of cells per gram of tumour whereby only those cells are counted which appear to have undamaged cell walls when examined with a microscope at a magnification of 400 times.

It is well-established, however, that true changes in mean cell volume are not the only cause of the decrease in cell yield shown in figures V-1 and V-4 [Barendsen and Broerse, 1969; 1970]. During protracted exposures, cells will be eliminated from the tumour due to normal cell loss which occurs even in unirradiated tumours [Hermens and Barendsen, 1969; Hermens, 1973] and probably also due to increased cell loss as a result of the irradiation. Furthermore, some of the cells present at the end of a protracted exposure may be damaged to such an extent that they cannot remain intact during the trypsinization treatment applied in the dispersion procedure. This latter factor in particular introduces an uncertainty into the final result of the assay for the fraction of clonogenic cells, because some of the cells eliminated by the trypsinization treatment might have retained the capacity for proliferation if they had been left undisturbed in the irradiated tumour. Nevertheless, the yield from the cell dispersion technique shown in figures V-1 and V-4 has been used as an approximate measure of the correction to be applied for the complex factors mentioned. It can be deduced from figure V-1, e.g., that the correction factor applied is relatively small as compared to the factors by which the numbers of clonogenic cells are reduced due to irradiation, and, consequently, the influence of its uncertainty should not be of major importance. For instance, after 20 hours irradiation at a dose rate of 100 rad/h, the correction factor is 0.7, whereas the number of clonogenic cells is reduced by

a factor of about 40, i.e., the fraction of surviving cells is 2.2%, while without any correction at all it would have been 3.15%. Without the correction, the D_0 value of the exponential survival curve would have been 620 rad in comparison to the value of 580 rad derived with the correction.

4.2 *Survival of R-1 tumour cells*

For many mammalian cell survival curves, it has been shown that the high dose regions, below 10^{-1} survival level, can be described by a formula of the form $S = 1-\{1-\exp(-D/D_0)\}^N$, where D is the dose in rad; N, the extrapolation number; and D_0, the dose in rad required to reduce the fraction of surviving cells in this region by a factor $e^{-1} = 0.37$.

Curve 1 of figure V-2, fitted by eye, can be described by an extrapolation number, N, equal to 1.5 and a D_0 value of 520 rad.

The N and D_0 values for curve 1 of figure V-2 can also be calculated from the combined experimental data using a computer program that fits a straight line through the experimental points with the square root of the number of scored clones as a weighting factor. A D_0 of 580 (510–650) rad and an N value of 0.9 (0.5–1.7) can be found as parameters of the curve. These computer-fitted parameters are not significantly different from the parameters of the eye-fitted curve. The presence of an extrapolation number in excess of 1, i.e., a curve having a shoulder, is more likely, since, even for the survival curve obtained after fast neutron irradiation at a low dose rate, an extrapolation number in excess of 1 has been found. The survival curves obtained for protracted gamma irradiations of cultured R-1 cells showed also extrapolation numbers in excess of 1. The presence of a small shoulder in a survival curve obtained at a low dose rate can be explained as follows. During the hours following the first 8–10 hours of the treatment proliferating cells accumulate into G_2+M phase. In these phases cells are known to be more radiosensitive than cells in other phases. Due to this sensitizing effect the efficiency of cell killing per unit dose is increased and, as a consequence the slope of the survival curve will become steeper.

Curve 2 cannot be described by a single N and D_0 value, as discussed in paragraph 2.1. It has been shown that curve 2 can be described by N and D_0 values of survival curves of well-oxygenated R-1 cells and anoxic R-1 cells, respectively, taking into account the presence of a fraction of 15% anoxic cells in the tumour. These factors must be taken into account in the interpretation of the shape of the survival curve obtained at low dose rates.

As discussed earlier the initial slope of a survival curve which is obtained with acute irradiation might be ascribed to lethal damage produced by single events resulting from the fraction of the total energy deposited at relatively high local energy densities; i.e., from slow electrons with a relatively high LET produced by all types of electromagnetic radiation in irradiated material. With acute exposure, the determination of the survival curve parameters for the low dose region cannot be very accurate,

however, because of the experimental errors involved. Consequently, a detailed numerical analysis presents difficulties.

As discussed in paragraph IV-7, it may be assumed that, during protracted irradiation, sublethal damage can be repaired by the cells. As a consequence, the damage observed after a low dose rate treatment is determined by the energy deposited at high local energy densities; i.e., by electrons of low energy. Hence, assuming curve 1 of figure V-2 to be exponential ($N = 1$) with a D_0 of 580 rad, the slope is, to a first approximation, determined by the initial slope of the survival curve obtained at a high dose rate (curve 2 of figure V-2); i.e., the initial slope of the survival curve of well-oxygenated cells. Such initial slopes corresponding to D_0 values of 450 ± 100 and about 500 rad for acute X-ray exposure have been derived for T-1 cells [Barendsen, 1962] and HeLa cells [Hall et al., 1966].

The slope corresponding to a D_0 value of 580 rad for protracted gamma irradiation (or if an RBE factor of 0.85 for gamma rays from ^{137}Cs relative to 250 kVp X-rays is applied, 490 rad) is in the same range. But, although these D_0 values for protracted and acute irradiation are not statistically different, they are obtained with different cell populations and, hence, they cannot be directly compared with each other. This is reflected by the fact that the slope of the survival curve obtained after protracted irradiation of R-1 cells *in vitro* at 40 rad/h has a D_0 value of 422 rad when corrected for cell multiplicity. This value is considerably lower than the D_0 value of 580 rad found for the rhabdomyosarcoma. The tumour cell population exposed to protracted irradiation *in situ* differs from a cell population irradiated *in vitro* at high dose rate in several ways: 1. due to the protracted irradiation, cells are accumulated in certain phases of the cell cycle and, hence, their radiosensitivity will be altered as compared to the radiosensitivity of cells in a nonsynchronized cell population; 2. a fraction of severely hypoxic cells is still present in the tumour at the end of the exposure as discussed in paragraph 3.2, and, hence, the observed D_0 value will be influenced by these cells; 3. a fraction of about 50% of noncycling cells are present in the tumour. Because no data are available at present concerning the radiosensitivity and the capacity of these cells to repair sublethal damage and because the time sequence of the recruitment of Q cells into the compartment of P cells is still an unsolved problem, the influence of this fraction of cells on the final result is unknown. Therefore, a direct comparison between D_0 values of the initial slope of the survival curves *in vitro* and *in vivo* obtained at low dose rate can be made only if the relative contributions of these factors are known.

Survival curves have been published for two other experimental tumours irradiated at high and low dose rates. These are the transplantable mouse NCTC fibrosarcoma [Frindel et al., 1972] and the transplantable mouse KHT sarcoma [Hill and Bush, 1973]. The difference in the effectiveness per unit dose of continuous irradiation at 50 rad/h and acute irradiation on the NCTC solid tumour as determined by an *in vitro* assay technique is small. For the KHT sarcoma, for which a lung colony assay method was developed, the responses to doses between 1000 and 4000 rad was in-

dependent of dose rate over the range from 41.5 to 800 rad/h and was little changed as compared to acute irradiation. The conclusions drawn from the results on the KHT and NCTC tumours were 1. that the sensitizing effect of reoxygenation during continuous irradiation is balanced by the effect of repair of sublethal damage; and 2. progression and redistribution of cells in the phases of the cell cycle may partly explain the relatively high lethal effect of the continuous irradiation.

These conclusions concerning repair of sublethal damage, reoxygenation, and redistribution of cells in certain phases of the cell cycle agree well with the conclusions drawn from results obtained with the rhabdomyosarcoma. It must be emphasized, however, that, notwithstanding the extensive data obtained, a number of factors remain to be investigated, in particular about Q cells, before a complete analysis can be given and differences among different types of tumours can be evaluated.

4.3 *Reoxygenation*

From the experiment described in paragraph 3.2 in which the fraction of hypoxic cells in the tumour was determined at the end of the protracted irradiation, it can be concluded that the oxygenation status of cells in the tumour did not change during the low dose rate treatment. From irradiations of rhabdomyosarcomas with 300 kV X-rays at daily doses of 300 rad, it was also concluded that reoxygenation must take place during the daily intervals [Barendsen and Broerse, 1970]. It is now clear that this rhabdomyosarcoma is a relatively rapidly reoxygenating tumour. A similar conclusion was drawn from experiments with a mouse KHT sarcoma. Even after 3000 rad low dose rate irradiation, this tumour still contained surviving well-oxygenated cells [Hill and Bush, 1973]. A rapid reoxygenation was also observed after acute irradiation of the KHT sarcoma and the KHJJ mammary carcinoma [Kallman, 1972] which contained about 16 and 20% hypoxic cells, respectively. The hypoxic fraction had returned to a value very close to the pre-irradiation level in both tumours as early as one hour after a dose of 1000 rad. This was followed by fluctuations and a possible rise at some time after 12 hours (figure V-11, panel a). Thomlinson [1969] has plotted the reoxygenation curves for three other tumours showing a wide range of response; namely, a rat fibrosarcoma (RIB_5), hypoxic fraction 17%; a mouse mammary carcinoma, hypoxic fraction 12%; and a mouse osteosarcoma, hypoxic fraction 14%. These tumours had been treated with a single dose of 1000 rad or 1500 rad (figure V-11, panel b). In fibrosarcoma RIB_5, at least during the first post-irradiation day, reoxygenation occurred in a similar fashion as in the two mouse tumours, KHT and KHJJ. Later, however, the hypoxic fraction increased and, after approximately two days, it reached a peak which Thomlinson attributed to deterioration in the tumour blood circulation. In the case of the mammary carcinoma studied by Howes [1969], the hypoxic fraction declined somewhat slower after irradiation but fell below the pre-irradiation level. The curve for the hypoxic fraction as a function of postirradiation time for this type of tumour showed a combined decrease reaching a minimum of

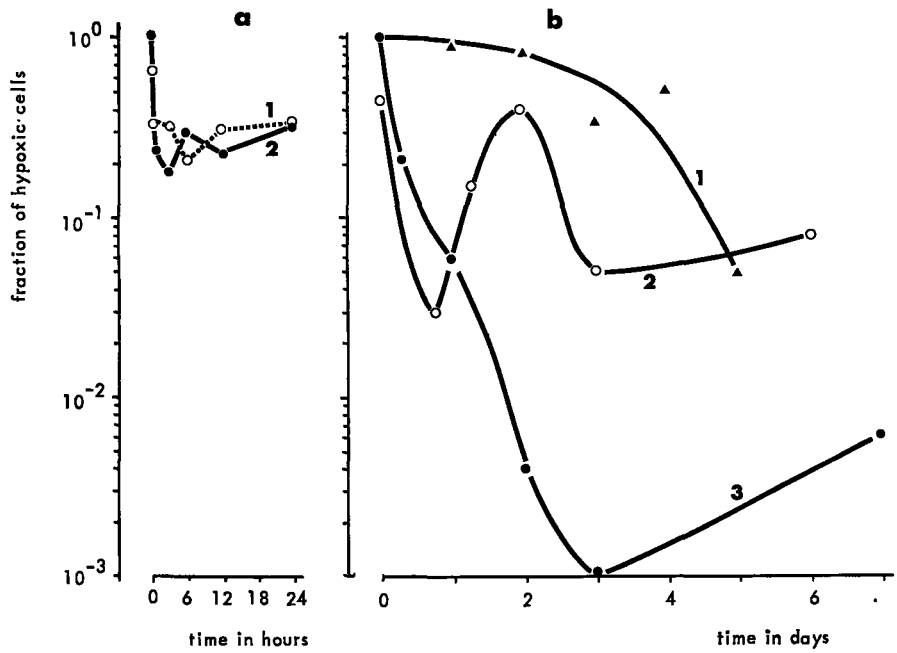


Fig. V-11. The fraction of hypoxic cells in experimental tumours as a function of time after irradiation with a single acute dose. Panel a: the fraction of hypoxic tumour cells during the first day after irradiation for the mouse KHJJ, curve 1, and the mouse KHT tumour, curve 2, after a dose of 1000 rad [Kallman, 1972]. Panel b: the fraction of hypoxic cells as a function of time for the mouse osteosarcoma, curve 1 [Van Putten, 1968], the rat fibrosarcoma RIB5, curve 2 [Thomlinson, 1969], and the mouse mammary carcinoma, curve 3 [Howes, 1969] after doses of 1000, 1500 and 1000 rad, respectively. See also Thomlinson [1969].

about 1/1000 the normal pre-irradiated value at 4–5 days. In contrast to these tumours, a mouse osteosarcoma, for which a reoxygenation curve was determined by Van Putten [1968], is an exceedingly slow reoxygenating tumour. The tumour still appeared to consist primarily of hypoxic surviving cells as late as 4 days after irradiation with a single dose of 1000 rad of X-rays.

These reoxygenation curves were obtained by different methods. The criteria for radiobiological effect being the rate of tumour regrowth *in situ* [Thomlinson, 1969], changes in TCD_{50} (50% curative dose) [Howes, 1969], and the TD_{50} end-point dilution method [Kallman, 1972; van Putten, 1968]. It is highly unlikely that the various patterns of reoxygenation observed are related to the different methods employed. Therefore, one may generalize that the course of reoxygenation following a single test dose may differ greatly from tumour to tumour irrespective of the pre-irradiation proportion of hypoxic cells.

4.4 *Changes in cell volume and DNA content distributions*

As discussed in paragraph III-5.1, cell volume distributions can be described by several parameters; i.e., mean or modal cell volume, skewness, and kurtosis. The differences observed in these parameters are evident for cell volume distributions obtained with cells from a monocellular suspension derived from cell cultures after certain treatments. For cell suspensions obtained from tumours, however, where cells of the connective tissues and leukocytes are usually present, differences observed in the distribution parameters are more difficult to interpret as compared to the differences observed for single cell suspensions from cultures. This is indicated in figure V-12 where cell volume distributions of R-1 cells cultured *in vitro* and R-1 cells of the rhabdomyosarcoma are shown. In panel a distributions of unirradiated cultured R-1 cells, curve 1, and irradiated R-1 cells 16 hours after a dose of 2000 rad of ^{137}Cs gamma rays, curve 2, are shown. The mean values are indicated by arrows. In panel b, two cell volume distributions are shown, one for cells from an unirradiated tumour, curve 3, and one for tumour cells 15 hours after a dose of 2000 rad of X-rays, curve 4. A small difference in mean channel numbers is observed; the ratio of the two values of the mean volumes is only 1.04. This small ratio is a reflection of the presence of: 1. normal cells which could not be completely removed by the trypsinization technique and which are little or not at all affected by the irradiation treatment, since they are nonproliferating cells; 2. noncycling tumour cells, which

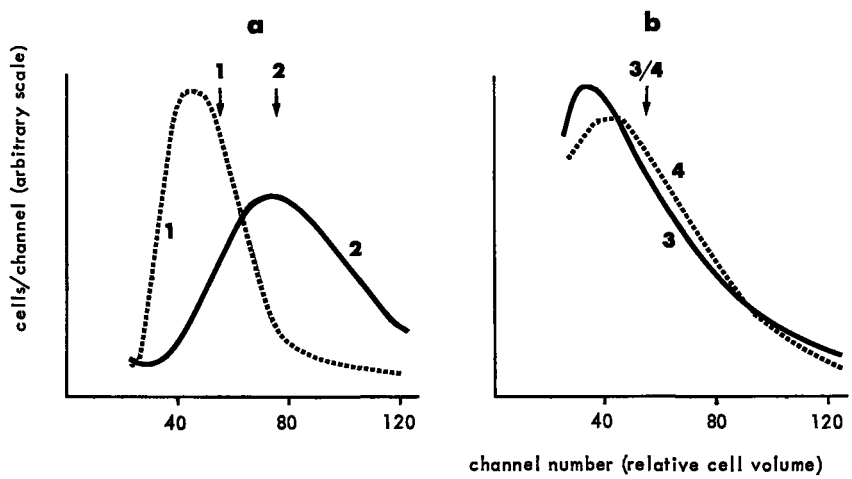


Fig. V-12. Cell volume distributions of rhabdomyosarcoma cells. Numbers of cells counted per channel are plotted on an arbitrary scale as a function of the channel number, which is proportional to the relative cell volume. At least 50,000 cells are counted for each curve. Arrows denote the mean cell volumes. Panel a: R-1 cells *in vitro*; curve 1: control cells; curve 2: R-1 cells 16 hours after a dose of 2000 rad of ^{137}Cs gamma rays. Panel b: cells from R-1 tumours; curve 3: cells from a control tumour; curve 4: tumour cells 15 hours after a dose of 2000 rad of 300 kV X-rays.

represent about 50% of the total number of tumour cells in this rhabdomyosarcoma; and 3. severely hypoxic cells which are assumed to proliferate at a reduced rate as compared to well-oxygenated cells after being reoxygenated [Koch et al., 1973].

Because of this small discriminative capacity of cell volume measurements of cells obtained from a tumour, ultrarapid cytophotometers which enable measurements of the DNA or RNA content per cell in large cell populations may be expected to give more information. Measurements of changes in DNA content with the ICP11 after irradiation of cells and tumours have now provided evidence concerning the phenomena causing the relatively small shifts in cell volume distributions [Kal, 1973a]. In figure V-13, panel a, curve 1 represents the DNA content distribution of unirradiated R-1 cells in culture, and curve 2 represents the DNA content distribution of R-1 cells 16 hours after irradiation with an acute single dose of 2000 rad of ^{137}Cs gamma rays. From a comparison of these curves, it can be concluded that, due to mitotic delay, the majority of the irradiated R-1 cells are accumulated into the $G_2 + M$ phase of the cell cycle. In figure V-13, panel b, curve 3 represents the DNA histogram of unirradiated cells growing in the tumour *in situ* and curve 4 represents the DNA histogram of cells 16.5 hours after irradiation of the tumour *in situ* with an acute dose of 2000 rad of gamma rays. The first peak of the curves of figure V-13, panel b, represents the

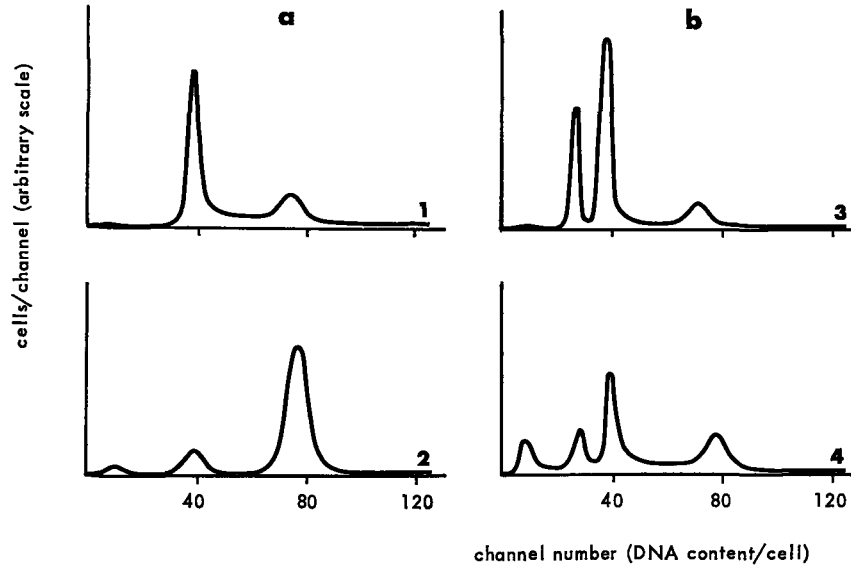


Fig. V-13. Distributions of DNA content of rhabdomyosarcoma cells. Number of cells counted per channel are plotted on an arbitrary scale as a function of the channel number, which is proportional to the DNA content/cell. For each curve 50,000 cells are counted. Panel a: R-1 cells *in vitro*; curve 1: control cells; curve 2: R-1 cells 16 hours after a dose of 2000 rad of ^{137}Cs gamma rays. Panel b: cells from R-1 tumours; curve 3: cells from a control tumour; curve 4: tumour cells 15 hours after a dose of 2000 rad of 300 kV X-rays.

DNA content of the normal diploid cells in the tumour as discussed in paragraph 3.4. It is clear from these two curves that, although more cells are found in the G_2+M phase in the irradiated tumour as compared with the control tumour, a considerable fraction of cells is present in G_1 and S phases. This is in contrast to the findings obtained with cultured R-1 cells as demonstrated in figure V-13, panel a. The finding that a considerable fraction of cells with a DNA content of G_1 and S cells is still present in tumours 16 hours after irradiation must be ascribed to the presence of noncycling cells in a resting phase of the cell cycle. From these DNA distributions it can be concluded that: 1. the normal cell population can be distinguished from the tumour cell population by their DNA content; 2. comparison of DNA histograms of cells cultured *in vitro* and *in vivo* indicates the presence of a fraction of nonproliferating cells in the tumour; and 3. proliferating R-1 tumour cells *in situ* are affected in a similar manner as are proliferating R-1 cells *in vitro*. Finally, recruitment of Q cells could not be demonstrated in the interval of up to 16 hours after irradiation [Kal, 1973b]. These findings clearly indicate that comparison of DNA histograms provides a useful tool for investigating the influence of various types of agents on the kinetics of cells in tumours and in culture.

4.5 *RBE values*

The original rationale for the clinical use of fast neutrons was that the oxygen enhancement ratio, OER, for fast neutrons is less than that for X-rays or gamma rays. Improved insight into various factors has led to the conclusion that the general criterion should be that the RBE for damage to the tumour must be larger than the RBE for damage to the dose-limiting normal tissue.

The influence of the dose rate on the RBE for 15 MeV neutrons for damage to the rhabdomyosarcoma will be discussed here and the influence of the dose rate on the RBE for damage to the skin in chapter VI. The following factors must be considered in order to understand why the RBE value of 3.5 ± 0.1 for protracted fast neutron irradiation of R-1 rhabdomyosarcomas, as derived from curve 1 of figure V-2 and curve 2 of figure V-5, is a factor of 1.2 to 1.5 larger than for acute fast neutron irradiation: 1. In general, fast neutrons are more efficient in damaging proliferating cells with respect to cell proliferative capacity by a factor of 2.5 to 3 as compared to X-rays or gamma radiation; 2. Because of the relatively low OER, hypoxic cells in the tumour are damaged more efficiently by fast neutron irradiation than by X-irradiation when compared with the same degree of damage to well-oxygenated cells; 3. Although the radiosensitivity of cells to fast neutron irradiation as a function of cell age was observed to follow similar trends as with X-rays, the difference between the most sensitive and most resistant phases is smaller for fast neutrons; i.e., sensitivity is less dependent on the phase of the cell cycle; 4. The fraction of the total dose deposited by fast neutron irradiation at relatively low LET values, causing sublethal damage, is much smaller than for X-irradiation. As a consequence, repair of sublethal damage is not an important phenomenon with fast neutron irradiation.

In conclusion, whether fast neutrons are administered at low or high dose rates, differences in effectiveness in causing cell inactivation are smaller than for X-rays. Due to the repair of sublethal damage during protracted gamma irradiation, the effectiveness per unit dose of low dose rate gamma irradiation is significantly smaller than the effectiveness of acute irradiation. As a consequence, the RBE for protracted fast neutron irradiation is larger than the RBE for acute neutron irradiation. For this rhabdomyosarcoma, this factor is 1.2–1.5, depending on the level of damage considered.

The RBE value for 15 MeV neutrons for radiation damage to the rhabdomyosarcoma equal to 3.5 cannot be compared with RBE values for other experimental solid tumours treated with fast neutrons at a low dose rate because no data have been reported yet in the literature. In order to evaluate whether the RBE of 3.5 is larger than the RBE for normal tissues, effects of 15 MeV neutrons have been measured on rat skin as will be discussed in chapter VI.

5 Conclusions

The results of experiments described in this chapter have indicated that during continuous gamma irradiation of the rhabdomyosarcoma at a low dose rate several factors play a role. The sensitizing effect of reoxygenation of severely hypoxic cells is partly balanced by repair of sublethal damage and cell progression and accumulation of cells into G_2+M phase and this may partly explain the relatively high lethal effect of the continuous irradiation at low dose rate. These findings agree well with those obtained by Frindel et al. [1972] and Hill and Bush [1973] who studied the effectiveness of continuous irradiation on a mouse NCTC and KHT sarcoma, respectively.

The RBE for 15 MeV neutrons at low dose rate for the rhabdomyosarcoma relative to gamma rays is close to 3.5. RBE values for 15 MeV neutrons for radiation effects on normal tissues must be evaluated before conclusions can be drawn about an eventual therapeutic gain in applying fast neutron irradiation at low dose rates.

CHAPTER VI

SHRINKAGE OF THE AREA OF TRANDSPLANTED RAT SKIN AFTER ACUTE AND PROTRACTED IRRADIATION

1 Introduction

For the evaluation of effects of acute and protracted irradiation on a normal tissue, the skin of the rat appeared to offer a suitable system because arguments have been advanced indicating that dose-response relationships of skin in laboratory animals show several similarities to that of man [Fowler, 1971; Barendsen, 1971; Field and Hornsey, 1971]. A transplantation technique was employed in order to eliminate the influence of damage produced in subcutaneous tissues which otherwise cannot be entirely avoided. After whole-body irradiation, pieces of white skin of the female WAG/Rij rat were transplanted onto the back of brown $(WAG/Rij \times BN/Bi)F_1$ hybrid rats as discussed in chapter III. The areas of grafted skin remaining 5 months after transplantation and irradiation with different doses were measured relative to controls. These ratios depend on the dose given and can be used as a measure of the damage produced by irradiation of the donor skin. Each point in the dose-response relationships represents the ratio for one dose delivered to at least 4 pieces of skin in one experiment. If two or more points for one dose or for doses which differ by less than 13% were obtained in different experiments, the mean value of these data is presented as a single point with bars representing the standard error of these data and the spread in the doses.

2 Shrinkage of transplanted skin area exposed to acute 300 kV X-rays and protracted ^{137}Cs gamma rays

Results of the determinations of the mean fractional areas of skin relative to controls as a function of the dose are shown in figure VI-1. Curves 1 and 2 represent the mean fractional area of skin after acute X-ray and protracted gamma irradiation which were applied at dose rates of 40 rad/min and 85 rad/h, respectively.

The curves of figure VI-1 show large shoulders. Consequently, a dose-rate effect cannot be analyzed quantitatively at doses of up to 600 rad of acute X-rays. An estimate of the dose-rate effect can be made with sufficient accuracy for the dose region where a reduction in the skin area to a fraction below 70% is obtained. The ratio of the doses of protracted gamma irradiation and acute X-ray irradiation required to obtain a reduction in mean skin area of 90% is 2.05. If an RBE of 0.85 for gamma rays with respect to X-rays is taken into account, this dose rate effectiveness ratio, DRER, is 1.75. It is clear that this ratio depends on the level of damage considered; i.e., on the percentage of the remaining skin area. The choice of the 10% level to be discussed in

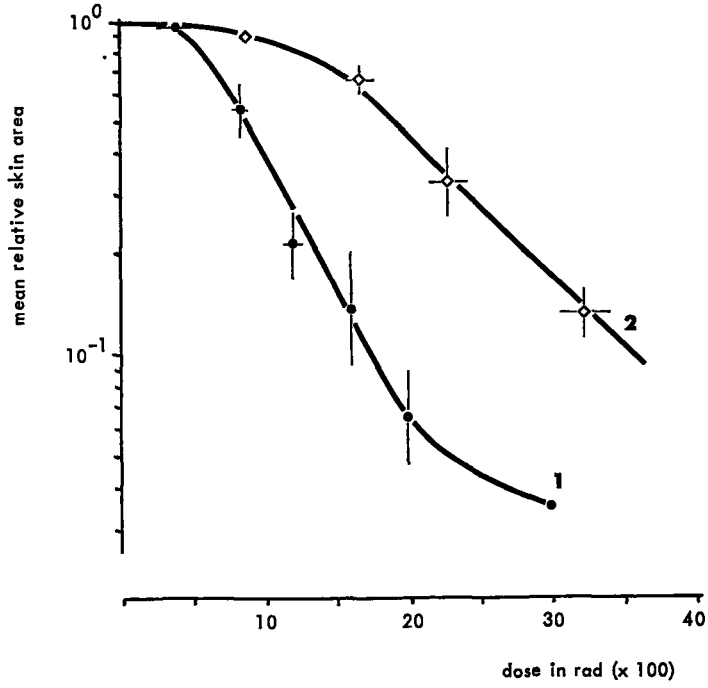


Fig. VI-1. Dose-effect relationships for reduction in the area of skin grafts for 300 kV X-rays applied at 40 rad/min (curve 1) and for ^{137}Cs gamma rays at a dose rate of 85 rad/h (curve 2).

more detail in section VII-5 was made because the dose required to reduce the mean skin area by a factor of 10 is of similar magnitude as the Ellis nominal single dose (1300–2100 rad) for normal human tissue tolerance. For this dose range a considerable amount of data is available [Ellis, 1968].

These dose-effect curves for skin area reduction can be described as a first approximation in the same manner as survival curves obtained after irradiation of single cells. Curve 1 of figure VI-1 can be described by an extrapolation number of 2.4 (1.3–5.9)* and a D_0 value of 570 (390–1100)* rad. The D_Q value, the dose obtained when the exponential part of the curve intersects the 100% level, is about 450 rad. The parameters for curve 2 of figure VI-1 are: $N = 3.3$ (2.6–4.6); $D_0 = 1030$ (760–1460) rad, and $D_Q = 1200$ rad.

It must be taken into account that these values are obtained for a multicellular system and that they cannot be directly compared with the parameters obtained for monocellular systems. In all systems where multiplicity of cells in units or clones plays a role, "survival curves" exhibit an initial slope equal to zero. For instance, after a dose which kills 50% of the cells a single division of all surviving cells

* numbers in parentheses denote 95% probability interval.

would cause complete repopulation [Sinclair and Morton, 1965]. In the skin transplantation system, e.g., the D_0 value of the slope of the initial part of the curve in the low dose region is zero. This is in contrast to D_0 values for the slopes of survival curves of single cells where D_0 values between 400 and 500 rad are found for the low dose region.

3 Shrinkage of transplanted skin area after acute and protracted irradiation with 15 MeV neutrons

Results of determinations of the mean fractional area of skin relative to controls as a function of the dose of 15 MeV neutrons are shown in figure VI-2. Curves 1 and 2 of figure VI-2 represent the mean fractional area of skin after protracted and acute 15 MeV neutron irradiations. The dose rates used were 48 rad/h and 20 rad/min, respectively. As was found for X-rays and gamma irradiation, these dose-effect curves show large shoulders with D_Q values of 440 rad and 460 rad for continuous and acute irradiation, respectively. The extrapolation numbers are 3.0 (1.2–7.4) and 5.6 (1.6–19.1). The D_0 values for the exponential part are 390 (315–465) rad and 270 (220–320) rad, respectively. The D_0 values are significantly different ($p < 0.05$).

At the 10% level, the ratio of the doses for protracted and acute fast neutron

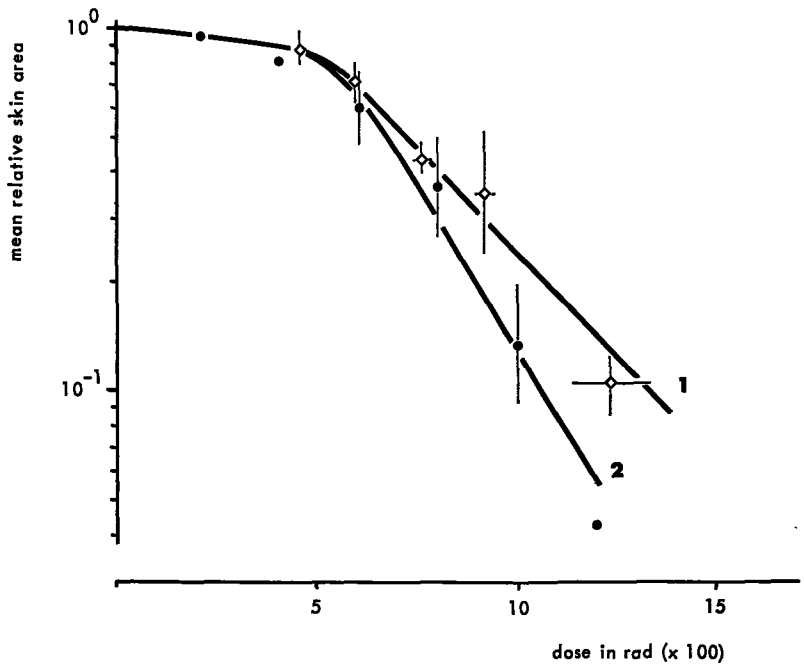


Fig. VI-2. Dose-effect relationships for reduction in the area of skin grafts for 15 MeV neutrons applied at 48 rad/h (curve 1) and 20 rad/min (curve 2).

irradiation is 1.26. This dose rate effectiveness ratio, DRER, is considerably lower than the DRER obtained from data for protracted gamma rays and acute X-ray exposures which was 1.75.

It can be concluded that a sparing effect on skin has been obtained with fast neutron irradiation at a reduced dose rate. The sparing factor is smaller as compared with ^{137}Cs gamma rays. However, for 15 MeV neutrons and ^{137}Cs gamma rays a smaller sparing effect was observed in the rhabdomyosarcoma as discussed in paragraph V-2.2.

4 RBE values for 15 MeV neutrons for shrinkage of rat skin area

For the application of fast neutrons in radiotherapy, it is important to evaluate the responses of normal tissues in comparison with responses of experimental tumours after fast neutron, and X-ray or gamma irradiation. The RBE values derived for the rhabdomyosarcoma for protracted neutron irradiation have been discussed in chapter V; the RBE values for 15 MeV neutrons for the shrinkage of rat skin will be discussed here. An estimate of the RBE for 15 MeV neutrons for the shrinkage of skin can be made with sufficient accuracy only for the dose region where a reduction in the skin area to a fraction between 70% and 10% is obtained. For this region, the RBE depends on the level of damage considered; i.e., on the percentage of remaining skin area. For the 70% level, the RBE for acute fast neutron irradiation is 1.14 as derived for the acute neutron dose of 570 rad and for the acute X-ray dose of 650 rad. For the 10% level, the RBE is 1.63 as derived for the neutron dose of 1060 rad and the X-ray dose of 1730 rad, respectively.

RBE values with respect to shrinkage of rat skin area for 15 MeV neutrons administered at low dose rate are as follows: for the 70% level, the RBE for 15 MeV neutrons with respect to X-rays is 2.1 as derived for the neutron dose of 580 rad and the gamma dose of 1450 rad (assuming an RBE of 0.85 for gamma rays with respect to X-rays). For the 10% level, the RBE relative to X-rays is 2.27 as derived for the neutron dose of 1330 rad and the gamma dose of 3560 rad. Thus, the RBE value for protracted neutron irradiation is larger by a factor of 1.4 than the RBE value for acute neutron irradiation at the 10% level.

5 Discussion

5.1 *Shrinkage of transplanted skin area after irradiation*

With the skin transplantation system, dose-effect curves are obtained with shapes that are assumed as a first approximation to be determined by the dose-survival curves obtained for single mammalian cells. However, the shape is complicated because of the multicellularity of the system. The D_0 values found for the acute and protracted irradiation of skin are larger by a factor of about 5 than the D_0 values

derived from mammalian cell survival curves. This is assumed to be a reflection of the complexity and multicellularity of the system.

Large D_0 values have been reported for survival of cells in other biological systems. A value of 850 rad was derived for visual cells in the retina of 4-day old mice after acute X-ray exposure *in vivo* [Lucas and Mole, 1965] and a D_0 value of 2440 rad for potential tumour cells in the mouse epidermis was calculated from data on tumour induction after superficial external beta irradiation [Hulse et al., 1968]. It should be pointed out, however, that these large D_0 values are also derived from multicellular systems which exhibit a dose-response curve of a type basically different from that of single cell responses. In particular, in contrast to results with single cell systems at very low doses, the initial slopes of the curves obtained after acute and protracted irradiation of the rat skin will be zero. This can be simply related to the fact that, after low doses, a few proliferation cycles of the surviving cells will suffice to replenish the lost cells.

5.2 *Therapeutic gain in dose as derived from a comparison of skin and rhabdomyosarcoma responses*

As discussed in chapter V-2 for the R-1 tumours irradiated *in situ*, the effectiveness of continuous gamma irradiation applied at dose rates of 75, 110, and 150 rad/h is lower by a factor of 1.7 as compared with 300 kV X-irradiation at 340 rad/min. This decrease in effectiveness is due to repair of sublethal damage during protracted irradiation, but is partly counteracted by the sensitizing effect of reoxygenation of severely hypoxic cells and synchronization of cells into a more radiosensitive phase of the cell cycle.

For the rat skin the effectiveness of continuous gamma irradiation applied at a dose rate of 85 rad/h is larger by a factor of 2.05 as compared with acute 300 kV X-irradiation. This factor is larger than found for the rhabdomyosarcoma. This can be related to the fact that the fraction of severely hypoxic cells in the skin is negligible with respect to the fraction of severely hypoxic cells in the tumour. Thus, reoxygenation which partly balanced out the effect of repair of sublethal damage in the rhabdomyosarcoma does not play a significant role in the rat skin. Furthermore, the fraction of nonproliferating cells in the skin is larger than it is in the tumour, hence, the observed increase in radiosensitivity of proliferating cells due to redistribution of the cell ages might play a smaller role in the skin. The results observed for the rat skin suggest that repair of sublethal damage is the most important factor during the continuous exposure.

Due to the differences in responses of skin and the rhabdomyosarcoma to continuous irradiation a therapeutic gain in dose can be calculated.

The acute X-ray dose of 1730 rad which reduces the skin area to 10%, decreases the surviving fraction of clonogenic tumour cells to 6×10^{-3}. A dose of 2950 rad of gamma rays administered at a low dose rate will also cause a reduction in surviving

fraction to 6×10^{-3}. In the combination of skin-rhabdomyosarcoma, however, where the skin is considered as a dose-limiting tissue, a dose of 3560 rad of gamma rays could be administered to the tumour in the protracted regimen to cause a reduction of the skin area to 10%. This dose results in a fraction of surviving tumour cells of 2×10^{-3}. This means that, due to the application of radiation at low dose rate, a gain in therapeutic margin can be attained equal to a factor of 3560/2950 = 1.2. This gain would cause a reduction from a surviving fraction of 6×10^{-3} to a fraction of 2×10^{-3}; i.e., a reduction in survival fraction by a factor of about 3. However, this gain due to low dose rate treatment for this tumour would not be sufficient to obtain cure of the tumour at doses tolerated by skin.

Frindel et al. [1972] irradiated the NCTC transplantable fibrosarcoma at 50 rad/h and reported that the difference in effectiveness of continuous and acute irradiation on the tumour is small. Hill and Bush [1973] irradiated the transplantable KHT sarcoma at 41, 175, and 800 rad/h. They compared the results obtained after irradiation at 6000 rad/h and also reported almost no dose-rate effect for the tumour. Assuming that in the mouse skin a similar sparing effect would be obtained for low dose rate treatments this would indicate that a much higher gain can be obtained for these tumours by applying a low dose rate regimen over a single acute dose treatment. For instance, the gain in dose that can be derived for the KHT sarcoma would be about 2.1. For doses in excess of 2100 rad no dose-response curve is known for the NCTC fibrosarcoma; hence, no gain in dose can be calculated. From these findings, it can be concluded, however, that gamma irradiation applied at a low dose rate has a larger sparing effect on skin than on tumour cells as compared with acute irradiation and this indicates that low dose rate treatments may have a therapeutic advantage over a single acute dose treatment.

5.3 *Therapeutic gain factor for 15 MeV neutrons*

As discussed in VI-3, the effectiveness of continuous fast neutron irradiation when applied at a rate of 48 rad/h on the rat skin is lowered by a factor of 1.26 at the 10% level as compared to acute neutron irradiation applied at a rate of 1200 rad/h. This factor is significantly different from 1 ($p < 0.05$). For the rhabdomyosarcoma, the effectiveness is lowered by a factor of 1.1. Due to the scatter in the fractions of surviving tumour cells, this factor of 1.1 is not statistically different from 1. It seems that a relatively small gain in therapeutic margin could be obtained over acute fast neutron irradiation when applying fast neutron irradiation at a low dose rate.

The advantage of a given treatment regimen with respect to a regimen with another type of radiation can be expressed quantitatively in the following way: For a specified degree of damage to one of the tissues in both regimens, the therapeutic gain factor is defined by

$$\text{TGF} = \frac{\text{RBE for tumour tissue}}{\text{RBE for normal tissue}}.$$

If the RBE for fast neutrons for tumour damage is larger than that for the corresponding degrees of skin damage, then treatment with fast neutron irradiation is advantageous. The TGF observed for fast neutron irradiation at high dose rates for daily doses of clinical interest is in the order of 1.1 to 1.3 [Barendsen, 1971; Field et al., 1968; Fowler et al., 1972]. For protracted fast neutron irradiation the RBE observed for the rhabdomyosarcoma is 4.3 relative to gamma rays. This value was deduced from the dose of 2650 rad of ^{137}Cs gamma rays and the dose of 615 rad of 15 MeV neutrons at the 10^{-2} survival level (see figure V-2 and figure V-5). The RBE for shrinkage of rat skin area relative to gamma rays is 2.7 as derived in VI-4. Hence, the TGF for protracted neutron irradiation can be calculated for these RBE values and is equal to $4.3/2.7 = 1.6$. This value is significantly larger than TGF values obtained for acute fast neutron irradiation which were in the range of 1.1 to 1.3. This result provides additional evidence for a possible advantage of the application of fast neutrons in clinical radiotherapy.

The RBE of 2.7 for protracted fast neutron irradiation of rat skin can only be compared with RBE values for other normal tissues obtained with ^{252}Cf neutrons because no RBE data of 15 MeV neutrons have yet been published. A comparison can be made when the difference in neutron energy dissipation patterns of 15 MeV neutrons and ^{252}Cf neutrons is taken into account. As discussed in paragraph IV-7, this factor might be 1.85. The calculated RBE value for ^{252}Cf neutrons at 48 rad/h with respect to ^{137}Cs gamma rays as derived from the RBE for 15 MeV neutron irradiation of rat skin would then be $2.7 \times 1.85 = 5.0$. This value is close to the RBE values of 5.2 for mouse jejunal crypt cells [Withers et al., 1971] and 6.6 for pig skin [Atkins et al., 1970]. It is also close to the RBE value of 4.1 obtained for $LD_{50/30d}$ in mice [Dean et al., 1972]. These RBE values are represented in figure VI-3 as a function of the ^{252}Cf dose rate (n+γ). Included in this figure is the RBE value obtained by Carsten [see Atkins et al., 1973] for effects on the haemopoietic system. This low RBE value of 2.44 is consistent with generally lower RBE values for haemopoietic tissues compared to other tissues as observed by Field [1969] and Broerse et al. [1971].

No RBE values for ^{252}Cf neutrons for effects on experimental solid tumours have yet been reported. In order to arrive at an approximate value the RBE of 4.3 for 15 MeV neutrons relative to gamma rays derived in paragraph V-2 might be extrapolated to a value of 8 for ^{252}Cf neutrons. This value is also included in figure VI-3, K3.

The RBE for ^{252}Cf neutrons for effects on normal tissues and *in vitro* cultured cells as a function of ^{252}Cf dose rate (n+γ) show several similarities to the RBE versus dose per fraction relationships for 15 MeV neutrons with respect to effects on normal tissues and cultured cells; i.e., the RBE for effects on normal tissues are larger than those for effects on cultured cells at a specific dose rate or dose per fraction [Barendsen, 1971]. It was also found that the RBE for 15 MeV neutrons at high dose rate for effects on tumours is larger by a factor of 1.1 to 1.3 than RBE values for effects on normal tissues. Assuming that similar relationships apply to RBE values for ^{252}Cf

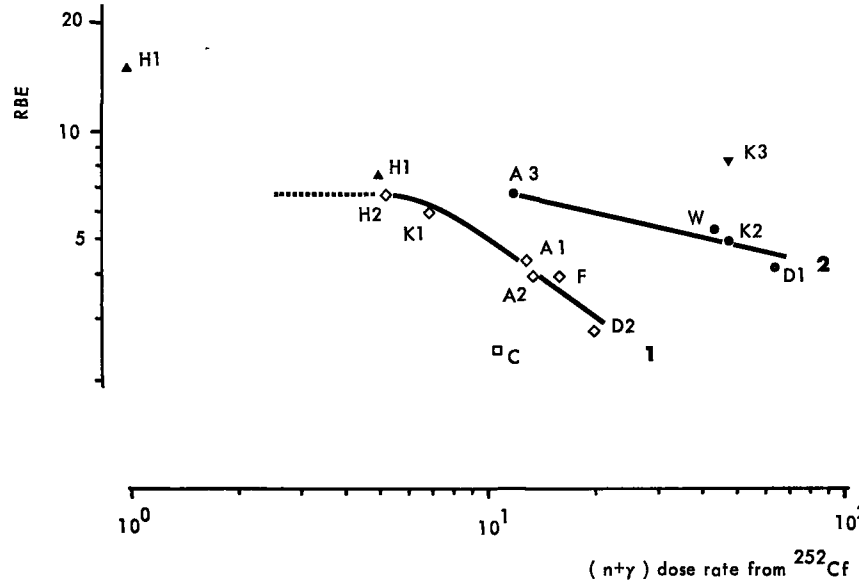


Fig. VI-3. RBE for ^{252}Cf neutrons as a function of ^{252}Cf dose rate (n+γ).

Curve 1: RBE as a function of dose rate for normal tissues;
Curve 2: RBE as a function of dose rate for cultured mammalian cells.

The letters represent the following studies:

A1: Atkins et al. [1973], survival of HeLa cells;
A2: Atkins et al. [1973], survival of Chinese hamster cells;
A3: Atkins et al. [1970], effects on pig skin;
C: Carsten [see Atkins et al., 1973], effects on haemopoietic system;
D1: Dean et al. [1972], acute mammalian lethality $LD_{50/30d}$;
D2: Djordjevic et al. [1973], survival of HeLa cells;
F: Fairchild et al. [1969], survival of HeLa cells;
H1: Hall et al. [1971], proliferation of Chinese hamster cells;
H2: Hall [1972], survival of Chinese hamster cells;
K1-3: present study; K1: survival of R-1 cells; K2: shrinkage of rat skin area; K3: effects on rat rhabdomyosarcoma (extrapolated values);
W: Withers et al. [1971], effects on mouse jejunal crypt cells.

neutrons for effects on experimental tumours, then a TGF of 1.6, as derived for the rhabdomyosarcoma and rat skin, might be obtained for fast neutrons applied at low dose rates.

6 Conclusions

The results of the experiments on rat skin indicate that a sparing effect can be obtained if gamma or fast neutron irradiation is applied at a low dose rate as compared with irradiation at a high dose rate. This sparing factor is about 1.75 for gamma rays and 1.26 for fast neutrons. For the rhabdomyosarcoma, these factors are 1.45 and 1.1,

respectively. From the ratio $1.26/1.1 = 1.14$ it can be concluded that a small gain in therapeutic margin could be obtained if only the effectiveness of fast neutron irradiation at low and high dose rate are compared. A larger gain could be obtained by applying ^{137}Cs gamma rays at a low dose rate as compared to a high dose rate. This ratio is $1.75/1.45 = 1.2$.

The therapeutic gain factor, TGF, for fast neutron irradiation at low dose rates is equal to 1.6 (deduced from $RBE_{tumour}/RBE_{skin} = 4.3/2.7 = 1.6$) and is significantly higher than TGF values obtained for acute fractionated fast neutron irradiation where TGF values are found to lie between 1.1 and 1.3 in the radiation doses of clinical interest [Barendsen, 1971]. For a comparison with published RBE values obtained with ^{252}Cf neutrons with a mean energy of 2.3 MeV [Stoddard, 1964], a factor of about 1.85 must be taken into account for the differences in the energy dissipation patterns of 15 MeV and ^{252}Cf neutrons [Broerse et al., 1968]. The extrapolated RBE value obtained for rat skin is close to 5 and agrees well with the RBE values for ^{252}Cf neutrons for effects on other normal tissues. If this extrapolation is made for the RBE for 15 MeV neutrons at low dose rates for effects on the rhabdomyosarcoma, then an RBE value of about 8 could be derived. Assuming that similar relationships apply to RBE values of ^{252}Cf neutrons for experimental tumours, then a TGF of 1.6, as derived for the rhabdomyosarcoma and rat skin might be obtained when fast neutrons are applied at low dose rates.

CHAPTER VII

DISCUSSION OF THE POSSIBILITY OF TUMOUR TREATMENTS AT LOW DOSE RATES IN CLINICAL PRACTICE

1 Introduction

A large amount of radiobiological data has been obtained in the past decades from radiation experiments on cultured mammalian cells, experimental tumours and normal tissues. On the basis of these data several phenomena which were observed in clinical practice could be interpreted. For instance, the occurrence of severe late normal tissue reactions in patients treated with fast neutrons [Stone, 1948] could be ascribed to overexposure caused by the fact that the RBE of a neutron dose fraction increases as the fraction size decreases. This phenomenon was unknown to Stone in 1939 at the time at which the clinical trial started. Furthermore the success of fractionated treatments used in clinical radiotherapy was found to be due to several factors such as the Elkind type of repair of sublethal damage in normal tissues and the occurrence of reoxygenation of severely hypoxic tumour cells in the time intervals between two successive dose fractions.

The radiobiological data obtained under laboratory conditions may serve as a guide for starting new clinical trials. Well-known are the clinical trials started a few years ago in which fast neutrons were first used for the treatment of various types of tumours in patients [Catterall, 1972]. (See also Proceedings of the 2nd meeting on fundamental and practical aspects of the application of fast neutrons in clinical radiotherapy, Europ. J. Cancer 1974, in press). The RBE values initially used in these trials were based on radiobiological data.

In the preceding chapters experimental data obtained from irradiation experiments on single cells cultured *in vitro*, cells in a solid tumour, and the skin of the rat have been discussed. Although these data refer only to one specific tumour and one normal tissue, it is of interest to speculate whether the conclusions drawn from these experiments support clinical findings or may be applicable in clinical practice. Therefore, the clinical experience obtained with continuous gamma irradiation as obtained by Pierquin et al. [1974] will be reviewed and the experience with ^{252}Cf neutrons in radiotherapy will be briefly discussed on the basis of data published by Castro et al. [1973] and Smith et al. [1974].

It seems likely that continuous irradiation will generally be too elaborate a procedure for routine therapy, since only one or a few patients per week per gamma source can be treated. Therefore, an alternative method of administration of a radiation dose which is equivalent to a treatment at low dose rate will be discussed.

A comparison will be made between the effectiveness of continuous gamma

irradiation and acute X-ray irradiation on rat skin, and the data will be discussed on the basis of relationships derived for tolerance levels of normal human tissues. When these relationships are found to yield reasonable results then methods can be indicated for the calculation of a partial nominal single dose of a low dose rate treatment. This may be important in situations where a radiation regimen can be divided into two parts; one part of the regimen consisting of a low dose rate treatment, the other part comprising a fractionated treatment or a low dose rate treatment in which a quite different dose rate is used. The partial nominal single doses for each part of the treatment can then be calculated and added to derive a guide with respect to the tolerance level.

Finally, advantages will be discussed of ultrarapid cytophotometers which may be used in the screening of a large number of patients suspected of having some types of malignant conditions. These instruments can also be used in monitoring patients during a treatment in which ionizing radiation and/or chemotherapeutic drugs are used.

2 Clinical experience with continuous gamma irradiation at low dose rate in radiotherapy

From the radiation experiments with continuous gamma irradiation of rat skin at low dose rate as described in chapter VI, it was concluded that skin could tolerate considerably larger doses applied at a dose rate of 85 rad/h as compared with acute irradiations. It was shown in chapter V that, although larger doses must be administered to the rhabdomyosarcoma at low dose rates as compared with acute doses in order to obtain the same response, the difference was smaller than for skin. The conclusion was drawn that a therapeutic gain could be expected when low dose rate regimens would be applied in clinical practice if similar differences existed between tumours and normal tissues in patients.

Pierquin and Baillet [1971] and Pierquin et al. [1974] described results of treatments of 22 patients, most of them having large squamous cell carcinomas of the oropharynx and hypopharynx in stages T3–T4. They used a telecobalt unit with an activity of 45 Ci at a dose rate of about 120 rad/h; approximately 800–900 rad/day was delivered in daily sessions lasting 6–9 hours. These patients received tumour doses of 3500–7000 rad in 5–12 days, with an interruption of 2 days during the week-end. They developed a mild skin reaction and a moderately severe to severe reaction of the mucosa, while the tumour rapidly regressed. About 45 days after the start of the treatment, the radiation reaction on skin and mucosa had subsided. In a later modification of this technique, a dose of 7000 rad was divided into two equal halves of 3500 rad which were separated by a 4- to 5-week rest interval. In this treatment the reactions on the mucosa were less severe than in the earlier irradiations where the dose of 7000 rad was delivered within 12 days. Preliminary results obtained from 4 patients were sufficiently encouraging to further pursue this method.

In a pilot study in the Rotterdam Radiotherapeutic Institute, several patients with carcinoma of the cervix and one with an osteosarcoma were treated with daily doses of about 500 to 1000 rad per day at dose rates between 100 and 175 rad/h. The impression was that the normal tissues could well tolerate these doses. A clinical trial will be started in the course of 1974 [van der Werf-Messing and Kuipers, 1973].

The rationale for this beam teletherapy at low dose rate is to combine possible advantages of low dose rate radium procedures with respect to the tolerance of the surrounding normal tissues with the convenience and absence of radiation hazards to staff as is characteristic of beam teletherapy.

It is too early to evaluate the preliminary results obtained so far. It seems likely that the mucosa is an important dose-limiting normal tissue in these treatments. It is expected that definitive conclusions can be drawn in 1975. Then, responses of patients treated 3 or more years earlier can be evaluated [Pierquin et al., 1974b). It can be noted from the clinical results of Pierquin, however, that the results described for the rat skin and the tumour in the present studies might also be applicable to human patients; that is, the radiobiological phenomena which were shown to cause the differences between tumour and skin in the rat might also play a role in tumours and normal tissue damage in patients, although an influence of other factors cannot be excluded, e.g., the slower growth of human tumours as compared with the rat rhabdomyosarcoma.

3 Clinical experience with ^{252}Cf neutrons in radiotherapy

From the experiments on rat skin and the R-1 rhabdomyosarcoma with 15 MeV neutrons applied at low dose rates, the conclusion drawn was that a gain in therapeutic margin could be expected when a low dose rate regimen were applied in clinical practice. Such regimens could be realized with the nuclide ^{252}Cf emitting fast neutrons at low intensities.

Recently, ^{252}Cf sources for interstitial and intracavitary use have been made available by the Atomic Energy Commission for evaluation in the treatment of human cancers [Castro et al., 1973]. Fifty-four sources were made available to the M. D. Anderson Hospital and Tumor Institute in Houston, Texas. These ^{252}Cf sources have been used to evaluate 1. basic dosimetry and health physics data; 2. a workable after-loading technique; 3. dose specification and computer dosimetry methods; and 4. a clinical impression with respect to tumour and normal tissue reactions and a clinically useful RBE factor for the neutron portion of the radiation dose.

With regard to the last point, Castro et al. used for clinical purposes an RBE value of 7.5 relative to radium gamma rays given at 40 rad/h. This was changed later to a value of 6.5 after treatment of 19 patients [Smith et al., 1974]. Although cell killing by ^{252}Cf is probably relatively independent of dose rate (except for the minor contribution to cell lethality of the gamma component), the effect of regeneration in rapidly proliferating tissues cannot be ignored. Therefore, it was found to be desirable to deliver the treatment with ^{252}Cf in about the same overall treatment time as would

be used with ^{226}Ra. A further reason might be that reoxygenation requires time and, if it is important in determining the response of human tumours, then better responses would be expected for protracted treatments. From data obtained on 19 patients who received either interstitial (17 patients, 18 implants) or intracavitary (2 patients) implants with ^{252}Cf as all or a portion of their radiotherapeutic treatment, the following conclusions were drawn: a. The clinical impression of 6.5–7.0 for the neutron RBE for ^{252}Cf appears to be correct; b. a number of patients with advanced disease have shown local control of the tumour, indicating that ^{252}Cf may be an effective interstitial source; and c. controlled clinical trials will be needed to prove or disprove a greater effectiveness of ^{252}Cf over that of gamma emitting sources such as ^{226}Ra or ^{192}Ir. With regard to points 1–3, the conclusions drawn were: 1. afterloading techniques are required to maintain a low exposure dose to personnel and to assure a satisfactory geometric implant with ^{252}Cf; 2. intracavitary use of ^{252}Cf is feasible and may be the easiest to standardize. A ratio of 0.45 μg of ^{252}Cf to 1.0 mg ^{226}Ra is suggested for gynaecologic implants; 3. very stringent precautions and care must be taken in handling ^{252}Cf and patients to prevent overexposure of health personnel; and 4. decay of ^{252}Cf may be a problem in maintaining a sufficient level of the required intensities, especially for gynaecologic implants. A source bank may be needed to replenish sources fairly frequently.

The preliminary results of Castro et al. [1973] and Smith et al. [1974] do not contradict the results derived from experiments on rat skin and tumour as described in chapters V and VI. It must be noted, however, that the small number of patients treated and the relatively short follow-up time of these patients do not allow a definitive conclusion. More patients have to be treated and evaluated before definitive conclusions can be drawn.

4 Multifractionation, an alternative for continuous irradiation at low dose rate?

It seems likely that continuous irradiation will generally be too elaborate a procedure for routine therapy since only one or a few patients per week per gamma source can be treated in view of the time consuming character of the treatment. It is worthwhile to evaluate whether an alternative treatment can be found with equal effectiveness. For this purpose, Hill and Bush [1973] compared the results of the continuous low dose rate irradiation experiments on the KHT mouse sarcoma with a fractionated course of treatment in which an acute dose of 470 rad was given every three hours (multifractionation), an average dose rate quite similar to the continuous dose rate of 175 rad/h. No difference was observed between the surviving fractions of cells in the tumours as determined by the assays of these two different experiments, indicating that this fractionation scheme is as effective as the continuous irradiation. No data are available at the present time on the effectiveness of multifractionation with respect to damage to normal tissues and the treatment of other tumours. However, it is known that for oxygenated cells both *in vivo* and *in vitro*, repair of radiation damage

occurs rapidly in the first few hours following irradiation [Till and McCulloch, 1963; Withers and Elkind, 1969; Elkind and Whitmore, 1967] and that reoxygenation in several experimental tumours is also a fast process occurring in the first few hours following irradiation [Barendsen and Broerse, 1970; Thomlinson, 1969; Howes, 1969; Kallman, 1972]. It seems worthwhile, therefore, to investigate in more detail whether multiple small dose fractions separated by a few hours can be used to simulate low dose rate treatments and to obtain a similar therapeutic gain as might be obtained from continuous low dose rate treatments.

5 Comparison of the effectiveness of protracted and acute regimens

In this paragraph, a comparison will be made between the effectiveness of continuous gamma irradiation and acute X-ray irradiation on rat skin in order to determine whether the relationships derived for tolerance levels of normal human tissues as derived by Liversage [1969b] and Shuttleworth and Fowler [1966] also apply to our animal skin system. Several arguments have been advanced indicating that dose-response relationships of skin in laboratory animals show similarities to that of man [Fowler, 1971; Barendsen, 1971; Field and Hornsey, 1971]. Therefore, quantitative data on human skin may be used for comparison with animal skin data. With respect to human skin, most quantitative data pertain to tolerance levels. Consequently the concept of the nominal single dose, NSD, of Ellis for connective tissue tolerance will be used as a reference dose for comparing the experimental data from treatments with continuous irradiation applied at low dose rates and with acute X-ray irradiation on the rat skin. For human skin tolerance, the NSD values range from 1300 to 2000 rad with a mean of 1825 rad [Ellis, 1968].

From curve 1 of figure VI-1, it can be derived that the mean fractional area for the rat skin is between 20 and 5% in the dose range between 1300 and 2200 rad. Therefore, the 10% level corresponding to a single dose of 1730 rad was chosen as a reference level. This dose is close to the mean value of the NSD for normal tissue tolerance [Ellis, 1968].

For continuous gamma irradiation at a dose rate of 85 rad/h, a total dose of 3560 rad was required to produce the same effect; i.e., a reduction in the mean area of rat skin to 10%. Thus, it can be derived that the effectiveness per unit dose of the low dose rate treatment is lower by a factor of 2.05 in comparison with an acute dose. The corresponding factor is 1.75 if an RBE of 0.85 is taken into account. The problem which will be analyzed now is whether this ratio can also be derived from relationships established for tolerance levels of normal human tissues. Liversage [1969b] has proposed a general formula for equating protracted and acute regimens of radiation. From this formula, it can be derived that, if a low dose rate technique employing continuous irradiation for t hours is replaced by a high dose rate fractionated regimen of N fractions given in the interval of t hours, the number of fractions which will result in the same biological response can be calculated from:

$$N = \frac{\mu t}{2\left[1 - \frac{1}{\mu t}\left\{1 - \exp(-\mu t)\right\}\right]}$$

where t is the time in hours and $\mu = 0.693/\tau$; τ represents the half-life of fading of the dose equivalent of sublethal damage [Lajtha and Oliver, 1961]. For t greater than 12 hours, this expression approximates to $N = t/4$ if τ is taken to be 1.5 hours. (A derivation of this formula is given at the end of this paragraph).

The overall treatment time may be extended in order to avoid the difficulty that N fractions according to the formula should be given in an interval of t hours. For each day that the overall time is increased, however, the dose of 3560 rad must be increased by C rad to correct for repopulation by proliferation of cells. The suggested value for repopulation, C, is in the order of 25–40 rad per day [Liversage, 1969b; Cohen, 1968; Dutreix et al., 1973].

The dose of 3560 rad of gamma rays required to reduce the skin area to 10% was given in 42 hours of continuous irradiation. According to Liversage's formula, the number of fractions required in the interval of 42 hours to obtain the same biological end-point is N = 10 fractions of 356 rad of gamma rays. If these fractions are given in an overall time of 12 days with 5 fractions per week, then the dose of 3560 rad must be increased by a dose, D_R, of $10 \times C$. For C equal to 35 rad per day, $D_R = 350$ rad. As a consequence, a fractionated treatment with daily doses of $(3560 + 350)/10 = 391$ rad of gamma rays would produce the same biological effect as a dose of 3560 rad given in 42 hours. According to the nomogram of Shuttleworth and Fowler [1966], a regimen of 10 fractions of 391 rad per day is equivalent to a single acute dose of 1820 rad of gamma rays. This single acute dose is equivalent to the Ellis nominal single dose, NSD. If an RBE of 0.85 for ^{137}Cs gamma rays is taken into account, the equivalent single dose will be 1550 rad of X-rays. This value differs by about 10% from the experimental acute X-ray dose of 1730 (1510–2170) rad found for the rat skin.

It appears that the low dose rate treatment is more effective by a factor of 1.1 than would be expected if the formula of Liversage and a correction for repopulation of 35 rad per day are taken into account. The formula of Liversage was derived, however, by considering only repair of sublethal damage. In the derivation of the formula for equating of protracted and acute regimens, an approximation was made (see VII-5.1) which leads to an error. This error, however, is usually less than $\pm 10\%$ for most cell-survival curves [Liversage, 1966]. Furthermore, from the experiments on the rhabdomyosarcoma, as described in chapter V, it is known that cells may become synchronized with respect to their age in the cell cycle during protracted irradiation and this effect was shown to result in an increase in effectiveness of the low dose rate irradiation due to an excess of cells in a more sensitive $G_2 + M$ phase. This effect might also play a role in low dose rate irradiations of skin. Although the error made due to the approximation in deriving the formula and the influence of the synchronization

effect is difficult to quantitate, it may well explain the 10% difference observed.

It may be concluded that the quantitative relationships derived from experiments on rat skin described in chapter VI are quite similar to those observed for human skin tolerance and that they can be described with a good approximation by the formulas of Liversage [1969b] and Shuttleworth and Fowler [1966]. In clinical applications, this method of calculation might be used to derive a partial nominal single dose, NSD, for that part of a treatment in which the dose is administered at a low dose rate. This may be of value in cases where a low dose rate treatment is followed by a fractionated regimen for which a partial NSD can also be calculated using the method of Ellis [1968].

5.1 *Derivation of the formula for equating protracted and acute regimens*

The effectiveness of a radiation treatment is strongly dependent on the dose rate at rates less than D_Q rad/h, but becomes less and less dependent as the dose rate increases above D_Q rad/h [Lajtha and Oliver, 1961; Oliver, 1964; Liversage, 1969b]. The slope of the survival curve obtained for low dose rate treatment after irradiation for t hours at d rad/h is assumed by Lajtha and Oliver [1961] to be the same as the slope of the corresponding acute cell-survival curve when a dose of D_E rad has been delivered; then $D_E = d(1\text{-}e^{-\mu t})/\mu$, where μ is the exponential decay constant of the dose equivalent, D_E, of the sublethal damage. D_E increases with irradiation times and, after about 10 hours, tends to reach a limiting value of d/μ. To make the method applicable to shorter irradiation times, the average slope of the survival curve obtained at low dose rate irradiation has been assumed to be equal to the slope of the acute survival curve at the point $D_{E(ave)}$, where $D_{E(ave)}$ is the average value of D_E over the irradiation time and is given by

$$D_{E(ave)} = \frac{1}{t}\int_0^t \frac{d}{\mu}(1-e^{-\mu t})dt = \frac{d}{\mu}\left\{1 - \frac{1}{\mu t}(1-e^{-\mu t})\right\}$$

The slope of the acute survival curve at $D_{E(ave)}$ is now assumed to be the same as the average slope of the curve from $D = 0$ to $D = 2\,D_{E(ave)}$. This is an approximation which leads to an error for the application of this slope to calculate the acute fractionated regimen which should produce the same surviving fraction of cells as a protracted irradiation. The error involved in this approximation is usually less than $\pm 10\%$ for most cell-survival curves [Liversage, 1966]. Within the limits of this approximation, if an acute irradiation dose is given in fractions of magnitude $2\,D_{E(ave)}$, the mean fractional reduction in the surviving fraction per rad should be the same as it is for continuous irradiation given at d rad/h for t hours. Thus, N acute doses of $2\,D_{E(ave)}$ rad should produce the same biological effect as continuous irradiation at d rad/h for t hours, provided the total doses given are the same in both cases. For this to be correct the following formulas should apply:

$$N \times 2D_{E(ave)} = td$$

or

$$N = \frac{\mu t}{2\left[1 - \frac{1}{\mu t}\left\{1 - \exp(-\mu t)\right\}\right]}$$

The formula suggests that the fractional radiation dose wasted due to repair of sublethal damage between N fractions will be equal to that wasted due to repair of sublethal damage during continuous irradiation lasting t hours.

6 Application of impulse cytophotometry in clinical practice

As a result of the introduction of ultrarapid cytophotometers such as the flow microfluorometer [van Dilla et al., 1969] and the impulse cytophotometer [Dittrich and Göhde, 1969], distributions of DNA and RNA content per cell can be obtained. As indicated in chapter III, this can be done by quantitative measurements of fluorescent light emission from cells stained with fluorescent dyes. Distributions for large numbers of cells can be obtained, usually several tens of thousands within a few minutes. Changes in the DNA content distributions as a result of various treatments can be analyzed. Such techniques have been used in studies of the effects of various chemotherapeutic agents on the cell cycle [Tobey and Crissman, 1972], phase progression of tumour cells after acute irradiation [Dittrich and Göhde, 1970; Kal, 1973b; Raju et al., 1974] and during continuous irradiation at low dose rate [Kal and Barendsen, 1973], and for comparison with cell volume changes [Kal, 1973a].

The number of possible applications of ultrarapid cytophotometers in clinical practice is increasing. Included among these are: 1. prescreening of gynaecological smears for the presence of malignant cells [Göhde et al., 1972]; 2. screening of material obtained from the stomach [Gibel et al., 1972]; and 3. follow-up of treatments of leukaemic patients and patients with skin tumours receiving cytostatic drugs [Hillen and Haanen, 1973; Maj et al., 1973; Schumann and Hattori, 1973]. Cytophotometers may be of value in the search for optimal dose schemes of drugs or doses of radiation during chemotherapy or radiotherapy. During the course of such treatments, the effectiveness of drugs or radiation doses can be monitored for individual patients if the DNA content per cell or the DNA/RNA ratio for tumour cells is different from that of normal cells.

7 Conclusions

From the experiments with R-1 cells cultured *in vitro*, it was shown that repair of sublethal damage took place during continuous irradiation at low dose rate and that cells were accumulated in the G_2+M phase of the cell cycle. These cells are known to be relatively more radiosensitive than cells in other phases and, therefore, the

effect of repair of the sublethal damage is partly counteracted by this synchronization process. Similar phenomena play a role in the tumour, but here it is complicated by the fact that reoxygenation takes place and that the influence of the recruitment of Q cells and the repair capability of severely hypoxic cells are not yet known. Nevertheless, continuous irradiation at a low dose rate is a less effective treatment as compared with acute irradiation. Probably because anoxic cells are not important, the effectiveness of low dose rate irradiation in skin is even smaller compared to acute irradiation than for the tumour; i.e., it was found that the dose rate effectiveness ratio, DRER, is larger for responses of the rat skin than for damage to the tumour. This implies that a larger differential effect between tumour and skin response can be obtained if a radiation treatment is carried out at a low dose rate. This differential effect was found to be larger for gamma rays than for 15 MeV neutrons. The advantage of a regimen with 15 MeV neutrons with respect to a regimen with another type of radiation, e.g., X-rays, can be expressed quantitatively with the therapeutic gain factor, TGF. The TGF for 15 MeV neutrons applied at low dose rate was found to be 1.6, which is considerably higher than the TGF for irradiation with daily doses of up to 200 rad of neutrons which lies in the range of 1.1 to 1.3. This high TGF provides additional support for a possible advantage of the application of fast neutrons at low dose rate in radiotherapy.

The skin transplantation system was found to be a suitable model for evaluating the effectiveness of a radiation treatment. The results obtained were found to be in agreement with data obtained for normal human tissues. This was demonstrated by the application of the formula of Liversage and that of Shuttleworth and Fowler for equating protracted and acute regimens.

The RBE of 15 MeV neutrons at low dose rate for skin when extrapolated to an RBE for californium neutrons leads to a value which falls within the same range of RBE values for californium neutrons as found for some other normal tissues. This finding also provides additional support for the assumption that the rat skin model is suitable for evaluating the effectiveness of a radiation treatment to be applied clinically.

The results obtained with the rhabdomyosarcoma and the skin of the WAG/Rij rat indicate which factors might account for the clinical observations that telecobalt therapy at a low dose rate can be well tolerated by human normal tissues and that the irradiated tumours respond quite well to the protracted irradiation.

The relatively large TGF found for fast neutrons at a low dose rate could not yet been compared with clinical data obtained with californium neutrons. The clinical impression is that normal tissues tolerate quite well the fast neutron irradiation at a low dose rate, assuming an RBE of 6.5. The number of patients treated (22), however, is too small to draw definitive conclusions whether the gain in therapeutic margin is as large as it is in the rat skin and tumour model.

An alternative for continuous gamma irradiation at a low dose rate may be a multifractionation regimen; i.e., a regimen in which multiple fractions are given at

high dose rate and spaced by an interval of 3 to 4 hours. In this way, the mean dose rate over the total treatment time is equal to continuous gamma irradiation at a low dose rate [Hill and Bush, 1973].

Finally, it is pointed out that the advent of ultrarapid cytophotometers and their use in clinical practice may be of value in attempts to obtain optimal dose schemes in chemotherapy and radiotherapy.

SUMMARY

Experiments described in this thesis were designed to obtain insight into factors which influence cell survival in tumours and normal tissues during and after continuous irradiation with ^{137}Cs gamma rays and 15 MeV neutrons at a low dose rate. These data have been analyzed to determine whether a gain in therapeutic margin could be obtained if an irradiation with gamma rays was carried out at a low dose rate instead of a high dose rate and whether the application of continuous fast neutron irradiation at low dose rate could provide an advantage in radiotherapy.

The requirement for a successful treatment is that the malignant tissue be eradicated while the surrounding normal tissues retain their integrity and function. Therefore, dosage schemes must be sought which offer an optimal differential effect between normal and malignant tissues.

One of the first considerations in the application of fast neutrons in radiotherapy was that the oxygen enhancement ratio for fast neutrons was smaller than the oxygen enhancement ratio for X-rays or gamma rays. This would lead to a more effective eradication of hypoxic tumour cells with fast neutrons as compared with X-rays or gamma rays. Improved insight into the several factors which influence cell survival has led to the conclusion that the general criterion must be that the RBE for tumour damage must be larger than the RBE for damage to the dose-limiting tissues. The presence of anoxic cells is only one of a number of factors which play a part. Therefore, in chapter II the RBE as a function of the type of radiation, the dose or dose per fraction, and the dose rate have been discussed as well as the basic aspects of the energy dissipation of different types of radiation which determine their effectiveness for inducing death of a cell.

The effectiveness of continuous ^{137}Cs gamma ray and 15 MeV neutron irradiations was determined on a tumour (a rhabdomyosarcoma) and on the skin of the WAG/Rij rat. The methods used are described in chapter III.

The rhabdomyosarcoma is transplantable in the inbred WAG/Rij strain and is the cloned tumour line from the BA-1112 rhabdomyosarcoma which was originally isolated by Reinhold in 1963 [Reinhold, 1965; Barendsen, 1968; Barendsen and Broerse, 1969]. For quantifying the effectiveness of an irradiation treatment, assay methods have been developed for both the tumour and the skin [Barendsen and Broerse, 1969; Barendsen, 1969]. For the tumour, this method consists of the *in vitro* clone technique and, for the skin, the transplantation of irradiated white pieces of skin onto an unirradiated brown host. Tumour cells can also be cultured *in vitro* allowing irradiation experiments to be carried out under well-defined conditions.

Results for cultured cells are described in chapter IV. It was shown that repair of

sublethal damage took place during continuous gamma irradiation of *in vitro* cultured cells at a low dose rate. This resulted in an almost exponential survival curve with a D_0 value of 420 rad when the irradiation was performed at a dose rate of 40 rad/h. It can be concluded that continuous irradiation at 40 and 75 rad/h had no effect on the progress of cells through the G_1 phase of the cell cycle, but produced a delay in S and G_2+M phase which led to an increase in cell cycle time. Consequently cells accumulate in the G_2+M phase of the cycle during the first 20 hours of the continuous irradiation. This phase is known to be very radiosensitive and, therefore, an acute dose given at the end of a continuous irradiation is more effective in cell inactivation than a single acute dose without pretreatment. This increased radiosensitivity cannot be explained by assuming that accumulation of sublethal damage could take place during continuous irradiation as described by the theory of Lajtha and Oliver [1961]. The presence of significant amounts of sublethal damage could not be demonstrated by a special fractionated treatment consisting of a first dose of 850 rad of ^{137}Cs gamma rays administered at a rate of 75 rad/h and followed at different time intervals by a single acute dose of 600 rad of ^{137}Cs gamma rays.

Results of irradiation of tumours are described in chapter V. During continuous irradiation of the rhabdomyosarcoma at a low dose rate, not only repair of sublethal damage and synchronization of cells into G_2+M phase, but also reoxygenation will take place. Reoxygenation results in hypoxic cells receiving a better oxygen supply which leads to increased radiosensitivity. Therefore, the decrease in effectiveness due to the repair of sublethal damage in low dose rate versus acute treatments will be balanced in part by the synchronization effect and by the reoxygenation process.

The effectiveness of a continuous ^{137}Cs gamma ray irradiation for damage to the tumours is smaller by a factor of 1.45 as compared to acute irradiation. For 15 MeV neutrons, this factor is about 1.1. The RBE of 15 MeV neutrons at 48 rad/h for the rhabdomyosarcoma, as determined from dose-survival curves, is about 4.3 relative to ^{137}Cs gamma rays.

In chapter VI results of irradiations of rat skin are described. The effectiveness of continuous ^{137}Cs gamma ray and 15 MeV neutron irradiation of skin is lower by a factor of 1.75 and 1.26, respectively, as compared with acute irradiation. A small gain in therapeutic margin could be obtained if the dose rate of the neutron irradiation was lowered, because the dose rate effectiveness ratio, DRER, for skin and tumour were 1.26 and 1.1, respectively. A larger gain can be obtained by lowering the dose rate of the ^{137}Cs gamma radiation, because the dose rate effectiveness ratios for skin and tumour were 1.75 and 1.45, respectively.

The RBE of 15 MeV neutrons at 48 rad/h for damage to rat skin relative to ^{137}Cs gamma rays is about 2.7 at the 10% level.

The therapeutic gain factor as derived from the RBE for 15 MeV neutrons of tumour (4.3) and skin (2.7) is 1.6 and is higher than the therapeutic gain factor for acute irradiation which lies in the range 1.1 to 1.3 [Barendsen, 1971]. This high gain factor of 1.6 provides additional support for a possible advantageous application of fast

neutrons at low dose rates in radiotherapy. This may be accomplished by use of the nuclide ^{252}Cf which emits fast neutrons at low intensities.

In chapter VII a discussion is presented indicating that radiobiological phenomena which were shown to cause differences between responses of tumour and skin in the rat might also play a part in tumour and normal tissue damage in human patients. However, the influence of other factors cannot be excluded; e.g., the slower growth of human tumours as compared with the rat rhabdomyosarcoma.

An alternative for continuous irradiation at a low dose rate is discussed which consisted of multiple dose fractions separated by a few hours.

Using the concept of the nominal single dose of Ellis for connective tissue tolerance as a reference dose, it is shown that the quantitative relationships derived from experiments on the rat skin are quite similar to those observed for human skin tolerance.

Finally, it is pointed out that the advent of ultrarapid cytophotometers and their use in clinical practice may be of value in attempts to select optimal dose schemes in chemotherapy and radiotherapy.

SAMENVATTING

In dit proefschrift worden experimenten beschreven die verricht zijn met het doel

1. inzicht te verkrijgen in factoren die bij geprotraheerde gamma- en neutronenbestraling de overleving van zoogdiercellen beïnvloeden;
2. na te gaan of een therapeutische winst verkregen kan worden bij een geprotraheerde gammabestraling en
3. te onderzoeken of toepassing van geprotraheerde neutronenbestraling winst zou kunnen opleveren in de radiotherapie.

De voorwaarde voor een succesvolle behandeling van kwaadaardige gezwellen is dat het maligne weefsel te gronde gaat en het omringende normale weefsel zijn structuur behoudt en voldoende kan blijven functioneren. Daarom moet naar doseringsschema's worden gezocht waarin de toegepaste bestralingsbehandeling een zo groot mogelijk verschil in effect tussen normale en maligne weefsels te zien geeft.

Eén van de eerste overwegingen voor de toepassing van snelle neutronen in de radiotherapie was dat het zuurstofeffect, kwantitatief weergegeven door de OER, voor snelle neutronen een factor 1,6–1,8 kleiner is dan bij röntgen- of gammastraling. Hierdoor worden hypoxische tumorcellen effectiever door snelle neutronen geïnactiveerd dan met röntgen- of gammastraling mogelijk is. Het toegenomen inzicht in de verschillende factoren die celoverleving beïnvloeden heeft echter tot de conclusie geleid dat de belangrijkste voorwaarde is dat de relatieve biologische effectiviteit (RBE) voor schade aan de tumor groter moet zijn dan de RBE voor schade aan dosislimiterende normale weefsels. De aanwezigheid van anoxische cellen is slechts een van de vele factoren die een rol spelen bij de beïnvloeding van de overleving van zoogdiercellen. In hoofdstuk II wordt besproken hoe de RBE als functie van de stralingssoort, de dosis en het dosistempo varieert en wordt nagegaan welke verschillen er bestaan in energie-afgifte van verschillende stralingssoorten die tot de dood van een cel aanleiding kan geven.

De effectiviteit van geprotraheerde gamma- en neutronenbestraling werd bepaald op een rhabdomyosarcoom en op de huid van de WAG/Rij rat. De methodieken die hierbij worden gebruikt worden besproken in hoofdstuk III. Het rhabdomyosarcoom is transplanteerbaar in de ingeteelde WAG/Rij rattestam en is de gekloonde tumorlijn van het BA-1112 rhabdomyosarcoom dat oorspronkelijk door Reinhold in 1963 werd geïsoleerd [Reinhold, 1965]. Voor de tumor en voor de huid zijn technieken ontwikkeld [Barendsen en Broerse, 1969; Barendsen, 1969], waarmee het mogelijk is de effectiviteit van een stralingsbehandeling te kwantificeren. Voor de tumor wordt de analyse uitgevoerd met behulp van de kloningstechniek van tumorcellen *in vitro*, en,

voor de huid met een transplantatiemethode waarmee stukjes bestraalde witte huid worden getransplanteerd op de rug van een niet-bestraalde bruine gastheer. De cellen van de tumor kunnen ook *in vitro* worden gekweekt waardoor bestralingsexperimenten met deze cellen in goed gedefinieerde omstandigheden kunnen worden uitgevoerd.

Uit de experimenten beschreven in hoofdstuk IV is gebleken dat tijdens geprotraheerde gammabestraling van *in vitro* gekweekte tumorcellen herstel van subletale schade optreedt. Als de bestraling uitgevoerd wordt met een dosistempo van 40 rad/uur wordt een praktisch exponentiële overlevingscurve verkregen met een D_0 waarde van ongeveer 420 rad. Geprotraheerde gammabestraling bij 40 en 75 rad/uur had geen effect op de progressie van cellen door de G_1 fase, maar de duur van de S en G_2+M fase werd verlengd. Gedurende de eerste 20 uren van de geprotraheerde bestraling werden cellen geaccumuleerd in de G_2+M fase van de cyclus. Deze fase staat als zeer stralingsgevoelig bekend, hoewel voor de R-1 cellijn geen exacte gegevens konden worden verkregen. Een acute dosis die meteen na een geprotraheerde bestraling wordt gegeven leidt tot een kleinere fractie overlevende cellen dan een zelfde acute dosis die zonder voorafgaande behandeling wordt gegeven. Deze toegenomen stralingsgevoeligheid kan niet geheel worden verklaard door aan te nemen dat tijdens de geprotraheerde bestraling accumulatie van subletale schade optreedt zoals de theorie van Lajtha en Oliver [1961] voorspelt. Aanwezigheid van een significante hoeveelheid subletale schade kon niet worden aangetoond met een speciale gefractioneerde bestralingsbehandeling bestaande uit een acute bestraling uitgevoerd op verschillende tijdstippen na beëindiging van een geprotraheerde bestraling bij 75 rad/uur.

In hoofdstuk V worden experimenten besproken waaruit blijkt dat tijdens geprotraheerde bestraling van de tumor behalve herstel van subletale schade en synchronisatie van cellen in de G_2+M fase ook reoxygenatie optreedt, d.w.z. hypoxische cellen krijgen tijdens de bestraling een gunstiger zuurstofvoorziening. Hierdoor wordt bij deze tumor het herstel van subletale schade gedeeltelijk teniet gedaan door het sensitiserend effect als gevolg van synchronisatie en reoxygenatie.

Bij geprotraheerde gammabestraling van tumoren is de effectiviteit een factor 1,45 lager vergeleken met acute bestraling. Voor 15 MeV (D−T) neutronen is deze factor ongeveer 1,1. De RBE voor 15 MeV neutronen bij 48 rad/uur voor het rhabdomyosarcoom, afgeleid uit dosisoverlevingscurven en betrokken op gammastraling, is ongeveer 4,3.

In hoofdstuk VI worden de effecten van geprotraheerde gamma- en neutronenstraling op de huid besproken. Geprotraheerde bestralingen met gamma- en neutronenstraling van de huid zijn een factor 1,7 en respectievelijk 1,26 minder effectief dan een acute bestraling. Omdat voor huid en tumor de waarden van de dosistempo-effectiviteit (DRER) 1,26 en 1,1 zijn, zou een kleine winst kunnen worden behaald als het dosistempo van een neutronenbestraling verlaagd zou worden. Een wat grotere winst wordt bereikt bij verlaging van het dosistempo bij gammabestraling omdat de waarden van de DRER voor huid en tumor 1,7 en 1,45 zijn.

De RBE voor 15 MeV neutronen bij 48 rad/uur voor de huid betrokken op gamma-

straling is ongeveer 2,7. Geëxtrapoleerd naar een RBE voor ^{252}Cf neutronen is deze waarde ongeveer 5. De therapeutische winstfactor (TGF), gedefinieerd als de verhouding van de RBE waarden voor 15 MeV neutronen voor de tumor (4,3) en de huid (2,7), is 1,6, en is hoger dan de TGF van 1,1–1,3 die gevonden is voor acute gefractioneerde neutronenbestraling [Barendsen, 1971].

Deze hoge TGF van 1,6 geeft aan dat geprotraheerde neutronenbestraling wellicht nuttig kan zijn in de radiotherapie. Een dergelijke bestraling zou mogelijk zijn met het nuclide ^{252}Cf, dat snelle neutronen uitzendt en al gebruikt wordt voor interstitiële en intracavitaire toepassingen in enige "clinical trials".

In het laatste hoofdstuk wordt aangetoond dat de radiobiologische verschijnselen die aanleiding geven tot verschillen in effecten van tumoren en huid ook een rol kunnen spelen bij tumoren en normale weefsels van patiënten. Een invloed van andere factoren kan echter niet worden uitgesloten zoals de lage groeisnelheid van menselijke tumoren vergeleken met die van de onderzochte rattetumor.

Geprotraheerde bestraling met ^{60}Co gammastraling zal in de praktijk veel problemen opleveren omdat per gammabron slechts enkele patiënten per maand zouden kunnen worden behandeld en de fysieke en mentale conditie van de patiënt goed moet zijn om de geprotraheerde bestraling te doorstaan. Daarom wordt een alternatieve behandeling genoemd die zou kunnen bestaan uit een gefractioneerde behandeling met intervallen van slechts enkele uren tussen twee opeenvolgende doses.

Uit de experimenten op de rattehuid worden voor een bepaalde fractie overgebleven huid (0,1) kwantitatieve relaties afgeleid voor de tolerantiedosis als functie van de tijd waarover deze gefractioneerd of geprotraheerd wordt toegediend. De fractie van 0,1 werd gekozen omdat de dosis die deze oppervlakte afname veroorzaakt vergelijkbaar is met de tolerantiedosis, de "nominal single dose", NSD, van Ellis voor normale weefsels. De relaties voor de rattehuid worden vergeleken met relaties die voor normale weefsels van de mens gelden. Hieruit wordt afgeleid hoe een partiële NSD waarde voor een geprotraheerde bestraling berekend kan worden.

Tenslotte wordt besproken dat het gebruik van zeer snelle cytophotometers in de kliniek van waarde zou kunnen zijn voor het vinden van optimale doseringsschema's in radio- en chemotherapie.

LIST OF SYMBOLS

symbol	description	page
^{252}Cf	nuclide emitting fast neutrons by spontaneous fission in 3.1% of the desintegrations (average neutron energy = 2.3 MeV; modal energy $\cong$ 1 MeV). Its half-life of 2.6 years is determined by alpha decay, which occurs 96.9% of the time	15
D	absorbed dose = $\Delta E/\Delta m$, in which ΔE is the energy transferred by particle of energy, E, in moving through a distance ΔL and Δm is the small element of mass	23
D_E	dose equivalent of sublethal damage	57
D_0	dose in rad required to produce a decrease in the surviving fraction of cells in the exponential region of a survival curve by a factor of e^{-1}	12
D_Q	quasi threshold dose; i.e., the dose at which an extrapolation of the exponential portion of a dose-survival curve intersects the 100% level of survival	88
DRER	dose rate effectiveness ratio, ratio of doses required to give equal effects for low dose rate irradiation and acute irradiation.	67
Δ in LET_Δ	cut-off energy transfer; i.e., energy transfers less than Δ are considered part of the main track, those greater than Δ constitute a separate delta track	21
LET	linear energy transfer, a measure of the spatial distribution of the energy deposition, defined by the quotient of dE/dL, where dL is the distance traversed by the ionizing particle, and dE is the mean energy loss due to collisions	13
N	extrapolation number, the ordinate relative to 1 intersected by an extrapolation to zero dose of the exponential region of a survival curve	23
NSD	nominal single dose introduced by Ellis for normal human tissue tolerance	101
OER	oxygen enhancement ratio, ratio of doses required to give equal effect for irradiation of anoxic and well-oxygenated cells	13
P cell	proliferating cell, a cell in a tissue or tumour which proliferates and produces daughter cells	14

symbol	description	page
Q cell	quiescent or nonproliferating cell, a cell in a tissue which rests in a particular phase of the cell cycle. Repeated injections with ^{3}H-TdR can be used to label P cells in order to distinguish these cells from Q cells	14
RBE	relative biological effectiveness, ratio of doses required to give an equal effect for irradiation with a standard radiation and another type of radiation	19
TGF	therapeutic gain factor, ratio of the RBE_{tumour} and $RBE_{normal\ tissue}$	92
τ	half-life in hours in which the dose equivalent of sublethal damage decreased exponentially. τ is about 1.5 hours for most mammalian cell lines	57

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