Biological Properties and Response to X-rays of a Multiple Generation C_3H Mammary Carcinoma

by

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ABSTRACT

A multiple generation C_3H mammary adenocarcinoma was serially transplanted at intervals of 2-3 weeks over several years, using a tumour cell suspension. The growth pattern may be described by a Gompertzian pattern, with the doubling time increasing from 0.5 days at 2 mm mean diameter to about 3 days at 10 mm mean diameter. Histologically, the tumour appeared poorly differentiated with areas of necrosis; an appearance suggestive of extensive hypoxia. The very high TCD_{50} of 67 Gy was compatible with this suggestion.

A characteristic pattern and rate of tumour regression existed after irradiation with X-rays alone and in combination with the hypoxic cell radiosensitizer "Misonidazole". The rate of regression seemed to reflect only the rate of removal of the doomed tumour cells, cell debris and the efficiency of the mechanisms responsible for clearance after irradiation. The similar pattern of regression that emerged following non-curative and curative doses of radiation strongly suggested that the rate of regression is a poor indicator of radiation curability. However, in completely regressed tumours, a high probability of local control was observed, once an incidence of complete regression of more than 60 per cent was attained. In the present work, there was a definite growth restraint of recurrent tumours as a result of radiation damage to the tumour bed rather than an intrinsic cellular alteration in the surviving tumour cells. Clearly, this tumour bed effect should be considered in the analysis of the growth delay of these tumours. The magnitude of this effect, however, was

difficult to assess from the growth studies of recurrent tumours. Furthermore, growth studies of transplanted tumours at the sites of previously cured tumours and at pre-irradiated sites offered only limited help in this assessment. The results obtained at these sites, with their discrepancies, pointed to the need for further studies before a possible extrapolation to the situation of recurrent tumours.

The recovery capacity from sublethal damage (SLD) was found to be similar to other tumour systems. However, the values obtained using the cure data differed from those using the delay in growth data. The latter also showed the Elkind pattern (peaks and troughs) characteristic of the split dose experiments. These observations indicate the caution needed in the interpretation of the results using either set of data. This is especially true when comparing the recovery from SLD of various tumours. Although Misonidazole was used mainly to understand the gross response of the tumours following irradiation, its potency in radiosensitization of the hypoxic tumour cells was also confirmed in the present work.

The immunological studies of the tumour system have drawn attention to the possibility of an immuno-suppression rather than an immuno-stimulation status of tumour bearing mice. There appeared also to be a possibility of growth enhancement when attempting immunization of the animals.

Finally, comparative analysis of the biological characteristics of the present tumour and its first generation counterpart, clearly showed that serial transplantation led to several morphological and biological changes.

PREFACE

The aim of the work described in this thesis was to investigate the biological and radiobiological characteristics of a multiple generation C_3H mammary adenocarcinoma. This was done to determine the feasibility of establishing a suitable solid tumour system for further radiobiological and other research studies, at the Radiobiology Research Unit at the Glasgow Institute for Radiotherapeutics and Oncology.

As no previous studies have been carried out on this tumour, it was, therefore, essential to investigate the basic characteristics of the tumour and the methods most suitable for quantitation of its response to irradiation.

The thesis consists of four sections, the first is a review of some of the background literature concerning the biology and radiation biology of experimental animal tumours.

The materials and methods are described in section two and the problems of both tumour transplantation and irradiation are mentioned in some detail. The results of the biological and radiobiological studies of the tumour system are presented in section three. Finally, in section four, the experimental findings are discussed and interpreted in the light of present radiobiological knowledge, with mention of possible directionsin which further studies may extend.

Papers based on this thesis have been published or are in press. Two papers based on this work have also been read to the Royal Society of Medicine and the 20th Annual General Meeting of the British Association for Cancer Research.

CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

Radiotherapy has evolved mainly as a result of an empirical search by clinicians for doses and treatment schedules that would be just tolerated by the normal tissues, while inflicting maximum damage to the tumour cells to a level at which the probability of regrowth is negligible. Present radiobiological knowledge has demonstrated the multiplicity and complexity of the factors involved in the response of tumours, following irradiation.

An understanding of these factors will help the radiotherapist both to arrive at a more considered judgement of the prescribed treatment and to use this treatment more strategically and efficiently.

The clinical response of malignant tumours, following irradiation is the most obvious and easiest monitor of radiation treatment. Hence it is of interest and importance to explore this response, as described in this thesis, with the hope that such studies will guide cancer therapy. It is clear that a considerable amount of experimental radiobiological data about tumour response following irradiation is required before a more definite general conclusion can be drawn and extrapolated without hazards to the clinical situations. This entails studies of a wide variety of tumours of different biological properties.

First generation transplants of spontaneous C3H mouse mammary carcinoma have been used extensively for radiobiological investigations at the Gray Laboratory (1). The multiple generation counterpart of this tumour, described in this thesis, was originally obtained from a spontaneous tumour-

bearing C3H mouse from the Gray Laboratory. The biological properties of this tumour have departed from those of the original one, due to serial transplantation. Hence, it was possible to compare several of the properties of both tumours, despite differences in the techniques of transplantation and irradiation.

The use of a cytosieve tumour cell suspension for tumour transplantation has facilitated study of the take rate, the latent period and the pattern of growth in routine transplants, in cured and irradiated sites. This was performed in an attempt to find out the magnitude of growth restraint as a result of damage to the normal stroma associated with the tumour, following irradiation. However, in this thesis, normal tissue will not be of prime interest.

The aim of destruction of all tumour cells is hindered by the existence of radioresistant hypoxic cells in the tumours, which require an increase in the given dose. On the other hand, this requirement of an increase in dose is tempered by awareness of the limitations of normal tissue tolerance. In order to demonstrate the effect of hypoxic cells on the observed response in our tumour system, the hypoxic cell radiosensitizer (Misonidazole) was used with single doses of X-irradiation.

1.1 Tumour Transplants Using Cell Suspension Technique:

This technique has been used widely in animal transplants (2,3,4,5,6,7). The general methodology of the preparation of the tumour cell suspension can be

summarized as follows:

i - The tumour is excised and freed from non-tumour debris and grossly necrotic material;

ii - It is finely minced and gently passed through a stainless steel mesh;

iii - The crude filtrate is then subjected to one or more of several steps including: trypsinization and other enzymatic treatment, to free tumour cells from non-tumour debris; centrifusion for several minutes and resuspension of the pellet in fresh medium or sedimentation by gravity for periods varied from three to thirty minutes.

ALL these procedures are carried out under fully aseptic condition and in the cold (2-5°C) except enzymatic treatment.

Silobrcic and Suit (4) obtained, without the use of enzymatic treatment, a cell suspension with about 90% of the cells suspended singly and 10% in small clumps of 2-5 cells and 20-45% viable cells. On the other hand, Kallman et al. (5) and Hewitt et al. (6) were able to obtain single cell suspension of tumour cells, by the enzymatic treatment of the crude filtrate. A single cell suspension was needed by these investigators for the dilution assay method which required a known number of single viable tumour cells in each inoculum.

1.1.1 The Injections and Sites of Transplants:

Briefly, lightly anaesthetized mice were used to ensure

accurate placing of the inocula (5,6). There was a degree of leakage of cell suspension from the needle track and skin infiltrations (8,9). To avoid this, Thomlinson (8) used spherical tumour sausage containing alginate pellets prepared from the tumour suspension and Silobrcic and Suit (4) passed the needle through the upper thigh muscles and then into the subcutaneous tissue of the right flanks.

By using a cell suspension for tumour transplants, there is a degree of uncertainty about the number of viable cells injected in each mouse for several technical reasons:-

i - Variations in cell counting and the inclusion of non-viable but morphologically intact tumour cells;

ii - Loss of some part of the inocula through the needle tracks;

iii - Decrease of the number of tumour cells in successive inocula due to sedimentation and clumping of some cells in the syringe during the transplant procedure.

iv - The possible decrease in viability of the tumour cells from the time of killing the tumour bearing mouse and preparation of the cell suspension until the time of last injection.

1.1.2 The Latent Periods: (days after injections until the detection of the earliest tumour masses)

Silobrcic and Suit (4), using spontaneous mammary tumours, showed a spread of the latent periods, with most of the tumours appearing at 30-40 days and no tumours appearing after 80 days. Hewitt et al. (6), using squamous cell carcinoma showed that the latent period was dependent on the number of tumour cells injected. In this tumour, the longest mean latent period value was 27 days, with a decrease of 37h in the mean latent period for each doubling of the inoculum size.

1.1.3 TD_{50}:(number of tumour cells to produce 50% tumour take) Porter et al. (10) made a mathematical analysis of the transplantation kinetics of tumour cells, bearing on the fact that some tumours can be initiated by a single cell while others can not. Several investigators have reported variation of TD_{50} values in a variety of murine tumours with:-

i - The type of tumour: Hewitt et al. (11) in an analysis of 27 different tumours of spontaneous origin, found that TD_{50} values ranged from close to 1.0 for several lymphoid tumours to over 18,000 for an osteosarcoma.

ii - The site of injections: The lowest TD_{50} values were found to be for both axilla and groin by the dilution assay method (5,6). Kallman et al. (5) concluded that the anatomical subcutaneous site of inoculation and hence the immediately

adjacent vascularity and other factors, could determine the fate of the inoculum.

iii - In many tumour systems, lowering of TD_{50} and shortening of the latent periods specially for small inocula have been observed after concomitant transplant of normal tissues or lethally irradiated cells (5,6,7,11,12,13,14,15). Hewitt et al. (7) and Peters and Hewitt (12) attributed this Révész effect to an increase in the proportion of viable cells and a thromboplastic effect at the site of injection.

iv - Immunological factors: Kallman et al.(5) considered that immunological problems can lead to a variation in TD_{50} and irregular tumour take. These investigators demonstrated this possibility in sarcoma KHT and KHG, transplanted in immunized recipients. On the other hand, Hewitt et al. (11) showed a reduction of TD_{50} by immunization and also prior exposure of the recipients to sublethal doses of whole body irradiation. They considered that a high TD_{50} or the reduction of TD_{50} by the addition of heavily irradiated tumour cells or whole body irradiation do not imply immunological factors.

1.2 Tumour Growth:

The growth of many solid tumours in animals departs at an early stage of growth from a simple exponential

growth function to a growth pattern which can be described by a Gompertzian function. In this function, the exponential growth constant itself decreases exponentially, with a corresponding increase of the "doubling time", as growth progresses. The Gompertzian growth is also applied to normal biological systems: human fetus (16), avian and mammalian embryos (17), birds and mammals (18). The Gompertz equation itself is one of a number of possible empirical equations used for mathematical description of the growth pattern of animal tumours. Its more popular, integrated form is:

$$N_t = N_o e^{\alpha/\beta(1-e^{-\beta t})}$$

where N_t represents some measure of tumour size (i.e. cell number, tumour volume or tumour weight) at time t;

N_o is the initial tumour size at time zero for the period of observation;

α represents the exponential growth parameter;

β represents the retardation growth parameter.

The doubling time of the Gompertz curve is the doubling time of the exponential tangent at the point of interest and can be calculated from the following equation:

$$\text{Doubling time } (T_d) = \frac{\ln 2}{\alpha - \beta \ln (N_t/N_o)} \text{ i.e. } = \frac{0.693}{\alpha - \beta \ln (N_t/N_o)}$$

The retardation process of normal growth may be explained by the capacity of normal stem cells to differentiate and their sensitivity to normal regulatory

and homeostatic mechanisms. Laird (19) pointed out that tumours grow as a community of cells or a single organism derived from a single cell, rather than a population of dissociated individual cells. She (20) also suggested two explanations for the retardation process in tumours; either an ageing effect of the population of cells with increase in mean generation time of cells without a change in the proportion of reproductive cells, or a loss of the reproductive cells without change in the mean generation of time of the cells.

Hermens and Barendsen (21), in a study of rhabdomyosarcoma in rat, concluded that the decrease in growth rate of this tumour was due to a lengthening of the cell cycle toward the centre and a decrease of the growth fraction in both the central and the peripheral part of the tumour, as growth progressed from small tumours.

Frindel et al. (22) studied mouse fibrosarcoma (NCTC 2472) at various stages of its natural history, and found no change in the cell cycle but an increase of the cell loss and a decrease of the growth fraction as growth progressed. Tubiana (23) in a review article regarded the variations of the growth fraction and/or the cell loss factor as the main factor(s) for variation in the growth rate of a given solid tumour. Denekamp (24,25) in an analysis of the cell proliferation kinetics of animal tumours, found nearly

similar growth fractions in tumours (carcinomas and sarcomas) of wide differences in the degree of differentiation and doubling times. She pointed out that this might indicate some common limiting factor, such as the diffusion of metabolites from the vascular bed. A general tendency toward a higher cell loss was also noted with increase of the doubling times of tumours. Relatively slow growing carcinomas had a loss factor in excess of 0.70 while sarcomas of shorter doubling time had a cell loss factor less than 0.3. Denekamp concluded that the cell cycle times determined largely the overall growth of sarcomas, while the cell loss factor determined largely the overall growth of carcinomas.

McCredie et al. (26), in a study of spontaneous C3H mammary carcinoma and its syngeneic transplants, found that the relative tumour volume occupied by viable cancer cells decreased while that occupied by necrotic cells increased as growth progressed. These changes were more obvious in the rapidly growing 900th generation than the spontaneous tumours which had a relatively uniform distribution of blood vessels. These investigators stressed the importance of the blood supply in determining retardation in the rate of tumour growth.

Burton (27), in a theoretical analysis of the growth of a sphere of cells, showed that the Gompertzian growth of tumours with central necrosis would be explained by

the exponential decrease in time of the proportion of generating cells, forming the peripheral rim and the limited diffusion of nutrients including oxygen.
Summer (28,29) concluded from a mathematical model of tumour growth in time, that the kinetics of tumour growth would be determined by the vascular growth and the nutritional support from the host.
Laird (19) showed by extra polation of the tumour growth Compertzian curves back to one cell, that the number of doublings of size required for the tumour to grow from one cell to the theoretical upper limit of growth was essentially constant for a variety of tumours in two animal species (mouse and rat), but was of larger value for rat than mouse. Furthermore, Brunton and Wheldon (30) in an analysis of the published data on tumour growth in three species of animals (mouse, rat and hamster) showed that the observed positive correlation between the two parameters α and β of the Compertz equation was constant for a given species regardless of tumour type. These investigators favoured a host mediated control of tumour growth rather than an inherent self-limiting property of the tumour. They suggested that the supply of necessary nutrient(s) to the proliferating tumour population might be host limited in a specific way, either by oxygen diffusion or by the capability of the species to develop and maintain an adequate vascular supply to the growing tumour.

The existence of a positive relation between the two parameters α and β of the Gompertz equation described by several investigators (26,30,31) would suggest a general feature of tumour growth i.e. tumours with an initially rapid growth (large value of α) have marked retardation (large value of β) and vice versa.
The importance of metabolic and nutritional support from the host tissue (tumour bed) has been favoured by several theoretical and experimental workers (26,27,28, 29 + section 1.5) as a possible determinant of the kinetics of tumour growth. This would also be supported by evidence for the modification of tumour growth rate as a result of pretransplant X-irradiation of the tumour bed, discussed in section 1.7.
Other factors which might also influence tumour growth, include: host immunological factors (32,33), various nutritional deficiencies (34,35,36,37), metabolic and tumour non-specific by-products (38,39,40), tissue specific factors such as Chalones (41) and/or contact inhibition phenomena (42,43,44).

1.3 The Effect of Successive Transplants of the Tumour:
It is well-known that a variety of tumour properties can change during serial transplantation within syngeneic recipients. In general, malignancy and growth rate tend to increase with successive passages, till a relatively constant pattern is reached.
Wexler et al. (45), using 3 chemically induced and 2

spontaneous tumours in mice, found variations in the time of appearance and the rate of growth of the primary tumours. Tumour growth increased and became more predictable with serial transplantation. Steel et al. (46) in a study of a spontaneous rat fibroadenoma (BICR/A9) found acceleration but more uniform growth of the tumour by serial transplantation. The volume doubling time decreased, within the first 10 transplant generations, to almost one-twentieth of the doubling time of the primary tumour. This was associated with progressive shortening of the cell cycle time and reduction of the cell loss factor and an increase in the growth fraction. McCredie et al. (26) in a comparative study of C3H spontaneous mammary carcinoma and its first and 900th generation syngeneic transplants, found that the initial rate of growth and the retardation which subsequently occurred, increased with transplantation. These changes were correlated with the observed histological changes in the relative volume of cancer cells and necrosis that occurred with transplantation.

The changes in tumour properties and behaviour, with preservation of the original histological type have been explained by possible cell selection of cells having the greater potentiality for rapid growth even under poor nutritional supply, or on the basis of changes in the tumour specific antigens by successive transplants (47,48,49,50). On the other hand, the

tendency to change from carcinoma type to sarcoma type has been reported in some animal tumours and cultured cells (51,52,53,54).

Rockwell et al. (51) described the diamorphic histological appearance of KHJJ tumour, with carcinoma-like and sarcoma-like regions. However by the 30th transplant generation, the carcinoma-like pattern was only retained, with increasing anaplasia, cellular variability, and rapid growth through the latest passages. These investigators succeeded in separation of the sarcoma-like element as the EMT6 tumour line.

Begg (52) has also observed that the serially transplanted mouse tumour NT1 changed from an epithelial appearance, through that of a mixed histology, to being of fibrous structure. These changes were associated with progressive decrease of the growth rate, an increase of the mean cell cycle time and different response to irradiation. The decrease in growth rate of that tumour seems to be an exception to the findings of other investigators.

The change from carcinomatous type to sarcomatous type has been explained by an actual transformation of the tumour cells from one type to the other or on the basis that the primary tumour is diamorphic with carcinoma-like and sarcoma-like components, with subsequent random selection in favoour of the sarcoma-like components.

In general, there are changes in the tumour kinetics

during successive transplants and hence the obtained results of experiments performed at different times. This would entail the use for radiobiological experiments, either of early transplants with the least deviation from the spontaneous state or the use of late tumour subpassages after reaching the relatively constant pattern of growth and kinetics.

1.4 Antigenicity of Experimental Tumours:

The immunological problems in experimental tumours have been mentioned by several investigators, with respect to the dilution assays (5,6), tumour growth (16,20,32) and the biological behaviour through successive transplantations (45,47,48,49,50). Hewitt et al. (11) and Kallman et al. (5) raise the question of the validity of using tumours other than those without antigenicity for biological and radiobiological experiments to avoid immunity as a modifying factor of the obtained results.

This antigenicity is well documented in virus-induced and chemically-induced tumours (55,56). But despite reported immunity to a number of spontaneous tumours (57,58) most workers could not find evidence of immunogenicity in tumours of spontaneous origin (11, 59,60).

C3H mammary carcinoma is known to be of viral origin and to carry a specific antigen(s) which evokes only, a feeble immunological response in mammary tumour agent

negative mice (MTA-) and an even weaker or no response in MTA positive mice (61,62,63). Immunization was possible in MTA- mice but not in MTA+ mice (61). However, Suit and Kastelan (64) found that weak antigenicity in MTA- mice had no effect on the number of tumour cells to be killed by radiation to achieve local control of the tumour, i.e. the TCD_{50}s were the same in non-immunized and immunized MTA- mice (5205 and 5216 rads respectively).

1.5 Tumour Vascularity

Histologic; microangiographic, Algire chamber, chick chorioallantoic membrane and rat dorsal air-sac technics (65,66,67,68,69,70,71,72) have been used for intensive studies of the vascular architecture of tumours. Rubin and Casarett (65,66) concluded, after analysis and reviewing these technics that the microcirculation of tumours represents an active growth from the pre-existing host blood vessels with a specific vascular arborization pattern for each tumour which is inherent to the tumour and not the site of growth. The following anatomical patterns of tumour circulation have been suggested by Rubin and Cassarett:-

i - peripheral vascularization with penetrating vessels;

ii - peripheral vascularization without penetrating vessels;

and iii - central vascularization.

Tumour vascularity is imperfect (65,66,67,68) and mainly consists of irregular, incomplete channels with arteriovenous shunts and sinusoidal spaces and although it may appear adequate anatomically, it is less effective functionally leading to slow, sluggish circulation with multiple areas of necrosis. The proportion and distribution of these areas reflect the state of tumour oxygenation and hypoxia in different parts of the tumour (73). The absence of necrosis in slow growing spontaneous C3H mammary carcinoma has been attributed by McCredie et al. (26) to its relatively uniform distribution of the blood supply, in comparison with the rapidly growing syngeneic transplants which outgrew their blood supply.

The oxygenation status of a tumour depends on the balance between the tumour demands for the available oxygen and the functional adequacy of tumour vascularity. The latter is a function of the numbers of the patent blood vessels per unit volume of the tumour and the adequacy of the blood flow in them. The blood flow can be impaired by compression and occlusion of the blood vessels or excess capillaries than the blood supply (68). The response of tumour blood supply to vasoconstrictor and vasodilator drugs has been discussed by some workers (74,75) and the changes in the blood flow induced by stress and anaesthesia have also been reported (section 2.5.4).

The theoretical consideration and histological

observation of Thomlinson and Gray (73) and the careful studies of Tannock (76) concerning the rates of cell proliferation at different locations in the tumour cords have shown a limited range of about 150μ for the diffusion of oxygen with a falling gradient in the tumour cords away from the capillary wall. Beyond this range the tumour cells will be under hypoxic conditions, out of cell division and end in necrotic areas. Tannock (77), Tannock and Hayashi (78) suggested that the rate of tumour growth is limited probably both by loss of capillary function caused by blood stasis and the rate of proliferation of endothelial cells which are not derived from a faster proliferation precursor population with turnover time of a few months. Several transplanted tumours have been shown to survive and grow to a limited volume on simple diffusion. Greene (79) observed that small tumours implanted for more than a year in the anterior chamber of the guinea pig eye would not continue growth due to the absence of vascularization. When these tumours were reimplanted in the muscle of a rabbit, they grew to a large size due to vascularization. Folkman et al. (80) found that implanted tumours in isolated perfused organs grew to a limited size (3-4 mm). Using the Algire chamber technic (67,68), venous vascular invasion was noticed 3-5 days after transplant, followed by arterial supply in 1-3 days, coinciding with the start of growth. Knighton et al. (72), in a study of Walker 256 carcinosarcoma

implanted on chick chorioallantoic membrane, observed that the tumours remained avascular for 3-4 days after which they were surrounded and penetrated by new blood vessels from the host vasculature. The avascular phase supported the tumour up to 1 mm size, which consisted of central necrosis, surrounded by a peripheral rim of proliferating tumour cells. A phase of rapid growth began after the tumour became vascularized with the disappearance of the central necrosis at first. As the tumour approached 3-4 mm, central necrosis reappeared. Tumour induced vascularization appears to be a necessary event in the progression of the growth of malignant tumours. They establish the blood supply needed for further growth by secretion of tumour angiogenesis factor (TAF) which causes neoproliferation of host vascular endothelium and growth of host vessels into the tumour (70,71,81).

1.6 Histopathological Changes in Tumour Vasculature Following Irradiation:

Changes in permeability of the small vessels have been demonstrated shortly after irradiation (82,83,84,85). The blue flush over the irradiated depilated flanks appeared within two hours after injection of a vital dye (Pontamine Sky Blue) into the marginal vein of rabbits and was more apparent at intervals of 1, 9 and 16 days after 1200 or 2000 r superficial X-rays (82). Thomlinson (84) reported these changes in 3 hours after

4000 rads which cleared in 48 hours. Song and Levitt (85), Song et al. (86) using ^{51}Cr labelled red blood cells and ^{125}I-labelled plasma protein in studies of vascular changes in Walker 256 carcinoma of rats following X-irradiation, found increased permeability on the first day after irradiation with 2000 or 3000 R. This returned to the control level on the second day and continued to decrease to the lowest level by 12 days. Then gradual recovery was found from 15 days, with an increased permeability again at 27 days postirradiation. They also found a rapid decrease of the intravascular volume of tumours during the first few days, reaching a lowest level by 11 or 12 days during which time the tumours were undergoing regression then gradual recovery from 15 days; but the intravascular volume remained smaller than the control tumours at 27 days postirradiation. Merwin et al. (87), using mouse mammary carcinoma in the original type of Algire chamber, concluded that the endothelium was rarely able to produce new vessels within the irradiated volume of tumour after 2000 or 3000 rads at the time of regrowth. They also reported the reutilization of the pre-existing capillaries to support the regrowth with gradual stasis in these vessels followed by thrombosis and a new wave of necrosis of the tumour. Thomlinson and Cradock (88), using the chemically induced fibrosarcoma RIB5 in rats, observed capillary thrombosis associated in time with

the regrowth of the tumour, with a second wave of delay when the tumours grew up to and beyond the size at which they were irradiated. Rubin's conclusions (89) from his studies of the microcirculation of tumours are that the progressive destruction of tumour cells precedes the capillary changes, and months later capillary endothelium fails to proliferate when there are demands; hence vascular failure and another wave of cell death occurs. Brecher and Tessmer (90) reported that the late vascular damage became evident histologically about 2-3 months after a single dose of $> \approx$ 2000 rads or cumulative doses of similar magnitude. This damage includes endothelial swelling, hyaline thickening of intima, occlusion and fragmentation of blood vessels. The hypothesis of late expression of the vascular damage after irradiation is shared by several investigators (78,88,89) and strengthened by the fact that the endothelial cells are derived from a slowly dividing precursor population with a turnover time of a few months (77). No increase of thymidine labelling index was detected up to 3 weeks after 2000 rads nor up to 2 weeks after 4000 rads irradiation of muscle, skin or bone (78) but an increase was observed at 40 to 70 days after 2000 rads irradiation to the heart of rabbits (91).

Rubin and Casarett (66) using correlative microangiographic and histological techniques in transplanted rat tumours, have described a

'supervascularization' phenomenon 4 days after irradiation association with tumour regression. They also found that a dose of 1500 r produced more fragmentation and occlusion of the fine vasculature if it was given as a single dose than as 3 fractions (500 r x 3). Reinhold (67), using sheet-like mouse mammary tumour in a modification of the Algire chamber system, has also described increased vascular density and improvement of blood flow with daily fractions of 576 rads at the seventh day when the tumours have just regressed beyond the irradiated size. Sacki et al. (92), using microangiographic studies of C3H mouse mammary carcinoma, found that the vascularization pattern of either tumours transplanted into a tumour bed previously irradiated with 3300 rads or of tumours which recurred after 4000 rads, were basically the same but scattered and less rich and fine compared with the non-treated tumours. They concluded that the proliferative capacity of the endothelial cells was impaired by irradiation. McAlister and Margulis (93) studied the vascular changes in 5 different types of mouse tumours by angiography following various doses of X-irradiation and concluded that the timing and dose for vascular changes varied according to the type of tumours. Rubin (94) expressed the opinion that fractionated irradiation should lead to regression of tumours with preservation of vasculature, as rapid obliteration of vasculature can increase foci of hypoxic tumour cells

with increased radioresistance and incidence of necrosis and recurrences.

1.7 The Restraint of Transplanted Tumour Growth in Previously Irradiated Sites "Tumour Bed Effect":

Tumour bed effect (TBE) studies have also provided useful information on the complex interaction between tumour cells and normal tissue stroma in solid tumours and the effects of ionizing irradiation on the blood vessels.

Despite the possible differences between the effects of irradiation on already established proliferating tumour vascularity and unstimulated quiescent normal vascularity in previously irradiated graft sites, the following conclusions have been reached:

1 - In several animal experiments no significant TBE was detected with doses of 500 rads or less and reached maximum with single doses between 1500 and 3000 rads (95,96,97);

2 - TBE was detectable as early as a few days after irradiation (95,97) and persisted undiminished for many months afterwards (95,96);

3 - TBE did not increase the number of cells required to produce tumours in 50% of the sites (TD_{50}) (95,98, 99);

4 - TBE did not affect the latent periods (section 1.1.2) in some animal tumours (95), while it prolonged the latent period in others (97,98,100);

5 - TBE led to a slow rate of tumour growth (95,97,99, 100). Hewitt and Blake (95) found that the slope of tumour growth at the pre-irradiated sites was nearly half that of the controls. This was independent of the age of the host animal and the intrinsic growth rates of the tumours they studied. They also observed no growth restraint until the tumours approached a size at least 1 mm^3 and suggested that microtumours can obtain their nutritional requirements by diffusion from the existing vessels.

It appears that damage to the host vascularity and supportive tissue is the cause of TBE. Summers (29) concluded that the lower growth rate of tumours growing in pre-irradiated sites results from a decrease in the availability of the nutrients necessary for cell division. Clifton and Jirtle (99) concluded from their studies of MTG-B mammary adenocarcinoma grafted in C3H mice, that the inhibition of new vascular growth brought about by irradiation of the graft site was reflected in an increase in tumour cell death with little or no change in the proliferative rate within the viable tissue centres. They pointed out that many irradiated endothelial cells retained functional capability although they had lost reproductive capacity and that growth could occur along pre-existing, non-growing capillaries at reduced rate of growth. Jirtle et al. (101) found that the blood flow of MT-W9B mammary adenocarcinoma growing in rat mammary glands previously

exposed to 1500 rads X-rays, was 52% that of the same sized tumours in unirradiated host tissues. They suggested that this reduction in the tumour blood flow might be the cause of TBE.

1.8 The Hypoxic Tumour Cells:

Oxygen concentration strongly influences the radiosensitivity to low LET radiation. Anoxic cells are 2-3 times more radioresistant than well oxygenated cells and the radiosensitivity of these cells can be increased by oxygen (102-106). The anoxic cells are found to modify the shape and the slope of the radiation dose-response curve of a composite cell population of anoxic and oxic cells (107-111).

Hypoxia, specially when chronic or prolonged, can lead to a shift of the cells from the cyclic to the non-cyclic (76,112,113,114) and it seems also to impair their capacity for recovery from sublethal damage (115-118). On the other hand, hypoxic cells can repair potentially lethal damage (PLD) more efficiently than well oxygenated cells (119-121). Hence improvement of the oxygenation status operates in two opposite directions; it enhances killing of initially hypoxic cells by increased radiation sensitivity and impairment of repair of PLD, while it leads to more repair of sublethal damage and rapid proliferation of the surviving reoxygenated cells.

Numerous animal studies have confirmed the existence of

hypoxic cells in many, if not all experimental animal tumours (122). Most workers believe that a significant proportion of cells in a tumour would be likely to exist at levels of oxygen tension low enough to give them protection from radiation damage, yet remain clonogenic when irradiated. Such cells would be significantly more radioresistant than well oxygenated tumour cells and would be likely to survive doses capable of eradicating the rest of the tumour cells and repopulate and regrow tumours when their oxygen supply is restored. These hypoxic cells are considered to be one of the main factors affecting tumour curability by radiotherapy and the expected influence of a very small proportion of hypoxic cells on the cure probability of a tumour has been discussed by several workers. Suit (123) suggested that 1% hypoxic cells will raise the TCD_{50} from 5960 to 10,800 rads in a tumour of 10^6 cells treated by 30 equal fractions. Sambrook (124) suggested that 1% anoxic cells present in the tumour would soon dominate the response pattern and determine the ultimate surviving fractions. Thomlinson (125) estimated that a proportion of hypoxic cells of less than 1 in 10^6 would just not affect the size of the single dose necessary to sterilize all the cells in such a tumour. Thomlinson (126) calculated the upper limit of the proportion of hypoxic cells in a tumour which would reduce the chance of cure if exceeded, to be 1% for X-rays and 6% for fast neutron with an

OER (oxygen enhancement ratio) of 3 and 1.7 respectively. Field (127) in a schematic demonstration of the effects of hypoxic cells on the response of tumours to single doses of X-rays, showed that 1% hypoxic cells would raise the curative dose from 1500 rads, when all the cells are well oxygenated, to 3650 rads and only small further increases in dose are required for 10% and 100% hypoxic cells i.e. 4140 rads and 4410 rads respectively. Fowler et al. (128) in a theoretical model, calculated the single doses that are required to give 90% chance of "cure" of a tumour of 75 mm diameter to be 4300 rads if all the cells were well oxygenated, 8000 rads if 1% of the cells were hypoxic and 10,000 rads if all the cells were hypoxic. A single dose of 4300 rads would give this probability of cure of a tumour of 3 mm diameter with 1% hypoxic cells and 0.6 mm diameter if all the cells were hypoxic. Several mathematical analysis and models have been used for estimation of the proportion of hypoxic cells in various tumour systems, irradiated under hypoxic, air or high pressure oxygen breathing conditions. These analyses and models have been applied to local control "cure" data (9,129,130) growth delay curves (88,131), cell survival in vivo-vitro assays (132-134) and/or in vivo-vitro-vivo assays (108). Estimates of the hypoxic cell fraction in these tumour systems have ranged from 1% to greater than 50% (122).

Recently, several published papers have drawn attention

to the discrepancy in these estimates in the same tumour system, using different assays. McNally (132, 134) in studies on a rat fibrosarcoma, has shown that the modifying effect of oxygen and radiation quality on tumour growth delay and on cell survival in vitro after irradiation in vivo were not the same. McNally (133) in a study of sarcoma f in CBA mice found also quite a different estimate of the hypoxic fraction, using growth delay and tumour cell survival in vitro after irradiation in vivo. Estimates of less than 10% and in excess of 50% were found respectively. Hill and Fowler (131) in a study of squamous cell carcinoma D, growing in WHT/Ht mice, previously found to have 18% hypoxic fraction by in vitro assay (6), estimated a wide range of hypoxic fraction between 10% and 100% using 3 different methods of analysis of the growth delay curves of this tumour. McNally and Sheldon (135) in an analysis of the response of anaplastic MT tumour, using 3 different assays (tumour cure, cell survival in vitro after irradiation in vivo and growth delay) also estimated a wide range of hypoxic fraction between 5% and over 50%. The tumour bed effect, especially from vascular damage (sections 1.6,1.7) may contribute to the growth delay of the tumour after irradiation, which has been assumed to be primarily a reflection of the lethal effect of radiation on the tumour cells (88). There is also the possibility that the immediate post-irradiation environment critically affects the fate of irradiated

cells and the removal of these cells from in vivo to in vitro assays may change the course of events in the undisturbed tumours (132).

McNally and Sheldon (135) pointed out the erroneous conclusions from the use of a single assay of radiation response alone and the usefulness in using several methods of radiation response assays in the same tumour.

1.9 The Phenomenon of Reoxygenation:

A process of "Reoxygenation" occurs when the number of hypoxic cells in the tumour decreases more rapidly than would be expected on the basis of radiation killing of hypoxic cells. The importance of this phenomenon is related to the reduction of the number of hypoxic cells after the first dose of radiation due to improvement of the oxygenation status of the tumour. This will counterbalance the effects of repair of radiation damage and that of cellular proliferation after irradiation, leading to a more effective second dose of radiation and fractionated irradiation (1,9,122,126,129, 136).

The phenomenon of reoxygenation has been reported in several animal tumour systems (129,137-143).

The process of reoxygenation can be expected as the tumour shrinks following irradiation. The reduction of intercapillary distances and the relief of compression on the tumour capillaries improve the oxygenation status of the remaining cells, especially with the availability

of more oxygen for consumption after the metabolic inhibition and killing of many tumour cells.

Fowler et al. (1,136) showed that reoxygenation was the most important factor in determining the response of first generation C3H mammary tumour to a wide variety of fractionated X-ray schedules; while repair, synchronization and repopulation were of less importance. In that tumour system, reoxygenation coincided with the reduction of the tumour volume in 2-3 days after a single dose of 1500 rads (129). On the other hand, Thomlinson (126) in a study of RIB_5 sarcoma drew attention to an early phase of reoxygenation due to reduction of oxygen consumption as a result of the extensive cell death after irradiation. That tumour showed an early increase in size before shrinking.

The rate and extent of reoxygenation varied in different types of tumours (129,144) and in the same tumour, probably according to the initial dose and the schedule of fractionated irradiation (129,130,143,145,146).

Nias et al. (117) showed that the oxygen effect was fully operating on HeLa cells in vitro above 1000 rads while it might be minimal at doses below 200 rads.

Howes (129) and Howes et al. (130) in studies of first generation C3H mammary tumour, showed that reoxygenation was less apparent after a single dose of 300 rads than 1500 rads. A fractionated irradiation of total dose below 3000 rads was less effective than a

single dose of the same magnitude, due to the predominance of recovery processes over the reoxygenation effect at these dose levels. Hawkes et al. (147) in studies of both spontaneous and first generation isotransplanted mammary tumour in C3H mice, irradiated with 14 MeV electrons, found that two equal fractions at 24 hours interval were more effective than a single dose, when the total dose levels exceeded 2500 rads and 3000 rads; for transplanted and spontaneous tumours, respectively, in mice breathing oxygen. No difference was found below these dose levels or in mice breathing nitrogen. They suggested that reoxygenation became apparent after 2500 rads when the hypoxic cells predominated and recovery was an important factor in transplanted tumour below total dose of 2000 rads.

1.10 The Gross Response of Tumours Following Irradiation: Denekamp (25) in an analysis of the response of several animal tumours after single doses of 1-2 Krads of X-rays, noticed that the variation of individual tumours within any tumour type was remarkably small in the first few days, but this variation increased with time, specially during the phase of regrowth. She found that animal sarcomas continued to grow for 2 to 3 days before regression while carcinomas showed immediate regression. Denekamp concluded that the initial rapid shrinkage of carcinomas was not a direct result of radiation induced cell death, but unmasking of the normal rate of cell

loss by halting cell production by radiation induced mitotic delay. She attributed the difference in response between sarcomas and carcinomas to differences in the vascular architecture, mode of death or the relative amount of oedema from radiation injury. The initial increase in size of some tumours and the immediate regression of others have been reported by several investigators (21,25,85,88,130,148,149). Induced oedema due to increased permeability of the blood vessels (section 1.6) or the transient proliferation of the doomed cells (21) may be responsible for the initial increase in size of some tumours after irradiation.

The observed response of tumours, after irradiation is the net result of many dynamic processes, mainly cell death, cell loss and cell production and it seems a very poor indicator and inaccurate estimate for the number of tumour cells killed and the kinetic behaviour of the cell population. Both Suit (150) and Denekamp (151) commented that the observed response of tumours after irradiation would reflect the rate of removal of the dead cells and cell debris and also the efficiency of cell loss mechanisms. Thomlinson (152) pointed out that these mechanisms are radiosensitive and can be affected by radiation. Hermens and Barendsen (21), in a study of a rhabdomyosarcoma in rat, pointed to the discrepancy between two effects induced by a dose of 2000 rads of X-rays; namely, a decrease of the

surviving fraction of cells below 1% and a decrease in the tumour volume by only 25%. The regrowth of this tumour started 8 days after irradiation, reaching the pre-irradiation size 4 days later, while rapid proliferation of the surviving fraction at shorter cell cycle time commenced 4 days after irradiation, reaching the pre-irradiation value 5 days later. Denekamp and Thomlinson (148) did not find any conspicuous changes in the cell generation time, nor the growth fraction, 14 days after 1500 rads to C3H mammary tumour. Brown (153) did not observe big changes in the growth fraction but slight prolongation of the cell cycle time 1 to 3 days after 500 or 1000 rads to induced squamous cell carcinoma of the hamster cheek pouch. Szczepanski and Trott (149) in a study of transplanted adenocarcinoma 284 in C3H mice, found a transient doubling of the growth fraction 6 to 8 days after irradiation with doses of 600 or 1200 R, due to triggering of the resting cells (G_o cells) into cycle at normal or slightly slow speed. This tumour resumed growth 6 to 8 days after irradiation. The pattern of shrinkage and the time required for the tumour mass to regress completely after large doses of radiation, vary in tumours with different histological structure and within each tumour type. The regression rates tend to be similar over the range of large doses of radiation. This is despite the wide difference in the probability of local tumour control "cure" by these doses. These observations can be deduced from the

several published growth response curves for a variety of animal tumours following irradiation (88,131,154, 155).

1.11 The Processes Occurring in Split Dose Irradiation:

Several biological processes operate in the interval between two fractions of radiation, in vitro. These processes include:

1 - Recovery from sublethal damage (156,157)

2 - Division delay and partial synchronization of the cells surviving the first dose (158-161)

3 - Repair of potentially lethal damage (162,163)

and 4 - Repopulation by the surviving cells

Full recovery from sublethal damage occurs rapidly within 2 to 6 hours and is measured by reconstruction of the shoulder of a single dose survival curve or in terms of the parameter D_2-D_1, where D_2 is the total dose given in two fractions to produce the same biological effect as a single dose D_1. D_2-D_1 value depends on several factors including: the quality of radiation, the cell type and its capacity for recovery from sublethal damge, the magnitude of the doses delivered and the time between fractions.

The first dose sterilizes a high proportion of cells in the more sensitive stages of the early cell cycle, mainly mitosis late G_1/early S and G_2, leaving a high

proportion of the cells in the more resistant early G_1 and late S phases. Maximum division delay occurs if the first dose is delivered during the S and G_2 phases (158,159). The Elkind type of cyclic variation in radiosensitivity is then observed as the partially synchronized population progresses through the next cell cycle. A prompt rise in D_2-D_1 value in the first 2 to 6 hours is due to recovery from sublethal damage. This is followed by a dip and a further rise as the cells move to a sensitive then another resistant phase of the cell cycle. Radiation induced division delay appears to be dose dependent, hence, a longer division delay occurs as the magnitude of the first dose increases (approximately one minute for each rad). Young and Fowler (164) showed that D_2-D_1 value would increase with increase in the total dose, possibly due to a greater degree of induced synchronization. They also showed that the maximum value would be obtained by equal, rather than unequal fractions, while a lower or even negative value could be obtained by delivering a second dose of certain magnitude during the more sensitive state of the partially synchronized population. This means that synchronization may result in either more or less dose being necessary to produce the same degree of cell killing with two dose fractions, relative to a single dose. This is in contrast to recovery from sublethal damage and repopulation which necessitate an additional dose.

In vivo, the shoulder on a single dose survival curve cannot be determined but the additional dose increment (D_2-D_1) can be obtained. Alternatively the reduction in the biological effect from giving the same total dose in two fractions can be measured and compared with a single dose that produces the same level of biological effect. However, accurate estimate of recovery in tumours is difficult and complicated due to the interaction of the various processes mentioned earlier for in vitro irradiation, particularly the existence of hypoxic cells and reoxygenation in tumours (sections 1.8 and 1.9). Tannock (165) pointed out that tumour cells are not simply "aerobic" or "hypoxic" but exist in the tumour under a broad range of oxygenation state from fully oxygenated (cells located near a capillary wall) down to a severe hypoxic or even approaching an anoxic state. Several investigators (117,118,166-169) have found impairment or even loss of the recovery capacity in severe hypoxic state. Thus, the improvement of the oxygenation status of acutely hypoxic tumour cells at the end of hypoxic irradiation or chronically hypoxic tumour cells due to reoxygenation, would promote the recovery of these cells. However, since reoxygenation increases the sensitivity of the tumour cells to subsequent doses, it will obscure the observed cyclic variations seen in split dose experiments in vitro and will also underestimate the magnitude of the additional dose increment needed.

No additional dose will be needed if reoxygenation exactly balances recovery and even lower doses may be needed i.e. a negative D_2-D_1 value, if reoxygenation is more effective than recovery (142,147,170,171). In the latter case, two dose fractions would be more effective than a single dose.

For more accurate estimates of D_2-D_1 values for tumours, the effect of reoxygenation should be eliminated by making the cells either uniformly hypoxic or uniformly sensitive by a potent hypoxic cell radiosensitizer (Misonidazole) (172). The D_2-D_1 value obtained under hypoxic conditions is then divided by an assumed oxygen enhancement ratio (OER) of 2.5 to 3 to obtain the dose increment of recovery under well oxygenated conditions. Denekamp and Harris (172) pointed to possible artefacts in the measurement of the recovery increment of tumours by both methods; namely, the assumed OER and the possible cell damage and kill by hypoxia (144,173,174) and the cytotoxic effect of misonidazole and its enhancement ratio which is close to, but not similar to, that of oxygen.

Phillips (175) in a study of the published data, showed the variable recovery capacity of various normal tissues, with the highest values for the skin and gut (D_2-D_1 values of 500 to 900 rads). He also claimed a smaller recovery potential for tumours (D_2-D_1 values of 200 to 400 rads). The existence of homeostatic control and the absence, or near absence, of severely and

chronically hypoxic cells in normal tissues may play a part in the large recovery potential of these tissues. However, Withers (176) could not find significant differences in D_2-D_1 values between normal tissues and tumours. He concluded that the overlap in these values was too much to reach any conclusion.

Repair of potentially lethal damage (PLD) may contribute to the D_2-D_1 value. This repair has been shown in vitro in density inhibited plateau phase cell cultures (119-121) and in vivo in experimental tumours grown in both ascitic and solid forms (135,177,178). PLD seems to occur in poorly nourished, hypoxic and non-actively dividing cells, believed to exist in many tumours (section 1.8) and behaves in a manner similar to the cells in the plateau phase of a cell culture. This type of repair has been found to be completed in 4 to 6 hours and to be dependent on both the postirradiation environment and the dose, leading to decreased sensitivity of the cells in the high dose range.

The extent and rate of repopulation has been tested in multifractionated experiments of various schedules and in split dose experiments with intervals beyond 24 hours. Several investigators have shown that a smaller additional dose was needed to compensate for repopulation in the skin and tumours of experimental animals in comparison with the dose increment for recovery from sublethal damage (21,176,179-182). The delay in onset and effectiveness of repopulation has

been shown in several studies. Hermens and Barendsen (21), in a study of rhabdomyosarcoma in rat, found no cellular proliferation for about 4 days following a single dose of 2000 rads. Denekamp and Harris (170) in a study of carcinoma "NT" in CBA mice by two dose experiments, also found no appreciable repopulation effect up to 5 days. Fowler et al. (136) and Suit et al. (183) concluded that repopulation was less important in fractionated irradiation over short overall periods and with large doses per fraction. These investigators showed that repopulation became effective in early generations of C3H mammary tumours, if the overall period of fractionated irradiation exceeded 10 to 18 days.

Induced division delay plays an important role in halting the onset of repopulation for a period which is dependent on the magnitude of the first dose. However repopulation may commence at a faster rate due to shortening of the cell cycle (21). The presence of surviving cells among lethally irradiated cells might enhance repopulation of these surviving cells (section 1.1.3). This would be of greater importance after large doses when the surviving cells are mixed with a large number of killed cells.

1.12 Hypoxic Cell Radiosensitizers:

One of the most promising approaches to overcome the problem of hypoxia in tumours, is the use of electron-

affinic chemical radiosensitizers. Adams (184) summarized the properties essential for a hypoxic cell sensitizer:

1 - The compound must selectively sensitize hypoxic cells;

2 - It must be non-toxic to normal tissues at the therapeutic dose;

3 - It must be capable of diffusing readily into poorly vascularized areas of tumours, to the distant hypoxic cells;

and 4 - It must not be subject to rapidly metabolic breakdown.

These compounds have the following advantages over the use of hyperbaric oxygen and high LET radiation: the relatively low cost; the ease of administration; and the wider range of diffusion in comparison with 150 μ for oxygen. The vast majority of these compounds appear to be simple oxygen mimetics (185) and their efficiency of sensitization is related directly to the electron affinity of the compound. Successful sensitization in vitro and in vivo of mammalian cells was achieved by compounds containing the NO_2 group: paranitro-acetophenone (P.N.A.P.) and various analogues (186-192) and nitrofurans (193,194).

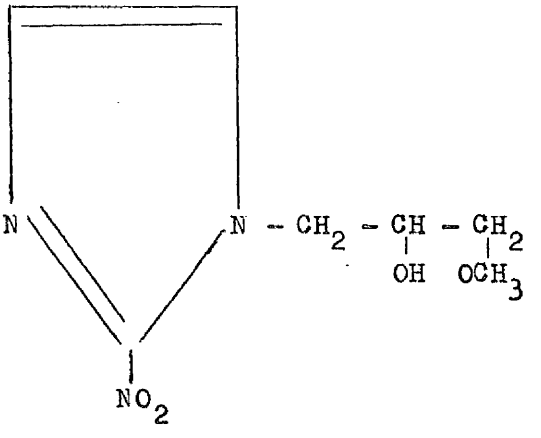
In 1973, attention was focused on nitro-imidazole compounds where the NO_2 group is attached to an imidazole ring. Following in vitro screening of eight chemical radiosensitizers on bacteria S. marcescens and

V79 Chinese hamster cells, under hypoxic and aerobic conditions, Asquith et al. (195), found that a 2-nitroimidazole compound, Misonidazole, with a code number RO-07-0582 was an extremely effective sensitizer of hypoxic mammalian cells and more potent than 5-nitroimidazole, metronidazole, marketed as "Flagyl". It became clear that the location of the NO_2 group at the 2 position of the imidazole ring, instead of the 5 position, has increased the radiosensitization potency.

1.12.1 Misonidazole

This compound was synthesized by Beaman et al. (196) as a protype for new trichomonicides. It has the following chemical structure:

1-(2-nitroimidazol-1-yl)-3-methoxypropan-2-ol



It is a small uncharged molecule which has an octanol: water partition coefficient of 0.4 (185) and is stable in solution in the absence of prolonged exposure to light for 41 days at 2 C° (197) and up to eight hours at 41 C° (198).

1.12.2 Radiosensitization Properties of Misonidazole

1 - Similar to other radiosensitizers, Misonidazole is a specific sensitizer of hypoxic cells but not well oxygenated cells. It has proved also to be more effective than other radiosensitizers both in vitro (189,195,197,199) and in vivo (200-206).

2 - Misonidazole sensitizes hypoxic cells to an extent which depends upon drug concentration with a dose modifying factor (DMF) which approaches that of oxygen by increasing drug concentration in vitro (195,197,199,207) and in vivo (204-206).

3 - It has been also shown that radiosensitization of hypoxic cells by Misonidazole and other electron affinic sensitizers, is independent of the position of cells in the mitotic cycle as in the case of oxygen (186,193,195,207,208).

4 - Moore et al. (197) showed that the radiosensitization property of Misonidazole was independent of the temperature in contrast to the cytotoxicity of this drug, discussed below.

1.12.3 Cytotoxicity of Misonidazole

Several in vitro studies (195,197,198,199,209,210) showed that Misonidazole was not only a specific radiosensitizer of hypoxic cells but also a specific cytotoxic drug to hypoxic rather than well oxygenated cells, dependent both on the concentration of the drug and the duration of exposure. Asquith et al. (197,208)

found that cytotoxicity occurred at a much higher concentration than that which was required for radiosensitization. For example, an enhancement ratio of about 2.5 occurred with 4 mM drug concentration, while less than 30% of the cells were killed at 50 mM drug concentration.

Several investigators (197,198,207,211,212) showed that cytotoxicity, but not radiosensitization of the drug was temperature dependent, being greater with increased temperature range from 22°C to 45°C.

Several animal experiments (201,202,213,214) have also suggested a cytotoxic effect on tumour cells which was more evident with administration of Misonidazole after irradiation. The cytotoxic enhancement ratios, in these studies varied from 1.1 to 1.4 and were small compared with the enhancement ratios observed with administration of the drug before irradiation. However, no cytotoxicity was observed in some tumours and even a small protective effect was seen (155,214).

Bleehen et al. (215) did not observe any significant cytotoxicity of Misonidazole on the EMT6 mouse tumour at 37°C but marked potentiating effect of hyperthermia on the cytotoxic effect of the drug at intra-tumour temperatures above 42.5°C for one hour.

1.12.4 The Pharmocokinetics of Misonidazole

Gas-liquid chromatography and polarographic studies have been used to measure the concentration of

Misonidazole as a function of time, in the serum and tumours of experimental animals and humans (216,217). Maximum tumour drug concentration in mice was generally 20-80% of the peak serum level probably because of the short biological half-life of 1 to 1.5 hours. In humans, drug concentration in tumours usually fell in the range of 50-100% of the peak serum level because of the much longer half-life of 12.5 hours.

Several investigators used radiobiological assays to determine the optimum time between administering the drug and starting irradiation. These assays are better indicators of the concentration of the drug at the critical hypoxic cells in the tumours at any time after its administration (218). Stone and Withers (204,219) found a greater probability of mammary carcinoma control, as the interval between I.P. injection of 1 mg/gm of the drug to irradiation was increased from 3 to 30 minutes. Brown (202) found that the surviving fraction of the EMT6 tumour was lowest at an interval of 30 minutes with doses of 0.3 and 1.2 mg/gm. Sheldon and Hill (220) observed greater sensitization of the anaplastic MT tumour at 30 minutes than 90 minutes for a low drug dose of 0.2 mg/gm; compatible with the decline of the serum concentration of the drug at 90 minutes. Sheldon and Hill (155) also found in another tumour (anaplastic MT1 tumour) an optimum interval of 45 to 60 minutes for maximum sensitization with the same low dose of the drug.

The maximum concentration of Misonidazole in the tumour mass and the period for which that concentration is maintained, would determine the effectiveness of the drug. The magnitude of radiosensitization can be small with low tumour drug concentration and intervals other than the optimum one. The cytotoxicity observed in animal tumours with administering the drug after irradiation, is also likely to be underestimated for the following reasons:

1 - A single or limited number of small doses of the drug are used, in vivo; not a continuous dosage for several hours as used in in vitro experiments. Hence, tumour cells are exposed to the drug for shorter periods due to the short half life of the drug in experimental animals.

2 - There is also the possibility of lower concentration reaching the tumour after irradiation due to vascular damage, especially after single doses of 20 Gy or more as discussed in section 1.6. However, because of the drug's longer half life in man, the interval between administering the drug and radiotherapy may not be so critical for either the radiosensitization or cytotoxicity.

1.12.5 The Use of Misonidazole in Experimental Animals and Humans:

The action of Misonidazole in any in vivo situation is likely to reflect a combination of its radiosensitization properties coupled with its

cytotoxicity which are selectively against hypoxic cells. The maximum effects of the drug, therefore can be expected in tumours whenever hypoxic cells predominate (218), provided that the drug has access to these cells and is present at the maximum concentration in the tumours. The effectiveness of a sensitizer is usually defined in terms of the "enhancement ratio" (ER) which is the ratio of X-ray dose required to produce a given biological effect with sensitizer to the dose required without sensitizer. In a wide variety of mouse tumours, with variable proportions of hypoxic cells and variable doubling times, Misonidazole was found to have enhancement ratios of 1.5 to 2.4 and 1 to 1.9 with doses of 1 mg/gm and 0.2 to 0.3 mg/gm, respectively (218). The drug was given immediately or several minutes before irradiation and different assay methods were used to measure tumour response: delay in growth (133,174,201), local control (i.e. cure) (206,219,220), cell dilution assays (133, 202) and loss of ^{125}IUdR from tumour cells (174). Some animal experiments have suggested a loss or decrease of efficiency of Misonidazole whenever well oxygenated cells predominate in the response to irradiation, as is the case with single doses below 2000 rads in sarcoma F (133) and 1200 rads in CBA carcinoma NT (201). At these low doses of irradiation, tumour response is mainly due to killing of well oxygenated cells. The ER was found to increase

approaching that of fully hypoxic population with increase in dose, due to the predominance of hypoxic cells after the killing of well oxygenated cells by the lower doses.

The decrease in efficiency of Misonidazole has also been demonstrated with fractionated irradiation in tumours which are known to reoxygenate rapidly, with decrease of the number of hypoxic cells between successive doses. This was the case with first generation C3H mammary carcinoma (206,221) where the enhancement ratio fell from 1.8 for single doses of irradiation to 1.1 or 1.2 for fractionated irradiation; and also in the case of CBA carcinoma NT (172) where the enhancement ratio fell from 2.1 for single doses of irradiation to 1.6 and 1.2 for 2 fractions in 2 days and 5 fractions in 9 days, respectively.

The loss of efficiency was less marked in the anaplastic tumour MT1, studied by Sheldon and Hill (220). Enhancement ratios of 1.7 and 1.5 were found for this tumour for single doses of irradiation and 5 fractions in 4 days, respectively. These investigators have attributed this slight loss of efficiency of Misonidazole to the minimal degree of reoxygenation. Johnson et al. (222,223) in studies of Midonidazole in primates, have also raised the possibility of some form of enzymatic drug induction which leads to a more efficient destruction of the drug, following repeated administration. This phenomenon seemed to differ among

species and was not observed with repeated doses in human (224).

It was concluded from early studies on patients, that Misonidazole showed good promise as a radiosensitizer in clinical radiotherapy (225). Since then, considerable interest has developed to assess the use of the drug in combination with radiotherapy in human solid tumours (see the proceedings of the 8th L.H. Gray Conference, Cambridge, 1977). These trials are designed to explore the best clinical application of the drug within the dose limitation imposed by the neurotoxicity of the drug mentioned below.

1.12.6 Toxicity of Chemical Radiosensitizers

Denekamp et al. (200) in their in vivo screening of some electron affinic compounds, found that LD_{50} (drug dose which is lethal to 50 per cent of mice) was: 0.2 mg/gm for N.D.P.P.; 0.3 mg/gm for Nifurpipone; 1.8 mg/gm for Misonidazole and 3.8 mg/gm for Flagyl. Despite the higher LD_{50} for the latter the therapeutic index of Misonidazole was about 4 times greater, indicating the particular effectiveness of Misonidazole compared with Flagyl.

A mild soporific effect of Misonidazole has been described in mice (200) which entailed slight reduction of the dose of anaesthesia (133). Sheldon et al. (206) described early death in mice with doses of 0.67 mg/gm

administered before daily fractionated irradiation due to Misonidazole "gut syndrome" i.e. the stomach was very full of dry food while the rest of the gut was completely empty. This occurred usually on the fourth or fifth day after starting irradiation and in 15 per cent of the 5 fractions in 4 days schedule. It did not occur in either the 3 fractions in 4 days or the 5 fractions in 9 days regimes. Therefore, it seems to be related to the cumulative daily dosage of the drug in a short period.

Schärer (226) described cerebellar degenerative changes and an acute syndrome in dogs in the form of ataxia, convulsion and death after repeated high doses of nitro-imidazoles. This toxicity was not observed in other experimental animals (mice, rats, guinea pigs or rabbits) and was considered a specific species sensitivity in dogs. Parkes (quoted 216) found that doses of 400 mg/kg of Misonidazole in monkeys, produced reversible severe muscular inco-ordination and incipient convulsions by day eleven from which the animals recovered within 24 hours after cessation of drug therapy. Small bilateral brain lesions were found at all dose levels even though no clinical signs were detectable with small doses.

In the human; mild insomnia and gastro-intestinal disturbances occurred with Misonidazole (216) and transient drowsiness, nausea and vomiting after Flagyl (227,228). Mild to severe neurotoxicity has been

described for both Misonidazole (224) and Flagyl (229) and on rare occasions convulsions have been reported after very high doses (230). Electron microscopy studies of affected peripheral nerves have revealed distal axonal degeneration with segmental demyelination, predominantly affecting small myelinated nerve fibers (231). This neurotoxicity is the main dose limiting factor for Misonidazole. It appears to be related to the total dose of drug administered and the time interval over which it is given. Various dosage regimes have been proposed on this basis including: once or twice weekly doses to a total dose of 18 g or 12 g/m^2 body surface area (231,232) and many small doses, combined with a convential course of multi-fraction radiotherapy (233).

CHAPTER II

MATERIALS AND METHODS

2.1 Host Animals:

Normal, healthy, virgin C3H mice aged between 8 to 12 weeks, of either sex were used in the experiments. The weight of the mice at this age varied usually between 20 to 25 g. Most of these mice were bred in our own Animal House by a conventional inbred mating system. For some experiments, C3H inbred mice from Bantin and Kingman Ltd., Hull, were used when the number of mice from our inbred colony was not enough. There was no difference between these and our inbred mice, regarding tumour growth and response to radiation. Mice were housed in polypropylene cages 192.5" in area, maximum of 10 mice per cage. This number was convenient from the point of view of the available space in the animal house and also avoidance of overcrowding of mice in each cage. The breeding, isolation and experimental rooms were kept at a constant temperature of $21 \pm 1\ C^{o}$ with relative humidity of $55 \pm 5\%$ and consecutive 12 hour periods of light then dark (i.e. light:- 7 am to 7 pm; dark:- 7 pm to 7 am GMT).

The animals were fed on formula 41 mouse diet (produced by Angus Milling Company, Perth and supplied by William Shearer and Company, Glasgow) and were provided with tap water ad libitum. The cages were cleaned twice weekly and the water was changed daily. Sterile precautions were taken in an attempt to reduce the risk of epidemic disease. These precautions included autoclaving all the cages and the water bottles and isolation for 2 weeks of

any mice other than those from our inbred colony.
The mice used for the present work shared the rooms with another strain of mice (DBA/2) used for other experimental works in the same laboratory.
Mice of either sex were used due to the short supply of animals. There was no evidence that sex has affected the tumour response in the present system despite those temporary differences reported by Fowler et al. (1).
No spontaneous tumours appeared in the experimental mice during the period of observation (100 days).
Furthermore, the mice which were considered as cured and were kept for more than 100 days showed no evidence of illness except for a few spontaneous tumours in some of the mice.

2.2 Animal Marking System:

In the initial pilot study, the ear marking system was used to identify the mice. However, this was found to be unsatisfactory due to the limited number of ear codes and also because on several occasions, it was difficult to identify the code due to loss of part of the ears.
A dye marking system was adopted using an aqueous solution of saturated picric acid. This was thoroughly applied to the skin and fur of the mice with a paint brush.
The dye system provided an adequate number of codes for the experimental animals. Furthermore, this system lasted at least for four months and no skin irritation

or carcinogenic effect of the dye were observed.

The marking system consisted of one, or a combination of the following nine basic codes: right ear; left ear, right forelimb; left forelimb; right hind limb; left hind limb; back; front and tail. (Nearly 60 different codes are available from this system.)

2.3 The Tumour:

C3H mammary tumour, originally obtained by A.H.W. Nias in May 1973 as a spontaneous tumour in a mouse from the Gray Laboratory (Angela Walder and J.F. Fowler) has since been maintained at our laboratory by subcutaneous transplantation every 2-3 weeks by cytosieve tumour suspension in syngeneic recipients.

By the time the study commenced in 1975, the transplanted tumour showed a definite departure from the histological features, growth rate and response to irradiation of the original tumour. These features have since remained constant. No previous experimental data were available before the present work except for a few histological sections of the early transplants of this tumour.

However the data obtained from this work can be compared with those published from the Gray Laboratory about the first generation transplant of this type of tumour.

2.4 Technique of Tumour Transplantation

2.4.1 Preparation of Tumour Cell Suspension:

ALL procedures were carried out at room temperature and under aseptic conditions.

1. A tumour of about 10 mm diameter was excised and freed from the non-tumour debris. This tumour should have begun to grow 7-14 days from the day of transplantation.
2. The tumour was then gently mashed and finally minced by passing it through a fine stainless steel mesh together with 1-2 ml of solid tumour culture medium (see below: 5), using a flat plunger from a 10 ml syringe.
3. The crude suspension was then aspirated through needles of serial sizes, the smallest size being 25 g, leaving the sediment at the bottom of the universal container each time.
4. A cell count of the tumour cell suspension was performed using a haemocytometer. The criteria for intact tumour cells were very similar to those used for squamous carcinoma by Hewitt et al. (6) under phase contrast microscopy:
   i. small rounded cells of cell diameter in the range 8-12 μ.
   ii. smooth outline.
   iii. yellowish tinge with darker outline and surrounded by a narrow halo of light.
   iv. the nucleus and cytoplasm could not be discriminated.

The suspension was found to consist of single cells and 10-15% small clumps of 2-5 cells.

The viability of the cells was tested by the Trypan blue dye exclusion method and found to be $59 \pm 1.7\%$ ($\pm$ s.e.m.).

5. A final tumour cell suspension of 10^6 cells in 0.05 ml was obtained by the appropriate dilution with the solid tumour culture medium. The latter consisted of:

500 ml MEM + Hank's salt solution
50 ml Horse serum
5 ml 200 µM glutamine
3 ml MEM non-essential amino acids
2 ml sodium hydroxide

The random variations in density and degree of dilution needed were due to:

i. slight variation of the size of the tumour used.
ii. the efficiency of hand mincing.
iii. the degree of dilution and washing for the tumour mince.

It was essential to gently shake the cell suspension during the process of injections as it was noted that the tumour cells tend to sediment forming large clumps.

2.4.2 The Injection and Site of Transplants:

10^6 cells in 0.05 ml were carefully injected,

subcutaneously, aiming at the midline of the back of non-anaesthetized mice, at the level of the abdomen. No shaving, or sterilization of the fur were done before the injections.

This site for transplantation was chosen, for the following reasons:-

i. Easy palpation and measurement of the 3 perpendicular diameters of the tumours.

ii. Easy irradiation of the tumour with shielding of the rest of the mouse body by a simple and practical jig, to avoid irradiation of important structures which can affect and modify the tumour response.

iii. No restriction of the normal mobility of the mouse and its ability to gain access to food and water, as we found occurred in those mice with tumours, especially larger sizes, in the thigh and the ventral aspect.

The whole procedure from the time of killing the mouse bearing the tumour till the last injection of the inocula, in not more than 40 mice was about 30 minutes. It was feared that centrifugation, trypsinization and other such lengthy procedures might damage and reduce the integrity and viability of tumour cells.

2.4.3 Transplantation in Cured and Preirradiated Sites:

The aims of these experiments were to study the effect of the tumour bed on the percentage take, latent period

and the growth of the tumour and also to find out any difference between the tumour bed effect in the cured and preirradiated sites.

The technique of transplantation was similar to that described in section 2.4.2 and mice with preirradiated or cured areas of only 1 to 1.5 cm diameter were used for these experiments. The injections were aimed at the centre of the treated areas; however a few deep, ulcerating, double and marginal tumours were obtained. These were discarded from the analysis.

The cured mice were collected from several irradiation experiments performed at different times. Hence the time between irradiation and transplantation varied between 100 and 250 days. These mice were collected in two groups: one group of mice received single doses from 60 to 85 Gy X-rays alone and another group of mice was treated with single doses from 25 to 45 Gy X-rays plus Misonidazole. A single dose of 70 Gy was chosen for skin irradiation (see section 2.5.5) and the time between irradiation and transplantation was between 100 and 110 days.

Despite these inevitable differences in the doses of X-rays, the timing between irradiation and transplantation and the age of the mice, the experiments were carried out on the assumption that neither the time nor doses of 25 Gy or more would have appreciable effects on the tumour bed effect as mentioned in section 1.7.

2.4.4 Transplantation Using Recurrent Tumours:

The aim of this experiment was to find out if the reduced growth rate of recurrent tumours was due to a tumour bed effect or a cell damaging or a cell selective effect of radiation on tumour cells. From the recurrent tumours following single X-ray doses of 50 and 60 Gy, a 10 mm diameter tumour was chosen from each dose and prepared into a cell suspension, using similar procedures as described in section 2.4.1. However, these tumours were difficult to excise and mince due to the irradiated surrounding tissues and the tough consistency of the tumours. This difficulty in mincing and the excess tumour debris led to a small yield of cell suspension, only enough for 5 recipient mice from each tumour after the dilution to 10^6 cells per 0.05 ml.

2.4.5 Other Transplantation Procedures:

The same transplantation technique described in section 2.4.2 was used for transplantation in immunized mice and outside the cured sites.

2.5 Technique of Irradiation

2.5.1 The X-ray Unit

X-rays were delivered from a Siemens Stabilipan I unit, operating at 250 kV and a filament current of 15 mA. Beam filtration was such as to give an experimentally determined first HVL of 1.85 $\pm$ 0.05 mm Cu and a dose rate of 110 rads/minute at 57 cm FSD. The X-ray tube was

positioned at the base of a shielded box, consisting of lead plywood supported on a simple metal frame with the X-ray beam directed toward the top surface of the box. The construction of the unit in that way was very convenient for tumour irradiation and other radiobiological studies (diagram 2.1 and plate 2.1).

2.5.2 Irradiation Procedures

In a pilot study, mice were restrained in perspex tubes which were positioned so that the tumour and the abdominal cavity were over a 1.5 cm wide slot cut in a 3 mm thick lead sheet. The aim of that method was the comparison of the radiation effects on the tumour and that of the gut. The method was abandoned since all mice died from the gastro-intestinal syndrome in 4-5 days after single doses of >1800 rads without any detectable effects on the tumour, and also since gut damage with lower doses might modify the tumour response. A final method was adopted for irradiation of the tumours at 6 ± 1 mm mean external diameter, with lead shielding of the mouse body. All the irradiation procedures were carried out at room temperature with mice breathing air and without anaesthesia.

Four cylindrical jigs have been designed for this irradiation technique. Each was made of a sheet of lead, 2 mm thick, 9 cm long and 8 cm wide. A slit of 1.5 mm width and 6 cm length was created where the edges of the lead sheet met, (plate 2.2). Plastic caps with multiple

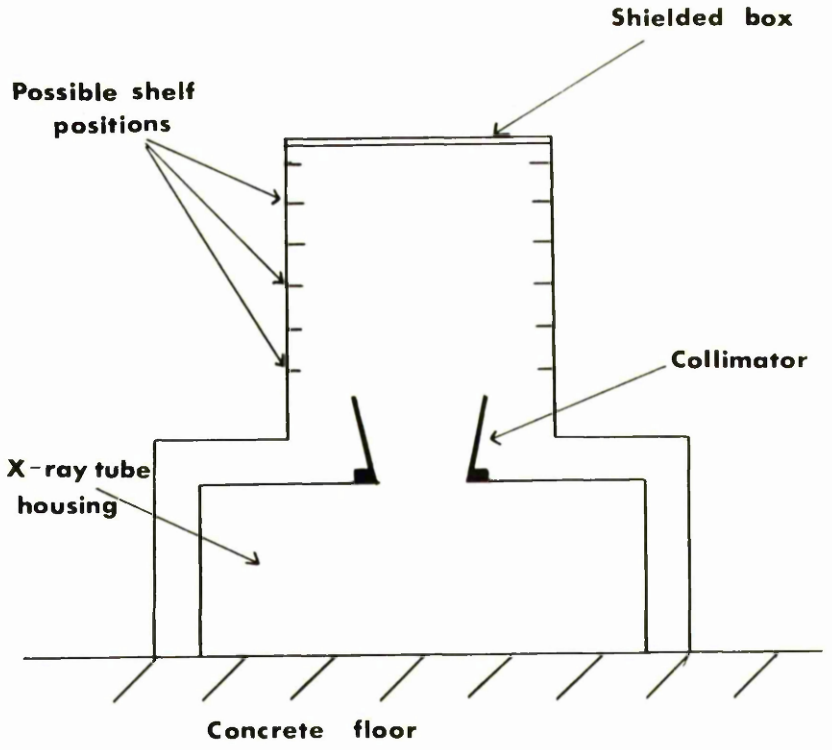


Diagram 2.1: Schematic plan of irradiation box.

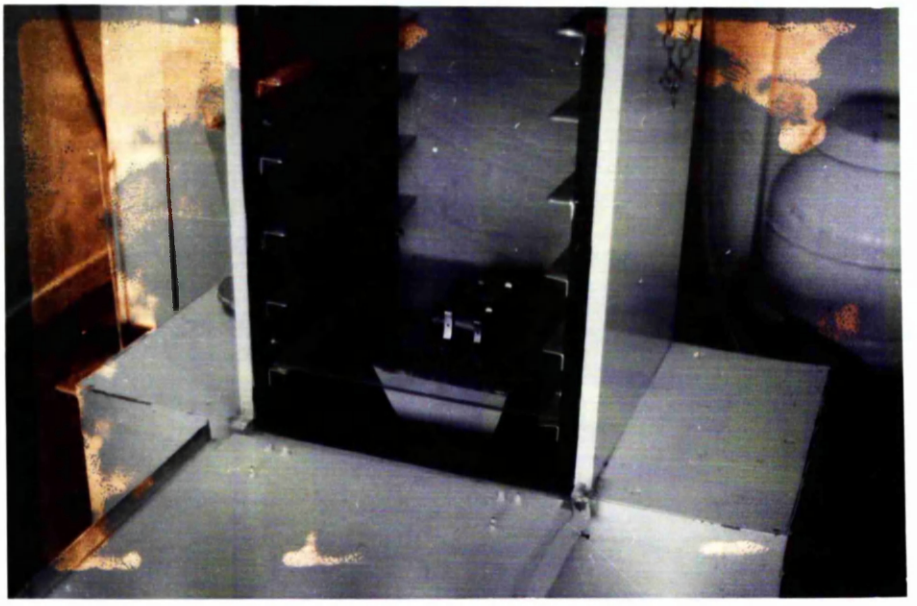


Plate 2.1: Set up for tumour irradiation.

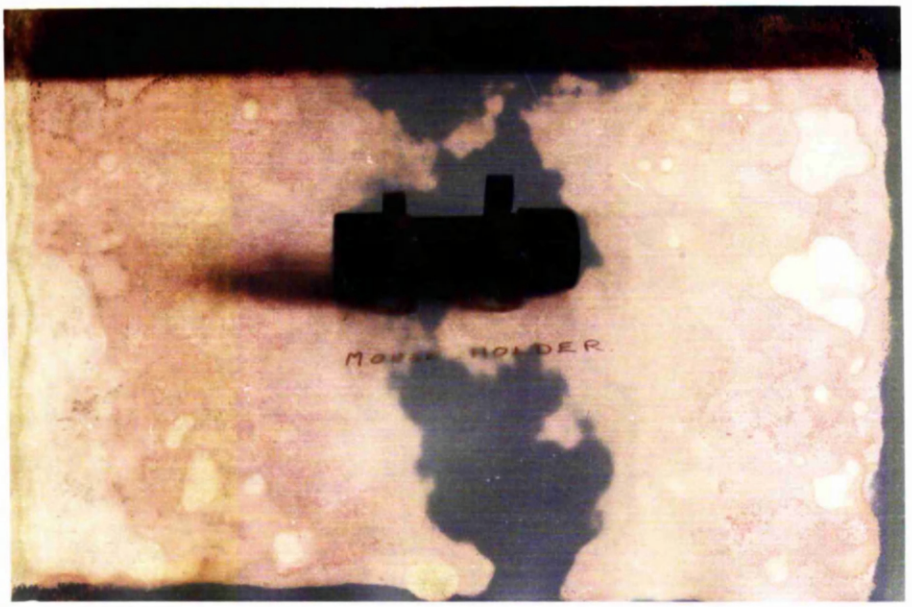


Plate 2.2: Irradiation jig.

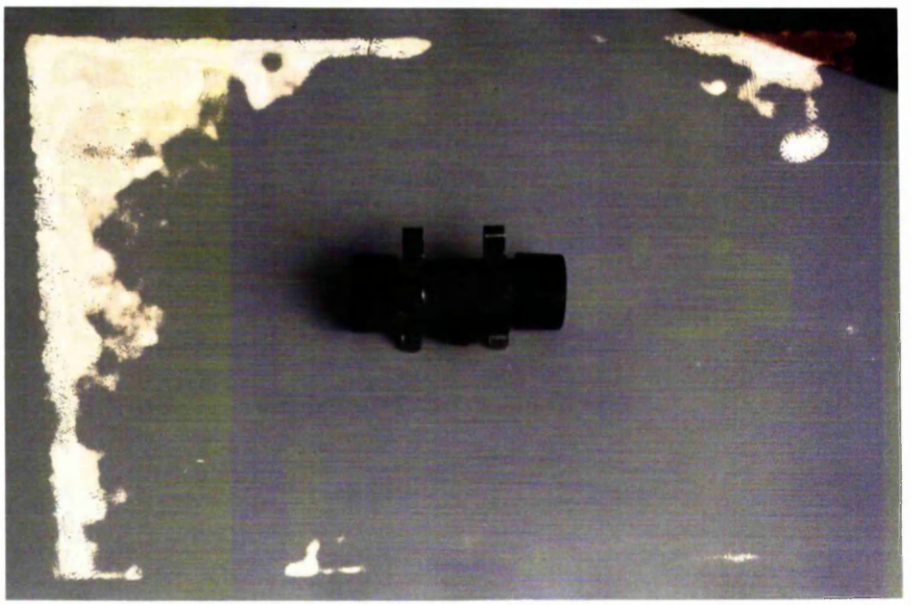


Plate 2.3: Tumour held outwith the irradiation jig.

airholes were fitted at each end to allow a plentiful supply of fresh air for breathing and to reduce perspiration during irradiation.

A mouse was introduced into each jig and the tumour was held outwith the lead shielding by gentle guiding of the tumour pedicle through the slit (plate 2.3). The width of the slit was such that only minimal pressure would be applied to the two skin layers.

A removable metal clip was placed at each end of the jig to support it on a perspex shelf during irradiation. Two rectangular lead sheets 3 mm thick and 1.5 cm apart were fixed to the undersurface of the shelf. This arrangement was designed to limit the width of field of irradiation to 1.5 cm, across which a maximum of 4 tumours could be irradiated simultaneously. The smooth upper surface of the shelf allowed free manipulation of the jigs for the appropriate positioning of the tumours over the narrow field of irradiation (plate 2.1).

The majority of the animals relaxed quietly in the jigs within a few minutes, but some of them nibbled on the anterior edge of the lead.

Irradiation was started soon after restraining the mice and setting the tumours in position. The tumours were irradiated with their centres 1 cm above the shelf or 57 cms from the target of the X-ray tube, in a tangential position to avoid any shielding by the lead jig. The tumours were kept in the same position and without 180° rotation during irradiation. Due to the

tumour site on the back, the mice were lying on their sides.

2.5.3 Dosimetry:

All the dose rate measurements were made using a Farmer-Baldwin dosemeter which had been calibrated against a standard instrument for the energy used. The dose rate was checked at frequent intervals.

The dose profile within the lead jig and the centre of the tumour was determined using LiF thermoluminescent dosemeter. Sachets of LiF μ-rods were placed within the jig which was filled with tissue equivalent material (standard bolus - $MgSO_4$/sucrose spheres) and in the centre of a tumour phantom made from the same material enclosed in a thin rubber covering.

The tumour dose determined by TLD dosimetry was in good agreement with the ionization chamber measurement. The absorbed doses to the shielded parts of the mouse were found to be approximately 1% of the tumour dose (A.M. Perry, personal communication).

2.5.4 General Comments on the Technique of Irradiation:

No significant difference was observed between the tumour response using the above irradiation technique and that of clamped tumours (section 2.5.5). This might indicate a hypoxic nature of the tumour, especially with the presence of multiple areas of necrosis (section 3.12.2). However, this obtained tumour response might

partially result from impairment of the tumour blood flow by the irradiation technique as suggested by the following:-

1 - the adverse effect of animal restraining on the blood flow has been claimed by Zanelli and Lucas (234). The restraining periods in the present system varied from 5 minutes to more than one hour according to the delivered dose;

2 - the tumour blood supply originated from the thin walled subcutaneous blood vessels. These vessels were easily distorted by slight pressure or minimal skin traction. This would occur with the tumour pedicle pulled out through the narrow slit of the irradiating jig and the attempts of the unanaesthetized mouse to pull the tumour in, during irradiation;

3 - impairment of Xenon-133 clearance from the tumour when the mouse was restrained in the jig (section 2.6).

Despite this possibility of impairment of the tumour blood flow during irradiation, this technique of irradiation has been adopted throughout the present work for the following reasons:-

1 - It was not desirable to use anaesthesia, as it impairs the blood flow of tumours (235-237). Furthermore, long treatment periods were needed, especially with the large single doses, due to the relatively low dose rate of the available X-ray

machine.

2 - It was essential to avoid any radiation induced animal deaths (gut; bone marrow) because of the short supply of mice;

3 - the technique has provided adequate restraining of the unanaesthetized mouse and adequate shielding of the gut and bone marrow during irradiation. The width of the slit was to prevent the inner part of the tumour from sliding back into the jig, whilst still avoiding complete obstruction of the tumour blood supply and the possible damaging effect of prolonged clamping mentioned in section 1.11.

In the preparation for tumour irradiation, the tumour, the surrounding skin and the subcutaneous tissue with the needle track were included in the field of irradiation to reduce the possibility of marginal recurrences due to invisible tumour foci. However, at the end of irradiation, some of these tumour pedicles were pulled in by the unanaesthetized mice. This was the most likely reason for some marginal recurrences in the system. These were excluded completely from analysis.

2.5.5 Irradiation Procedures for Clamped (Hypoxic) Tumours:

The same procedures as those described in section 2.5.2 were followed using 4 identical jigs. The only difference was that no slits were created where the edges of the lead sheet met. Hypoxia was achieved by

the squeezing effect of the blunt edges across the tumour pedicle on the application of the plastic caps. This method was enough to completely stop the blood supply of the tumours as suggested by the absence of Xenon-133 clearance (section 2.6). An interval of 10 minutes was allowed between the clamping off procedure and the start of irradiation.

To find out the effect of clamping itself, 12 unirradiated tumours were clamped for 1.5 hours; the longest time needed for irradiation of clamped tumours. No necrosis of skin, nor changes in tumour growth rate were noticed (table 3.6).

2.5.6 Irradiation Procedures for Skin Folds:

Irradiation of the skin folds was performed to provide enough preirradiated sites for the study of the tumour bed effect, the study of skin reactions in the absence of tumours and also for the study of the effect of radiation received by the mouse body inside the jig on the phytohaemagglutinin (PHA) index. The procedures for skin irradiation were similar to those described for tumour irradiation (section 2.5.2). A skin fold from the back of the mouse (the usual site for routine transplantation) was passed through the slit of the irradiating jig and fixed loosely with sellotape to the side of the jig nearer to the shelf. The diameter of this fold was about 1.5 cm. However, some of the mice pulled in part of the fold during irradiation.

A single dose of 70 Gy was chosen for irradiation of the skin. This dose was in the dose range that produced high tumour cure rates and was beyond the dose levels for maximum tumour bed effect (i.e. the plateau phase) as mentioned in section 1.7.

2.5.7 Split Dose Experiments:

The purpose of these experiments was to study the radiobiological processes occurring in the split dose experiments mentioned in section 1.11. This was in relation to intervals of 3, 12, 24, 48 and 72 hours in between the two fractions and also to the initial dose levels of 30, 35 and 50 Gy.

A total dose of 70 Gy was chosen in order to analyze the results using both the cure percentage and the delay in growth. Furthermore, the magnitude of the radiobiological processes would be at or near their maximum.

The minimal initial dose used in these experiments was 20 Gy which would be enough to kill all or most of the well oxygenated cells. Hence the obtained results would be mainly due to the hypoxic tumour cells. This dose would also be expected to inflict nearly maximum vascular damage; therefore, this damage would be the same at higher dose levels (i.e. a common factor in all the experiments) and would not influence the obtained results.

2.5.8 Misonidazole:

The hypoxic cell radiosensitizing drug "Misonidazole" (kindly supplied by Prof. G.E. Adams) was freshly made up as a sterile solution in 0.9% W/V saline at a concentration of 25 mg per ml. Incubation of the solution at 37° for half an hour helped the drug to dissolve completely.

The solution was then injected intraperitoneally 30 minutes before the start of irradiation at a dose of 1 mg/gm body weight for single dose experiments and 0.67 mg/gm in the split dose experiments.

The timing of injections and the doses used were chosen for comparison with much of the published literature (21,23,35,48,51,58,63,64). The optimal timing for administration of the drug was not tested; however, the obtained high enhancement ratio (ER) would suggest that 30 minutes was within the optimal timing for the present work.

2.6 Xenon-133 Studies:

Intratumour Xenon-133 injection technique (237,238) was used in this work to test any impairment of the blood supply to the tumour with the jig used for routine irradiation. It was also used to test complete occlusion of the blood supply with the jig used for tumour clamping.

There is little previous work and few quantitative data available on blood flow in tumours and various methods

have been used: systemic dyes (239-241); the oxygen cathode method (74,242); thermodynamic system (243); isotope indicators: potassium-42; rubidium-86; krypton-85 and xenon-133 (237,238,244-246). Hence, it is difficult to evaluate the accuracy of each method and it was felt that the xenon-133 clearance technique was suitable for a limited blood flow study as in this work.

With a radioactive inert gas like krypton-85 and xenon-133, the slope of the monoexponential clearance curve is usually assumed to be related to the blood flow in tissues, but only under certain conditions (viz: homogeneous tissue, removal only by constant blood flow; immediate diffusion equilibrium and no recirculation). However, tumours are not homogeneously perfused tissues (necrotic avascular areas and viable vascular areas). The clearance pattern, in this case is very complex and a multiexponential clearance curve would be expected. Both, Gillespie (247) and Strang (248) have drawn attention to the great care and the complex mathematical analysis of the clearance from non-homogeneous tissues. For this reason, xenon-133 was used in this work as a qualitative and not absolute measurement of tumour blood flow.

Radioisotope Technique:

Unanaesthetized C3H mice bearing tumours were used for this study; one mouse at a time. The tumour size was the same as that used for irradiation (6 ± 1 mm mean

diameter) and the fur covering the tumour was clipped before the xenon-133 injection. 33 μCi in 10 μl volume of an isotonic saline solution of Xenon-133 (10 mCi/3 ml) was injected at 3 sites along the needle track at the middle of the tumour, using a 50 μl syringe and 25-gauge needle. Precautions were taken to keep the solution free of air in both the vial and syringe.

The mice were placed immediately after the tumour injection in a plastic basket of inside dimensions 3 x 3 x 4.5 cm, or before the injection in the jigs used for routine irradiation or tumour clamping in a similar way to that described for tumour irradiation (sections 2.5.2, 2.5.5).

Radioactivity was detected by a scintillation counter model DM1-1 with a 1.5 inch NaI crystal, placed 20 cm from the mouse. The output of the detector was processed with a Scaler-ratemeter SR5 at a time constant of 10 seconds. Printouts of the data were obtained using Data Dynamic, Model 390 page printer (ASR 33 printout type 5160A). All these equipments were supplied by Nuclear Enterprises Ltd., Edinburgh.

Background activity was recorded before each Xenon-133 experiment and the counting was continued for approximately 30 minutes. The counts included the residual activity in the tumour with minimal contribution from the animal body and the expired air. After omitting some tumours where backtracking of Xenon was noticed along the needle track, 5-8 mice

were available for analysis in each group (basket, jig used for routine irradiation and jig used for tumour clamping).

For each Xenon-133 count, the appropriate background correction was performed and the counts were expressed as a proportion of the initial count.

Figure 2.1 shows Xenon-133 clearance for each group where the relative Xenon count is plotted against time on a semilogarismic graph paper. It can be seen that clamping effected complete stoppage of Xenon-133 clearance. There is also slow clearance for tumours with mice restrained in the jig used for routine irradiation. This is in comparison with Xenon-133 clearance for mice in the basket. These findings suggest impairment of the blood flow of the tumours in the jig used for routine irradiation. The possible mechanisms of this impairment have been discussed in section 2.5.8.

2.7 Immunological Studies of the Tumour System:

As yet, there is no satisfactory method for the assessment of immunogenicity nor for host immunization. This is partly due to the complex nature of the immune system and also the variation in techniques between laboratories. Furthermore, many of these methods and assays are not specific and of low sensitivity, leading to widespread doubts about their quantitative validity and clinical usefulness.

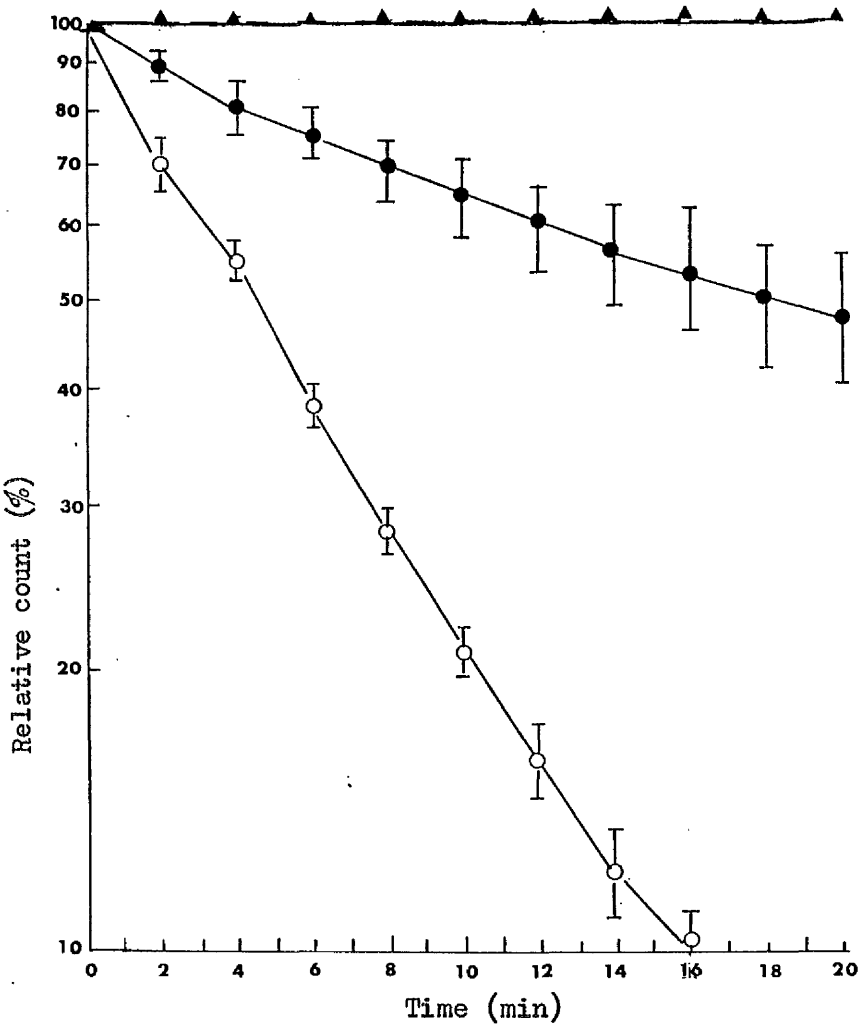


Figure 2.1 ^{133}Xe clearance from tumours.

▲ Clamped tumours

● Restrained mice in the irradiation jig with tumour outwith the slit

○ restrained mice in a basket

Error bars (1 s.e.m.) are shown every 2 minutes for clarity

In the present work, histological sections of the tumour were searched thoroughly for the presence of a host cellular reaction and the growth of the tumour was studied after immunization of the recipient mice with lethally irradiated cells.

The response of T-lymphocytes to the mitogen phytohaemagglutinin (PHA) was also used for its simplicity and its clinical use for cancer patients at our radiotherapy institute.

2.7.1 Immunization Procedures:

A tumour cell suspension of 10^6 cells/0.05 ml was prepared as in section 2.4.1; this was irradiated in a T.30 flask with a single X-ray dose of 90 Gy at a dose rate of 1.28 Gy/minute. Each mouse, then received 3 intraperitoneal injections, each of 2×10^6 lethally irradiated cells (0.1 ml suspension), at weekly intervals. The mice were challenged 3 weeks from the first injection with a tumour transplant using the same technique described in section 2.4.2.

Some of the immunized mice were designated for PHA studies instead of transplantation. The single dose of 90 Gy was beyond the 100% tumour curative dose and was expected to kill all the tumour cells in suspension. This was confirmed by the absence of ascites in both the mice challenged with tumours and the immunized mice without tumours, kept for more than one month from the day of the first injection.

2.7.2 PHA Studies: (performed by S.M. Chamberlain)

The mitogenic response is a simple in vitro test which gives a quantitative measure of the ability of T-lymphocytes to respond to a general antigenic stimulus, initiating DNA synthesis and cell division. In brief, one million lymphocytes from the mouse spleen were cultured in the presence of PHA for 4 days at 37°C. ^{14}C-thymidine (sp. activity 50 μCi/mM) was then used to label the lymphocytes, undergoing DNA synthesis and scintillation counts gave the measure of activity which was expressed as:

$$\text{PHA index} = \frac{\text{activity of PHA stimulated culture}}{\text{activity of unstimulated culture}}$$

PHA testing was done for the following groups of mice:

1 - Control mice bearing no tumour.

2 - Mice bearing spontaneous mammary tumours.

3 - Mice bearing transplanted tumours.

4 - Mice cured with radiotherapy.

5 - Immunized mice at the end of the third week following immunization.

6 - Mice with tumour transplanted in an irradiated skin fold (see section 2.5.5).

2.8 Tumour Measurements:

From the large amount of literature dealing with experimental animal tumours, most investigators obtained serial external measurements of the tumour diameters using Vernier calipers.

For the present tumour system, four specially designed plastic fan-shaped devices have been used for measurement of the three mutually perpendicular diameters of each tumour. Each measuring device consists of five rectangular plastic pieces of two millimeter thickness and of equal size joined at one end with slits of five different widths (1-5 mm, 6-10 mm, 11-15 mm, and 16-20 mm) at the free ends (plate 2.4). The tumour was retracted and held gently between the index finger and thumb while the tail of the mouse was held between the rest of the fingers and the palm of the left hand. With the appropriate measuring device held in the right hand, individually selected slits were slipped under the tumour and raised carefully until the smallest slit was found through which the diameter to be measured could pass. 90^{o} rotation of the tumour was essential for measurement of the perpendicular diameter to the body of the mouse (plate 2.5).

These measuring devices enabled one observer to measure the tumour diameters to the nearest millimeter in the shortest time possible and without the anesthesia which has been used by some investigators.

The effect of the skin thickness, enclosing the tumour, on volume calculations has been discussed by some workers (249,250), specially for tumours of small size. An average 1.25 mm double skin thickness was found by procedures similar to those of Dethlefsen et al. (250)

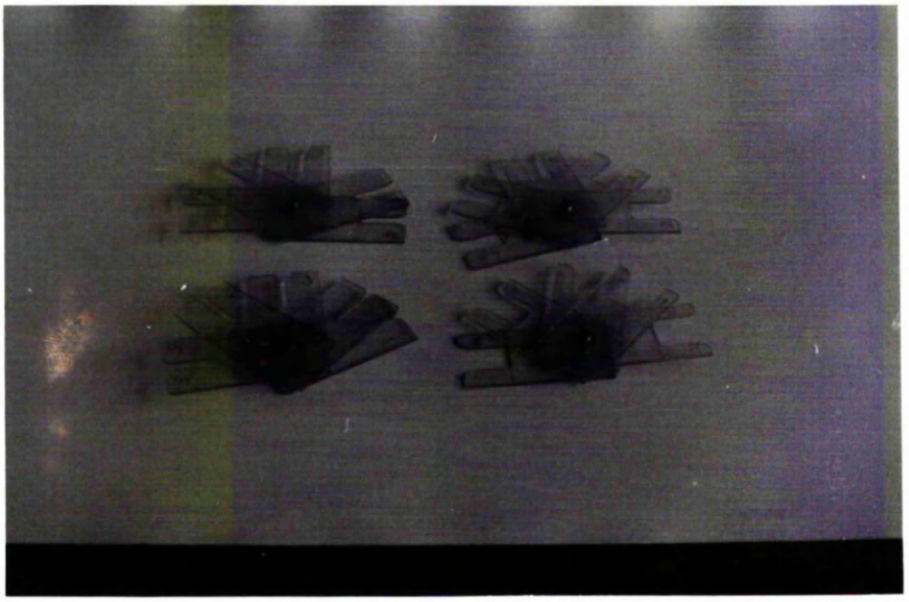


Plate 2.4: Measuring device.

Plate 2.5: Tumour measurement.

for non-tumour bearing male and female living mice and for subcutaneously implanted metallic spheres of various sizes in killed mice. For analysis of the radiobiological results, various investigators have used the three perpendicular diameters, mean diameter, longest diameter or the area enclosing the tumour to obtain the best correlation with the tumour volume or weight in situ, on the assumption that the tumours were typical spheres or ellipsoids.

For analysis of the results in this work, the average double skin thickness was subtracted from each arithmetic mean of the three measured perpendicular diameters. This was done in order to minimize its effect on the growth curves of the unirradiated tumours and the regression-regrowth curves of the irradiated ones. This was important as small tumours of 6 ± 1 mm were used for the radiobiological experiments.

2.9 Histological Studies of the Tumour System:

The following groups of tumours were subjected to histological examination:

1 - Unirradiated tumours of different sizes: 2 to 3 mm; 5 to 6 mm and 9 to 10 mm mean diameter.

2 - Irradiated tumours during the regression period at 8 to 10 days and 16 to 18 days following irradiation with single doses of 50, 60 and 80 Gy X-rays alone and 22.5 and 40 Gy of X-rays in combination with Misonidazole.

These doses were chosen to produce regression of the tumours and variable cure probabilities. They would also facilitate examination of the tumour mass in the presence of relatively few viable clonogenic tumour cells and in the absence of these cells (cure). The times for histology were chosen to be near the end of the slow regression (9 to 10 days) and the fast regression (16 to 18 days) periods (figure 3.7) for the purpose of finding out some of the biological processes behind tumour regression.

3 - Recurrent tumours of 5 to 6 mm and 9 to 10 mm diameter following single doses of 50 and 60 Gy of X-rays alone.

4 - Transplanted tumours in cured sites of 5 to 6 mm and 9 to 10 mm mean diameter.

The animals were sacrificed and the tumours with the overlying skin were immediately excised and fixed in 10% buffered formalin. Before fixation, the tumours were not bisected but the skin was trimmed around the tumours to allow easy access of the fixative. The tissues were embedded in paraffin wax and sections 5 micron thick were cut through the middle and the periphery of the tumours and stained with hematoxylin and eosin. The presence of erythrocytes within lumen assisted with the recognition of the capillaries and an eye-piece reticle was used to measure some of the tumour structures.

The fractions of tumour areas occupied by necrosis and

apparently viable tumour cells were determined using a graticule containing 300 equal sized squares in one eye-piece of the microscope and counting the number of squares of both viable and necrotic tissues. Several sections at the middle of the tumours (unirradiated, recurrent and transplanted in cured sites) were chosen and scanned methodically under a x 2 objective (total magnification x 80). This method gave useful, though crude, information about the proportion of both viable and necrotic tissues in the tumours.

CHAPTER III

RESULTS

3.1 Growth Characteristics of the Tumour System:

Careful tumour observations were made from the third day of the transplant by daily inspection and palpation of the injected sites for 5 to 6 days every week. The earliest detectable tumours were about one millimeter in diameter and the vast majority of them grew to large sizes. Problems similar to those reported in the literature, using a tumour cell suspension for transplantation, have been encountered also in this tumour system: ulceration of the skin with dermal rather than subcutaneous injections, deep and fixed tumours with deeper injections than the subcutaneous level and adjacent or amalgamated tumours along the needle track. Furthermore, the tumours obtained were not always at the midline of the back of the mice due to the use of manual restraint, of mouse movements rather than anaesthesia. However, with careful transplantation, the majority of tumours were single, freely mobile and either spherical in shape or one millimeter longer in one or two diameters. In a few instances, the detectable masses were firmer in consistency and did not grow progressively. These nodules were rejected from the present studies, although their neoplastic nature was confirmed microscopically (section 3.12.2).

3.1.1 Tumour Take Rate:

During the early part of this work (1975 and 1976), the tumour take rate was short of 100 per cent. From a

total of 654 mice used for 37 transplants, 58 mice did not develop tumours, i.e. a tumour take rate of 91 per cent. This was also associated with about 10 to 15 per cent unusable tumours (fixed and double). These failures could be traced to technical difficulties. Since then, however, the tumour take rate has reached 100 per cent, with fewer unusable tumours, due to more experience in the transplantation technique.

3.1.2 The Latent Period:

The minimal latent period was 5 days and the maximum was 28 days (figure 3.1). Statistical analysis of the latent period distribution showed a mean latency of 9.3 ± 3.48 days (table 3.10), where the errors quoted are plus or minus one standard deviation. It can be seen in figure 3.2, which shows the cumulative incidence of detectable tumours, that 50 per cent of the tumours developed by 7.5 days following transplantation.

3.1.3 The Average Pattern and Rate of Tumour Growth:

Tumour dimensions were recorded with time from the day the tumours became palpable until the tumour diameters approached between 13 and 15 mm, when the animal was sacrificed. The average growth curve of the tumour is shown in figure 3.3, where the mean diameter is plotted against the time on a linear-linear scale. Zero time is used for the reference size 0.75 to 1.75 mm (2 to 3 mm including the skin thickness). This average growth

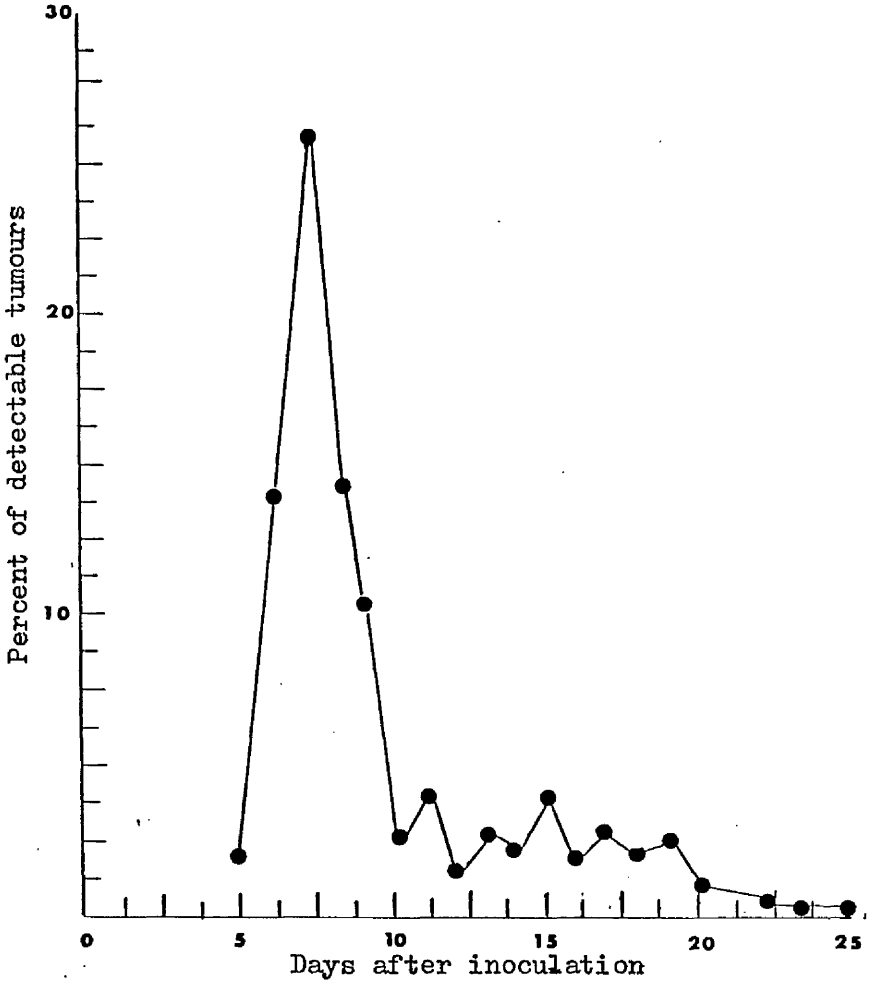


Figure 3.1: The time distribution of detectable tumours (o is the day of transplantation)

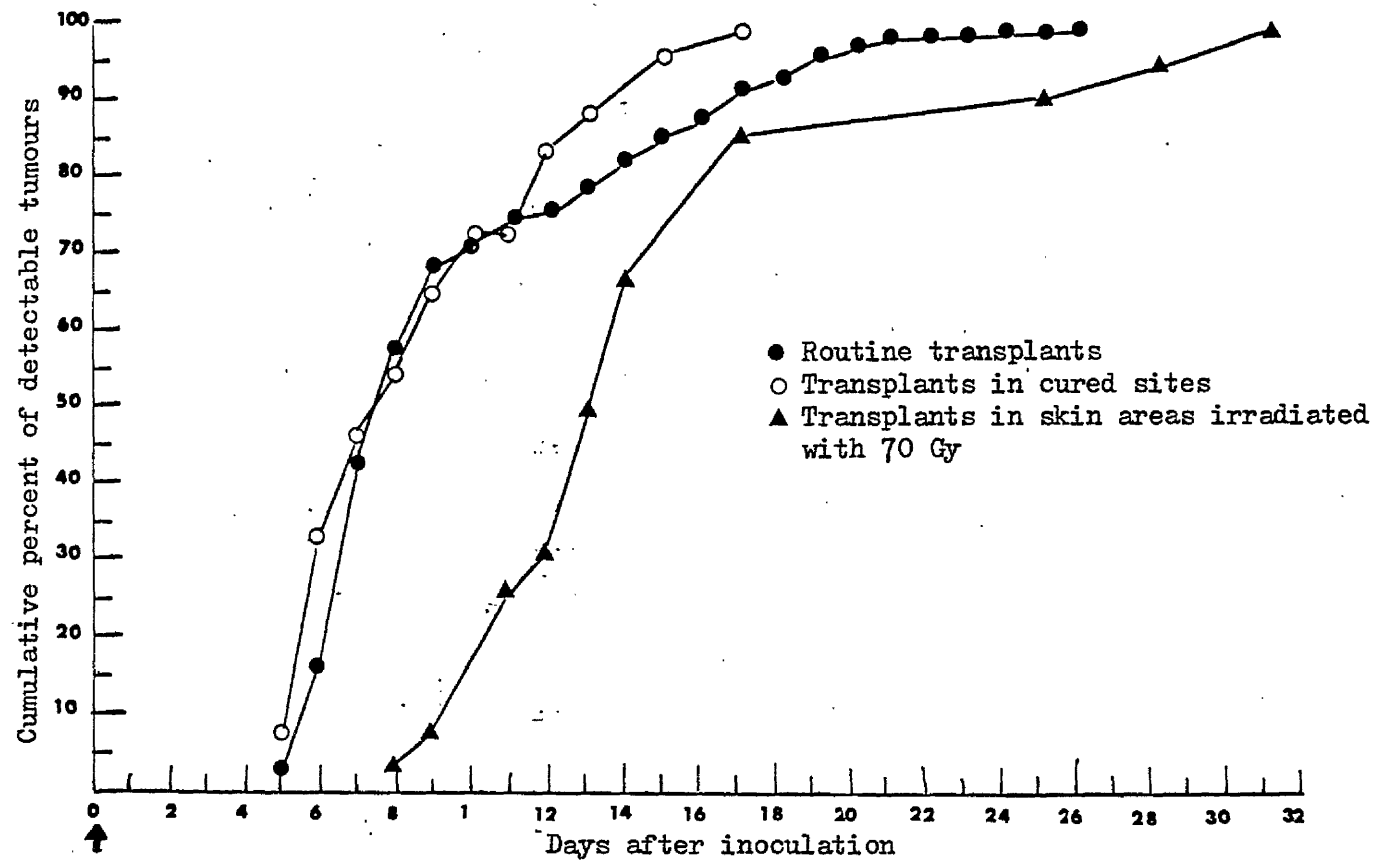


Figure 3.2: The daily cumulative percentage of detectable tumours
(↑) the day of inoculation

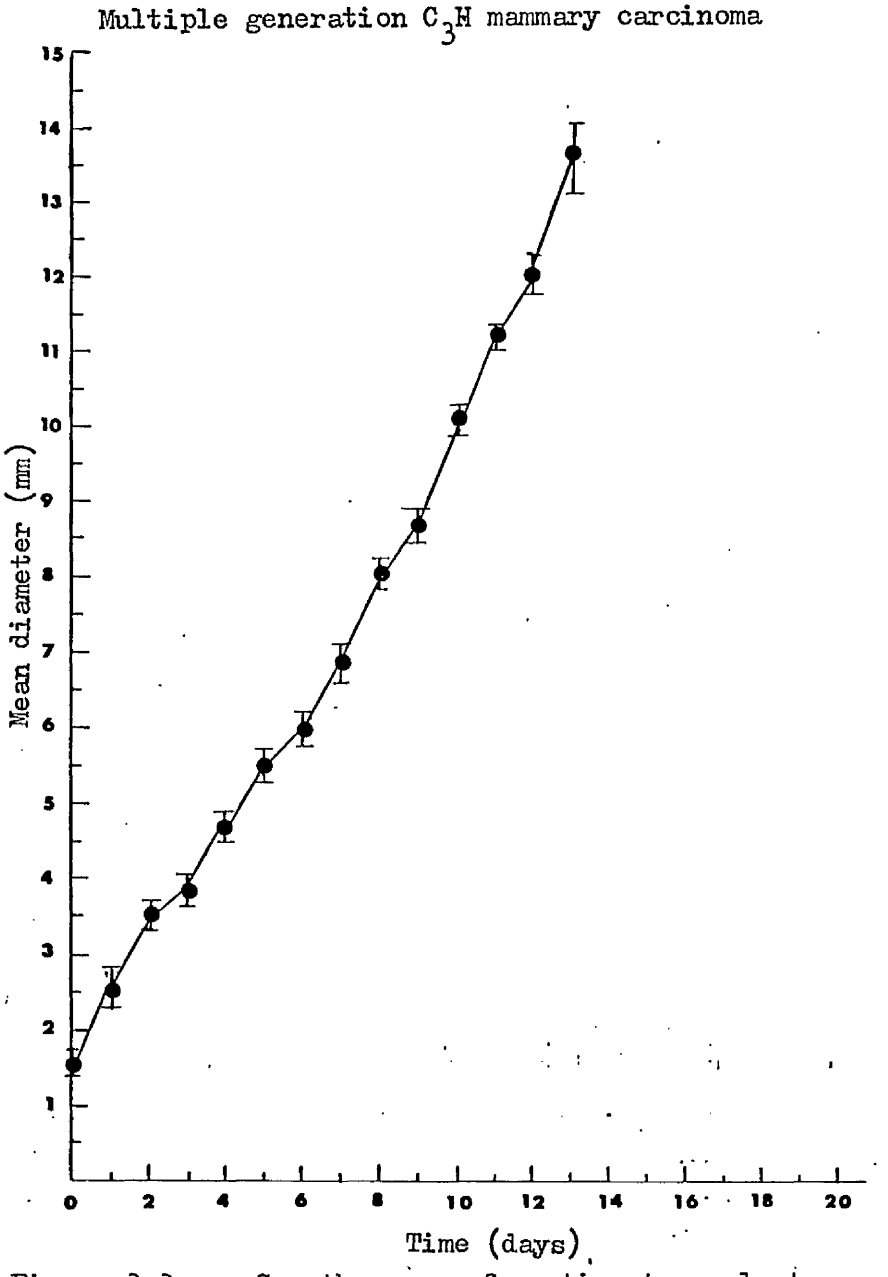


Figure 3.3: Growth curve of routine transplants.
Errors are ± 1 s.e.m.
The mean diameter does not include skin thickness (1.25 mm).

curve was obtained by moving the growth curves of individual tumours together in time so that they coincide at the reference size. This size was chosen since all the tumours grew progressively on reaching that size.

The tumour pattern and rate of growth were checked on several occasions and were found to be constant all through the present work. Furthermore, there was no significant difference in the latent period and growth rate of tumour transplants between mice of 8 to 12 weeks and older mice of 19 to 21 weeks. Therefore, the average growth curve in figure 3.3 was used as a common control throughout this work.

Mathematical analysis of this growth curve showed that the tumour growth above 1 to 2 mm mean diameter could be well described by a Gompertzian equation (section 1.2) of the following parameters:

Exponential growth parameter (α) = 1.64

Retardation growth parameter (β) = 0.225

Table (3.1) shows the calculated doubling times at various tumour sizes. This data is also used for the construction of figure 3.4 where the volume doubling times of the tumour are plotted against the mean diameters. The growth rate of this multiple generation C_3H mammary tumour decreased continuously with increase in tumour size. At the size chosen for irradiation 6 ± 1 mm (including the skin thickness), the tumour grew at a fast rate with about a one day volume doubling

Table 3.1 The instantaneous doubling time for the tumour at various sizes.

Mean diameter (mm) (without skin thickness)	Calculated doubling times (days)
1	0.42
2	0.50
3	0.66
4	0.81
5	0.98
6	1.20
7	1.46
8	1.81
9	2.28
10	2.98

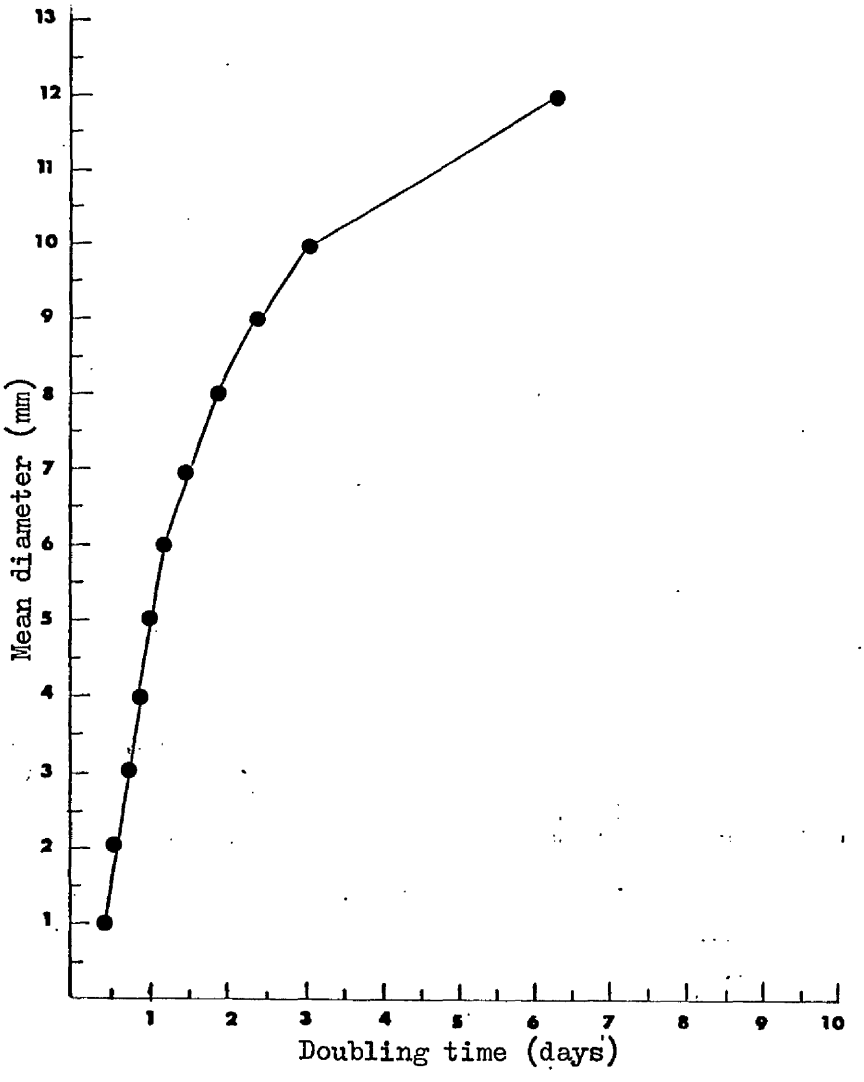


Figure 3.4: Volume doubling time of multiple generation C_3H mammary tumour. The mean diameter does not include skin thickness (1.25 mm).

time.

It was also possible to fit a straight line to the mean diameters for each individual tumour by means of the least squares method (see appendix II). The mean of the slopes of these lines was then calculated and was found to be 0.889 mm/day (table 3.11). This method was used to compare the rate of growth of untreated tumours with that of recurrent tumours; transplanted tumours in immunized mice and those in cured and pre-irradiated sites (tables 3.11, 3.12).

3.2 The Gross Response of Tumours to Irradiation:

Figure 3.5 shows the gross response of the tumours to various single doses of X-rays alone of 5 to 80 Gy. The mean diameter without the skin thickness is plotted against the postirradiation period. The symbol R indicates the day of irradiation and the number of tumours analyzed in each dose group are those in table 3.2. The variations in the gross response of individual tumours to each dose, increased with dose, but these variations were less obvious at low doses up to 40 Gy and also during the regression period with high doses of 50 Gy or more. Hence, it was possible to average the gross response of the tumours at the low dose levels and during the regression period. No significant difference could be seen in the growth of irradiated tumours from that of the control group for about two days. Thereafter, clear dependence of the tumour growth on the dose became evident with time. There was a progressive flattening of the early parts of the growth curves, which was more obvious as the dose increased towards 40 Gy, when the early part of the growth curve followed that of higher doses of 50 Gy or more for about twelve days before regrowth. Tumour regression was only evident after single doses of 50 Gy or more with a similar pattern of regression following both a non-curative dose, such as 50 Gy and a curative dose, such as 80 Gy.

Figure 3.6 shows the effect of Misonidazole on the gross response of the tumour to irradiation. The number of

Figure 3.5: The gross response of the tumours to single doses of X-rays alone. The curves plotted for the mean of all animals in each group.
The X-ray dose (Gray) is shown by each curve and the error bars have been omitted for clarity.
The mean diameter used, does not include skin thickness (1.25 mm).

MULTIPLE GENERATION C_3H MAMMARY TUMOUR

GROSS RESPONSE TO SINGLE DOSES OF X-RAYS

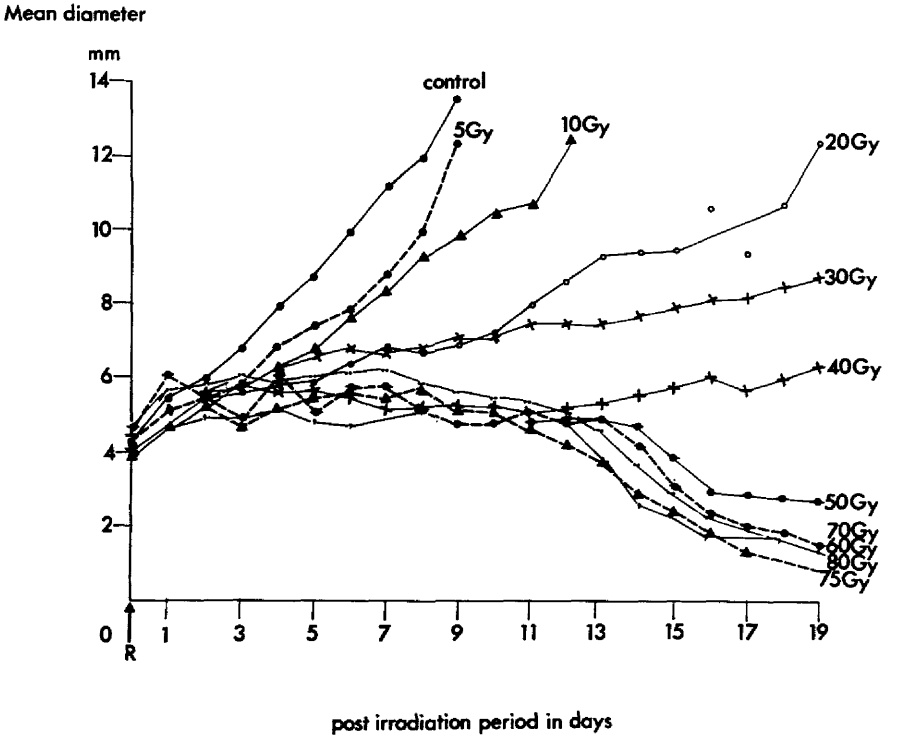
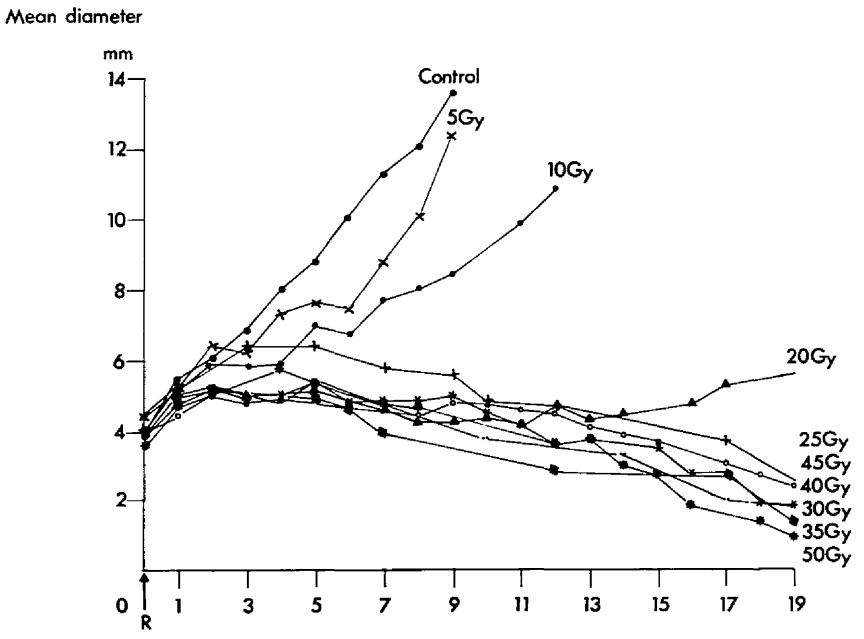


Figure 3.6: The gross response of the tumours to single doses of X-rays in combination with Misonidazole. The curves plotted for the mean of all animals in each group. The X-ray dose (Gray) is shown by each curve and the error bars have been omitted for clarity. The mean diameter used, does not include skin thickness (1.25 mm).

MULTIPLE GENERATION C_3H MAMMARY TUMOUR

GROSS RESPONSE TO SINGLE DOSES OF X-RAYS + MISONIDAZOLE (RO-07-0582)



tumours analyzed in each dose group are those in table 3.4. The response was similar to that of X-rays alone but with lower dose levels. A similar pattern of tumour regression could also be seen with doses of 25 Gy and up to 50 Gy.

3.2.1 The Characteristic Rate and Pattern of Tumour Regression After Irradiation:

Figure 3.7 shows the average regression patterns after single doses of 60 Gy or more of X-rays alone and with single doses of 25 Gy or more in combination with Misonidazole. The regression patterns were plotted up to nineteen days after irradiation, only, due to some difficulty in accurate measurement of the regressed tumours at later times, due to the radiation reactions mentioned in section 3.7.2.

The observed similarity of both regression patterns characterized the tumour response during regression after irradiation. The tumours continued to increase in size for about two days, then returned slowly to the pre-irradiated size about twelve days after irradiation. This was followed by a relatively faster regression towards a minimal size in about twenty days from the day of irradiation.

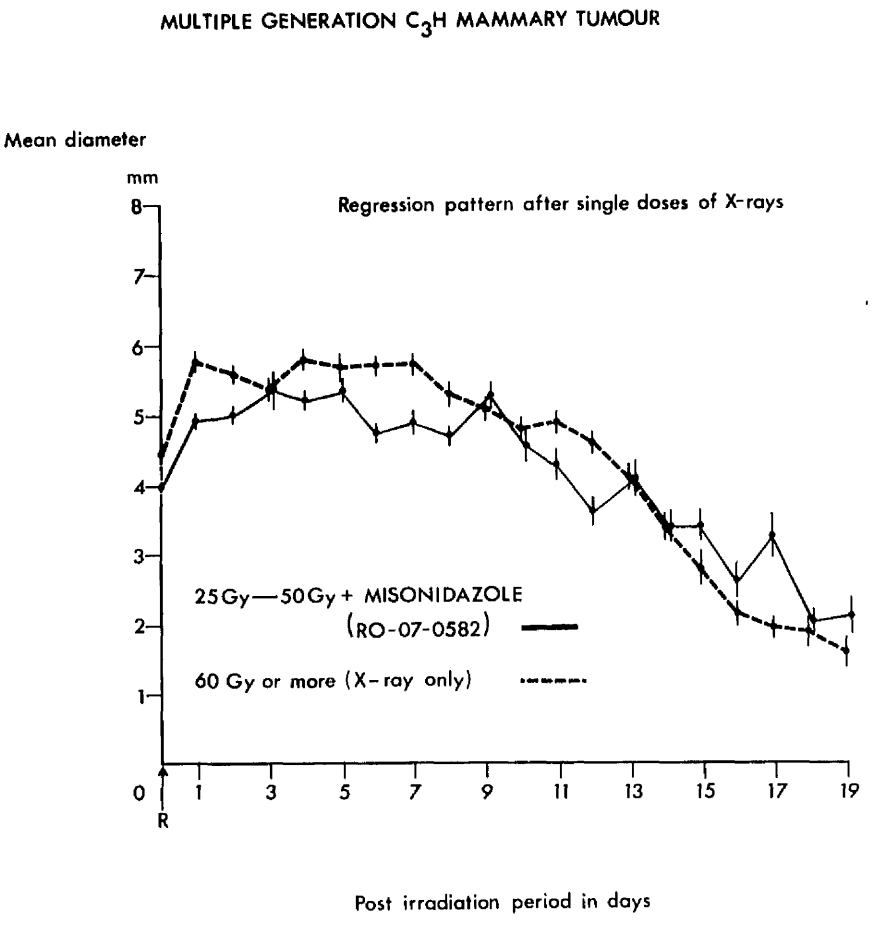


Figure 3.7: The regression pattern of the tumour after single doses of X-rays alone and in combination with Misonidazole. The mean diameter used, does not include skin thickness (1.25). Error bars are $\pm$ 1 s.e.m.

3.3 Tumour Local Control Analysis:

3.3.1 Choice of an Appropriate Time Period as an End Point:

Mice with locally controlled tumours were usually kept for more than 6 to 8 months after irradiation and were checked weekly. From 51 mice with locally controlled tumours at 100 days after single doses of X-rays alone (table 3.2), only two tumours recurred more than 100 days after irradiation. One tumour recurred at 120 days in the 70 Gy group and the other in the 80 Gy group recurred at 180 days.

Eight months after irradiation, two mice developed spontaneous tumours, near the neck outside the irradiated areas and five mice died twelve to fifteen months after irradiation presumably of old age or intercurrent disease.

Therefore, a 100 day period after irradiation was chosen as the most appropriate time interval for the end point of the tumour control experiments. This was mainly because the time distribution of recurrences indicated that few recurrences would be expected later than 100 days. The choice of this period also reduced the space occupied in the animal house and the expenses. No "losses" of experimental animals due to spontaneous tumours or death from old age were involved, therefore.

3.3.2 Criteria for "Controlled" or "Recurrent" Tumours:

All tumours which showed incomplete regression, eventually recurred, irrespective of the dose. On the

other hand, tumours that showed complete regression, either remained so at 100 days after irradiation or recurred. The failure of adequate irradiation of the skin margin with the needle track in some tumours led to a few marginal tumours. These tumours were detectable as small nodules adjacent to the irradiated tumour masses. Thus, the criterion for a definite local recurrence necessitated regrowth in the centre of the irradiated areas and the exclusion from the analysis of any tumour with marginal growth. Those tumours which regressed completely and remained so at 100 days after irradiation were considered to be locally controlled (cured).

3.3.3 The Results of Local Tumour Control Experiments:

For each dose group, the proportion of 'cures' observed 100 days after treatment was estimated as the number of 'cures' seen in the total sample of mice alive at that time together with those that have died or were killed, with unequivocally uncontrolled tumours previous to 100 days. 'Cured' mice which died before 100 days were excluded from the analysis and it was assumed that the estimated value of tumour 'cure' was independent of the death of 'cured' animals prior to 100 days.

Tables 3.2, 3.3, 3.4 give the details of the results of the experiments on the local control of the tumours at 100 days for single doses of X-rays alone (non-clamped and clamped tumours) and X-rays in combination with Misonidazole for unclamped tumours.

Table 3.2 Local control data of non-clamped tumours treated with single doses of X-rays alone.

Dose in Gy	Local tumour control at 100 days		
	male	female	total
5	0/7	0/4	0/11 (0)
10	0/6	0/7	0/13 (0)
15	0/4	0/5	0/9 (0)
20	0/9	0/8	0/17 (0)
30	0/10	0/3	0/13 (0)
40	0/15	0/14	0/29 (0)
50	0/10	0/10	0/20 (0)
60	1/10	1/8	2/18 (11)
65	2/7	3/8	5/15 (33)
70	12/16	4/8	16/24 (66)
75	3/4	6/7	9/11 (82)
80	9/9	10/10	19/19 (100)

The numbers in brackets denote percentages.

Table 3.3 Local control data of clamped tumours treated with single doses of X-rays alone.

Dose in Gy	Local tumour control at 100 days		
	male	female	total
5	0/12	0/13	0/25 (0)
10	0/13	0/13	0/26 (0)
15	0/10	0/13	0/23 (0)
20	0/15	0/12	0/27 (0)
25	0/10	0/5	0/15 (0)
30	0/15	0/16	0/31 (0)
35	0/10	0/10	0/20 (0)
40	0/8	0/8	0/16 (0)
50	0/8	0/5	0/13 (0)
55	0/3	1/12	1/15 (6)
60	5/14	-	5/14 (35)
65	9/17	-	9/17 (52)
70	8/12	-	8/12 (66)
75	3/3	7/10	10/13 (77)

The numbers in brackets denote percentages.

Table 3.4 Local control data of non-clamped tumours treated with single doses of X-rays in combination with Misonidazole.

Dose in Gy	Local tumour control at 100 days		
	male	female	total
5	0/9	0/9	0/18 (0)
10	0/6	0/8	0/14 (0)
15	0/8	0/12	0/20 (0)
20	0/7	0/8	0/15 (0)
25	1/10	0/7	1/17 (5.8)
27.5	1/11	0/5	1/16 (6.2)
30	5/6	3/7	8/13 (61.5)
35	10/15	11/13	21/28 (75)
37.5	5/5	9/9	14/14 (100)
40	6/6	6/6	12/12 (100)
45	9/9	10/10	19/19 (100)
50	5/5	8/8	13/13 (100)

The numbers in brackets denote percentages.

Table 3.5 TCD_{50}s at 100 days of multiple generation C_3H mammary tumour.

Group	TCD_{50} (Gy)
Non-clamped Tumours	67
Clamped tumours	65
X-rays + Misonidazole	31

Each table shows the number of tumours available for analysis and the number of tumours scored as Locally Controlled in each dose group as a proportion of the number analyzed (PC).

These data were also used to obtain figure 3.8 where ln (-ln pc) was plotted against the radiation dose using the log-log transformation analysis described in appendix I. The TCD_{50}s for local tumour control at 100 days are shown in table 3.5. The TCD_{50} obtained under fully hypoxic conditions (i.e. clamping) was 2 Gy less than those of non-clamped tumours. This difference was not statistically significant, implicating that non-clamped tumours had a relatively high proportion of hypoxic cells at the time of irradiation.

The TCD_{50} of non-clamped tumours was reduced from 67 Gy to 31 Gy with Misonidazole. The ratio of these TCD_{50}s was 2.15, indicating a high degree of radiosensitization by Misonidazole of the tumour response to single doses of X-rays. However, the ratio fell below the expected maximum range of 2.5 to 3 for the oxygen enhancement ratio (OER). This indicated that only a partial though substantial improvement was obtained with single dose treatment in combination with Misonidazole. The proportion of male and female mice varied from one treatment group to another (tables 3.2; 3.3 and 3.4). Hence, it was essential to find out if the local control of the tumours was influenced by sex distribution as reported by Fowler et al. (136) for first generation

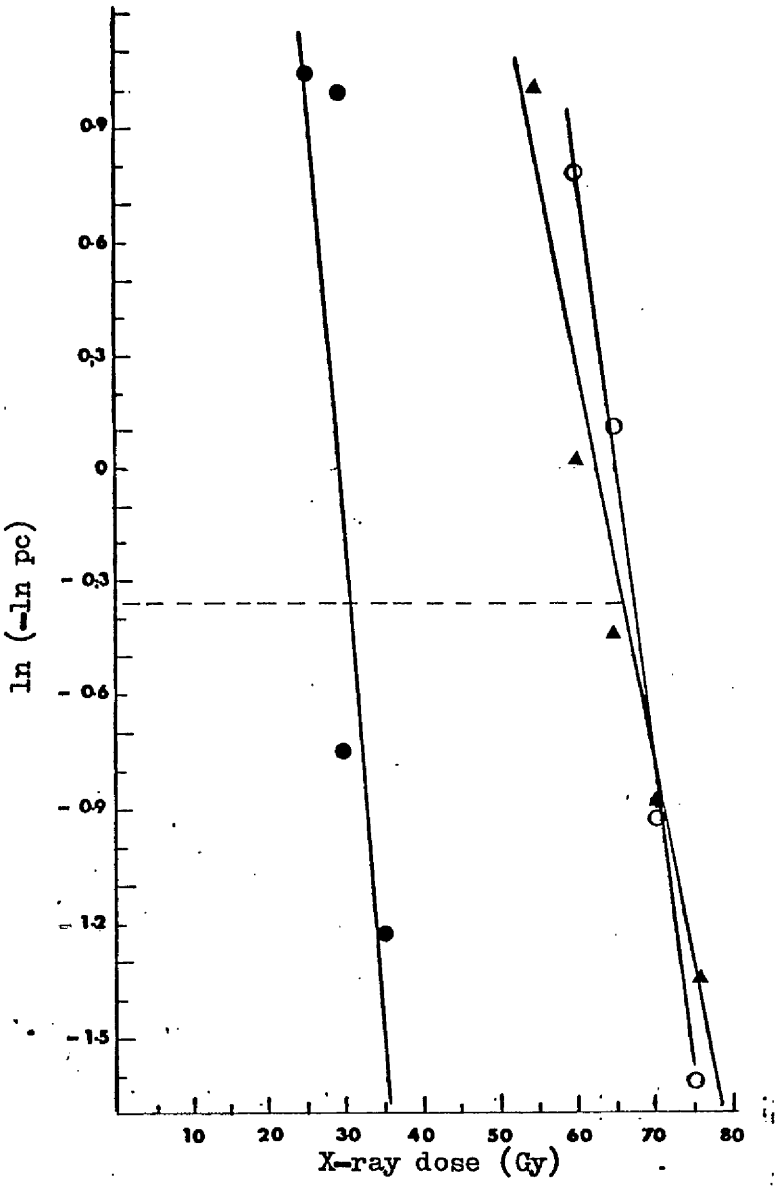


Figure 3.8: Analysis of local control of tumours at 100 days with single radiation doses using log-log transformation method.

- ○ Unclamped tumours
- ▲ Clamped tumours
- ● In combination with Midonidazole
- - - Level of TCD_{50}

PC is the probability of tumour control

C_3H mammary carcinoma. Statistical analysis, using X^2 test with Yates correction was performed and showed no significant difference in any of the groups ($p>0.1$). The tumours were irradiated within a narrow range of sizes (6 ± 1 mm mean diameter). The volume corresponding to 7 mm is 2.7 times greater than that at 5 mm. This difference in volume may alter the curability of the tumours. However, statistical analysis of all the cure data, using X^2 test with Yates correction showed no significant difference ($p>0.05$) in the probability of local control of tumours for sizes less than 6 mm and that for sizes more than 6 mm.

3.4 The Growth of Recurrent Tumours:

Many recurrent tumours grew very slowly with periods of no growth or even temporary regression, until the size at which the tumours were irradiated (6 ± 1 mm mean diameter). A final, relatively faster and more predictable phase of growth then followed.

Within each dose level, the main differences between the growth of individual tumours were in the interval between the end of regression (complete or incomplete) and the final phase of growth. Hence, it was only possible to average this final phase within each dose. This was achieved by moving the curves for individual tumours together in time at the size of 6 ± 1 mm mean diameter (figure 3.9).

Statistical analysis of the rate of growth during this final phase (table 3.12) has suggested that the rate of growth would not differ significantly from that of unirradiated tumours with single doses of 50 Gy or less. On the other hand, the rate of growth of recurrent tumours was significantly slower with higher single doses than that of unirradiated tumours ($p<0.01$).

The delay in time that each individual recurrent tumour took to regrow to a mean diameter of 10 mm was determined. The mean delay was then calculated for each group treated with single doses of X-rays alone (unclamped and clamped tumours) and X-rays in combination with Misonidazole as shown in table 3.6. These data are used for construction of the dose-response curves in figure

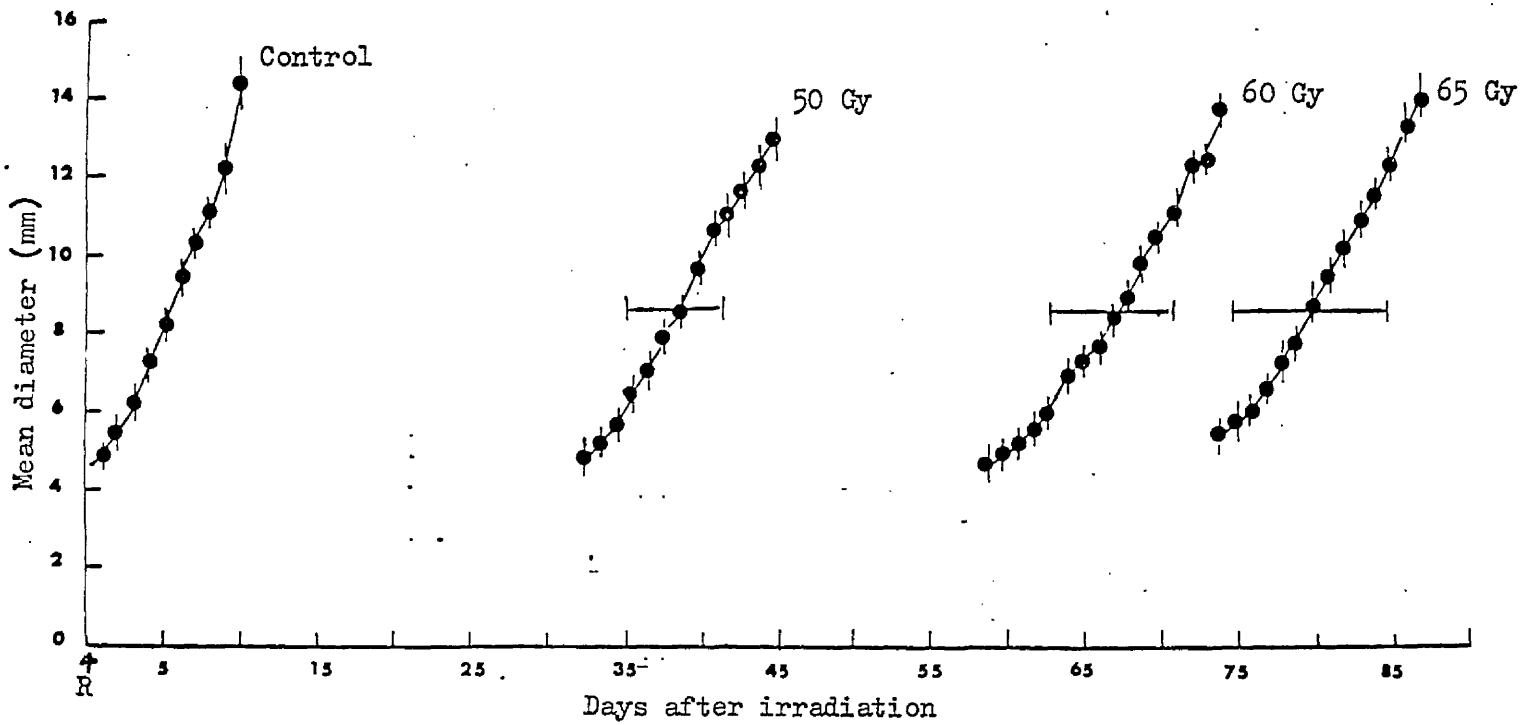


Figure 3.9: Regrowth curves of tumours recurring after single doses of 50, 60 and 65 Gy
R = day of irradiation
error bars are $\pm$ 1 s.e.m.

Table 3.6 Time taken for the tumours to grow to 10 mm after single doses of X-rays.

Dose	Mean time of delay in growth in days		
	Non-clamped tumours	Clamped tumours	X-rays + Misonidazole
0	5.4 ± 0.17 (20)	5 ± 0.20 (12)	5.2 ± 0.4 (10)
5	7 ± 0.50 (11)	6.4 ± 0.25 (25)	5.7 ± 0.31 (18)
10	7.9 ± 0.50 (12)	7.1 ± 0.20 (26)	9.14 ± 0.56 (14)
15	13.3 ± 1.30 (9)	8 ± 0.70 (23)	11.3 ± 0.02 (20)
20	17.1 ± 0.80 (17)	7.7 ± 0.40 (27)	32 ± 3.90 (15)
25	-	10 ± 0.70 (15)	37.6 ± 3.80 (16)
27	-	-	35.2 ± 3.23 (15)
30	23.1 ± 1.95 (13)	14 ± 1.20 (31)	66.5 ± 7.4 (4)
35	-	17 ± 1.0 (20)	61 ± 7.0 (6)
40	25.6 ± 1.65 (29)	24.1 ± 1.57 (16)	-
50	38.5 ± 2.97 (18)	35.5 ± 2.3 (13)	-
55	-	42.2 ± 4.4 (14)	-
60	66.5 ± 3.8 (16)	53 ± 2.5 (9)	-
65	78.5 ± 5.3 (9)	67.4 ± 5 (8)	-
70	87.4 ± 12.4 (7)	90.3 ± 3.5 (3)	-
75	-	70 ± 6 (3)	-

The number of tumours analyzed are shown in brackets.
Errors are ± 1 s.e.m.

3.10 where the mean delay in growth is plotted as a function of X-ray dose.

The curve for unclamped tumours irradiated in the absence of Misonidazole has a biphasic shape, with a break point at about 15 Gy. From this point the curve approaches the smooth curve for clamped tumours; both curves become indistinguishable from each other after single doses of 40 Gy or more. The biphasic pattern of the curve for unclamped tumours is suggestive of the presence of a significant proportion of hypoxic cells in the tumours which dominate the response after the break point.

The degree of radiosensitization achieved with Misonidazole is expressed as the enhancement ratio (ER) which is the ratio of X-ray doses needed in the presence and absence of the drug to produce the same biological effect on the tumour. This ratio can be read off horizontally at any level of effect in figure 3.10. It can be seen that the ER increases after the break point on the curve for unclamped tumours to a maximum of about 2, i.e. the same level as for the clamped tumours.

Figure 3.11 shows the results of all recurrent tumours after single doses of 50, 60, 65 and 70 Gy of X-rays alone. The delay in growth to reach 10 mm mean diameter is plotted against the size at irradiation. Within each dose level, there is no correlation between the size at irradiation and either the state of regression or the delay in growth. However, the importance of the dose

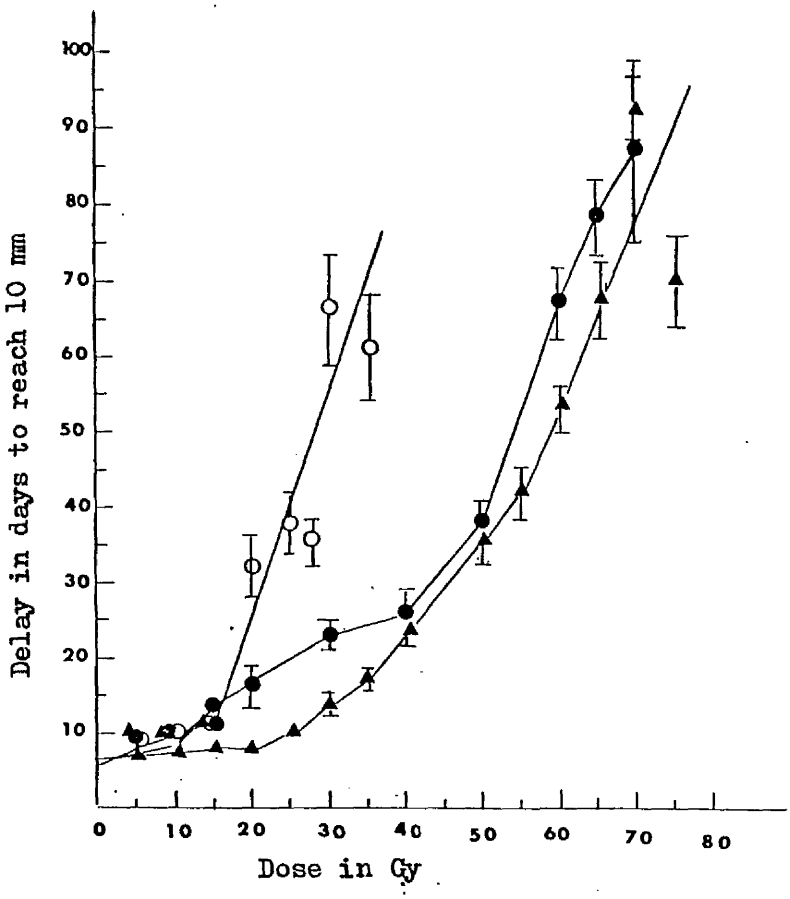


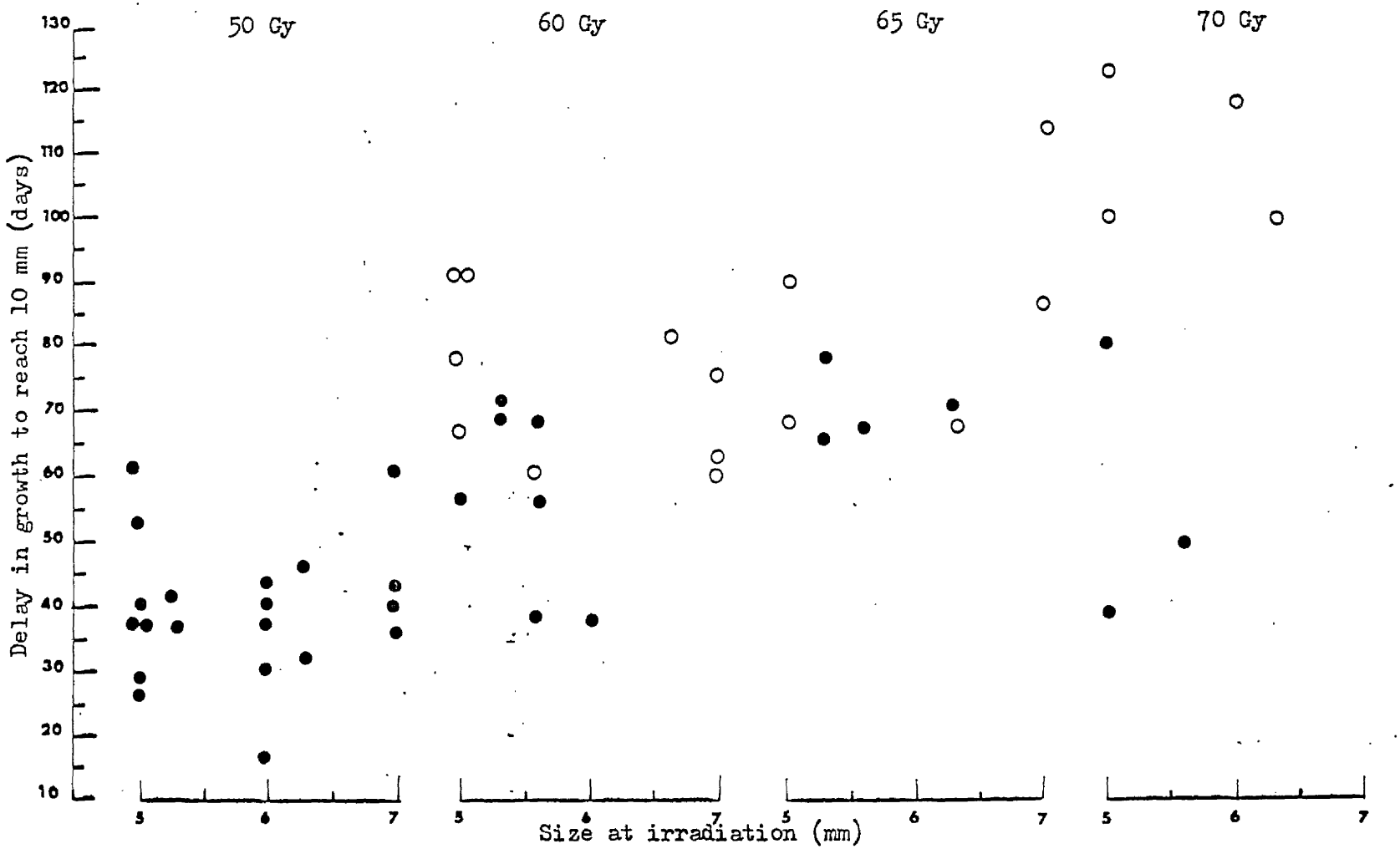
Figure 3.10: Dose response curves for recurrent tumours.

● X-rays alone ▲ Clamped tumours

○ X-rays and Misonidazole

Error bars are ± 1 s.e.m.

Figure 3.11: Delay in growth of recurrent tumours as a function of tumour size at irradiation.
Tumour size between 5 and 7 mm mean diameter) has no effect on the regression state nor the delay in growth.
○ = complete regression
● = incomplete regression


50 Gy
60 Gy
65 Gy
70 Gy
Delay in growth to reach 10 mm (days)
130
120
110
100
90
80
70
60
50
40
30
20
10
5
6
7
Size at irradiation (mm)

level, in effecting growth delay can be seen.

Six out of ten mice injected with a cell suspension from recurrent tumours (section 2.4.4) developed tumours. This tumour take appeared to be less than 100 per cent take observed for routine transplants. However, this could be due to the technical difficulties mentioned in section 2.4.4. The tumours obtained were detectable at latent periods (7,8; 8,10; 11 and 11 days after transplantation) which were within the range of that of the routine transplants. Furthermore, these tumours grew at a rate similar to that of routine transplants. This can be seen in figure 3.12 where the daily measurements of the six tumours are distributed along the growth curve of routine transplants.

These findings suggest that the slow rate of growth of recurrent tumours mentioned above is due to a tumour bed effect rather than an intrinsic defect in the tumour cells. However, a slow rate of cell division as a consequence of impairment of the oxygenation status, due to the tumour bed effect, can not be ruled out.

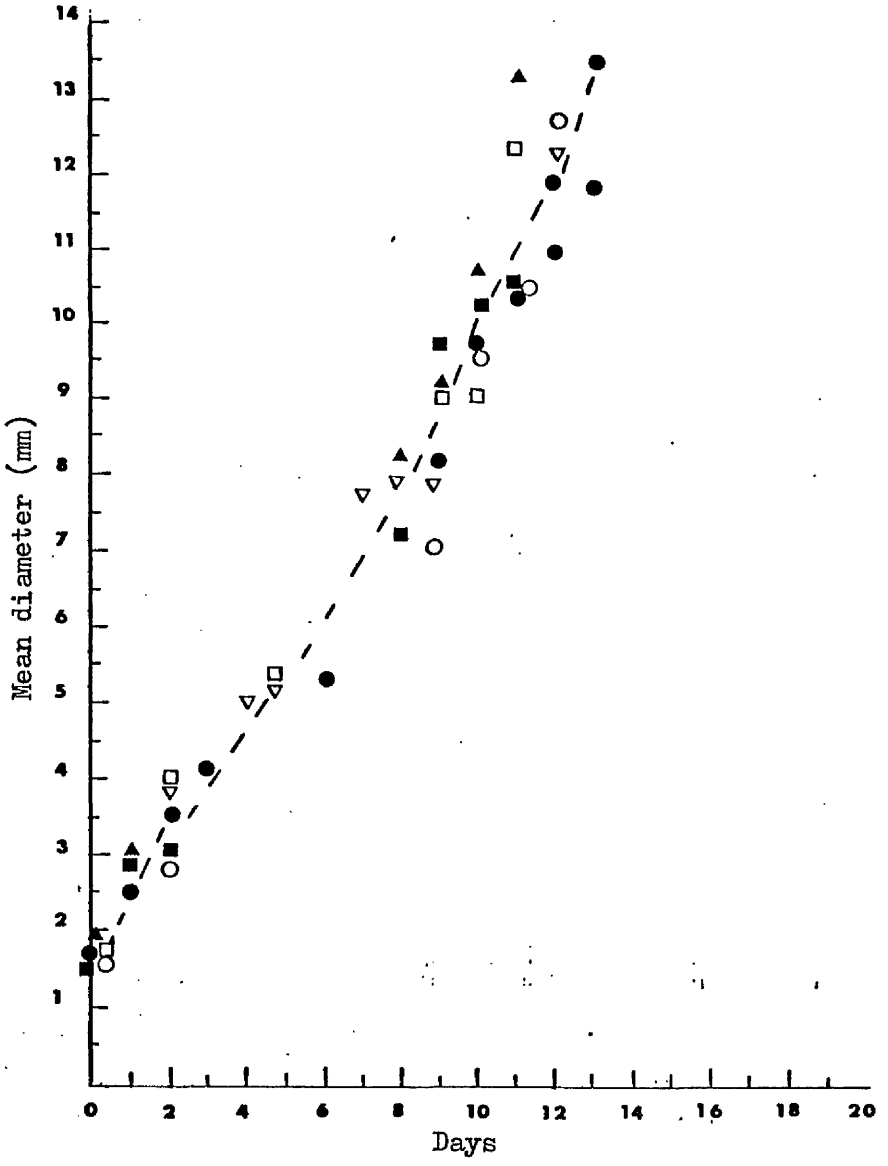


Figure 3.12: Tumour growth using 10^6 cells from recurrent tumours after 50 Gy (○, ●, ▲, ■) and 60 Gy (□, ▽), – – – control.
The mean diameter does not include skin thickness (1.25 mm).

3.5 The Results of Split Dose Experiments:

A series of experiments in which a 70 Gy total dose of X-rays, given as two fractions, were carried out with various time intervals between the two fractions. The experimental conditions including the time of day for the initial dose were similar in all the experiments. Both the local control probability of tumours at 100 days and the delay in growth to reach 10 mm mean diameter were recorded for each treatment. The equivalent single doses required to produce the same levels of tumour response in the range attained by all treatments can then be read from figure 3.8 for the local control probability and from figure 3.10 for the delay in growth, following single doses of X-rays alone.

The difference between 70 Gy and the equivalent single doses (i.e. D_2 and D_1 respectively) is a measure of the total recovery occurring in the irradiated tumours during the interval between the two fractions. This is assuming that the survival parameters for the two fraction experiments are the same as that for single dose experiments. The D_2-D_1 values from these series of experiments as well as the size of initial doses and the intervals between the two fractions are set out in table 3.7 for the cure data and in table 3.8 for the delay in growth data. These data are also used to construct figures (3.13, 3.14) where D_2-D_1 values are plotted against the intervals between the two fractions over the period of 0 to 72 hours for the initial doses of 20, 35

Table 3.7 Split dose experiments (data of local tumour control).

Intervals (hrs)	Local control	Equivalent single dose (D_1) Gy	(D_2-D_1) Gy
Initial dose of 20 Gy			
3	2/10 (20)	61.50	8.50
12	3/15 (20)	61.50	8.50
24	2/12 (16)	61.00	9.00
48	4/14 (28)	63.00	7.00
72	2/9 (22)	62.00	8.00
Initial dose of 35 Gy			
3	4/21 (19)	61.30	8.70
12	0/16 (0)	*	*
24	2/11 (18)	61.25	8.75
48	2/18 (11)	60.00	10.00
72	3/13 (23)	62.30	7.70
Initial dose of 50 Gy			
3	3/13 (23)	62.30	7.70
12	3/12 (25)	62.50	7.50
24	2/9 (22)	62.00	8.00
48	3/15 (20)	61.50	8.50
72	2/12 (16)	61.00	9.00
Misonidazole experiment			
3	7/10 (70)	33.75	6.25

The numbers in brackets denote percentages.

* D_1 is indeterminate.

Table 3.8 Split dose experiments (data of delay in growth to reach 10 mm mean diameter).

Interval	Mean time of delay in growth days	Equivalent single dose Gy	(D_2-D_1) Gy
Initial dose of 20 Gy			
3	62 ± 5.0 (8)	58.00	12.00
12	63.7 ± 2.7 (11)	58.50	11.50
24	75.6 ± 4.9 (10)	62.50	7.50
48	74 ± 3.1 (8)	63.00	7.00
72	60 ± 0.44 (5)	57.50	12.50
Initial dose of 35 Gy			
3	66.7 ± 3.6 (14)	60.00	10.00
12	66.7 ± 2.9 (13)	60.00	10.00
24	72.3 ± 4.6 (10)	62.00	8.00
48	81 ± 5.2 (12)	66.00	4.00
72	65 ± 6.4 (9)	59.50	10.50
Initial dose of 50 Gy			
3	64 ± 4.6 (10)	58.50	11.50
12	59 ± 4.7 (9)	57.00	13.00
24	70.5 ± 8.5 (6)	61.00	8.50
48	73 ± 7.5 (7)	62.50	7.50
72	51 ± 5.9 (8)	54.50	15.50
Misonidazole experiment			
3	62.6 ± 3.5 (3)	32.00	8.00

Errors are ± 1 s.e.m. The number of tumours analyzed are shown in brackets.

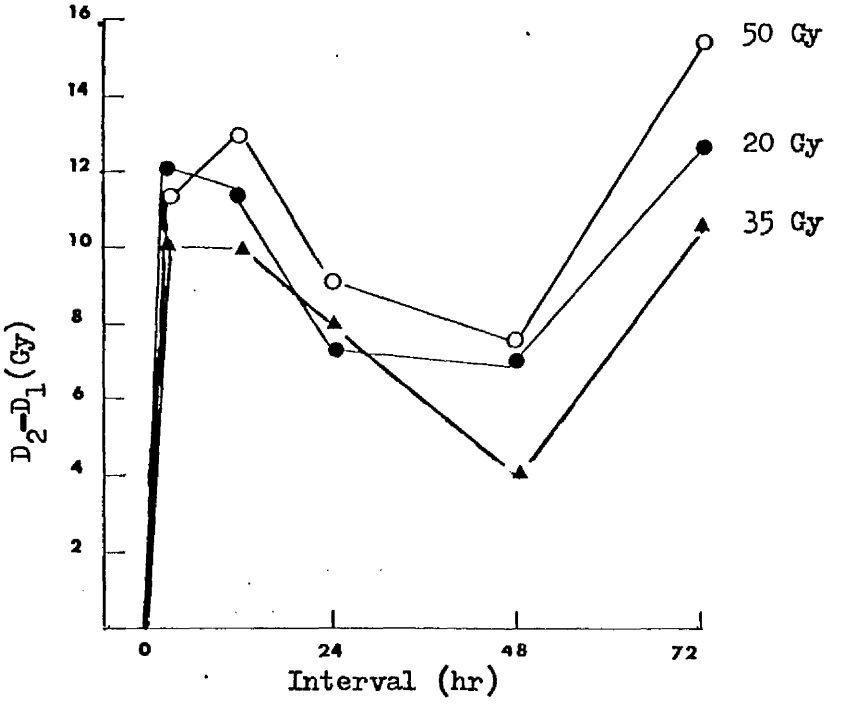


Figure 3.13: Split dose experiments using the delay in growth data.

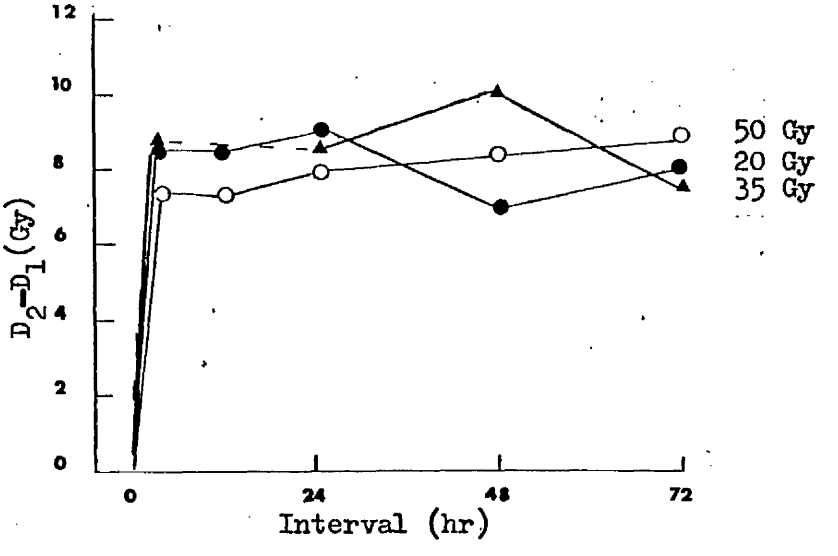


Figure 3.14: Split dose experiments using the cure data. Dashed line means D_2-D_1 is indeterminate.

and 50 Gy.

The fluctuations in the values of D_2-D_1 for cured tumours (table 3.7 and figure 3.14) are clearly not greater than would be expected from the experimental errors except for treatment with 35 Gy initial dose at 12 hours interval. However, in this case, with no cures, D_1 is indeterminate. On the other hand, the three curves for recurrent tumours in figure 3.13 show similarity in their peaks at 3, 12 and 72 hours and troughs at 48 hours interval and follow broadly the expected Elkind pattern of two fraction experiments. Both the local control and the delay in growth data are in agreement that recovery occurs rapidly in this tumour system (at least in 3 hours) and also that D_2-D_1 values are always positive. Furthermore, the D_2-D_1 values obtained from these experiments seemed independent of the magnitude of the initial dose. However, smaller values are observed at 3 hours interval for cured tumours, compared with that for recurrent tumours, 7.7 to 8.7 and 10 to 12 Gy respectively.

In split dose experiment at 3 hours interval, using 0.67 mg/gm Misonidazole 30 minutes before each fraction of 20 Gy (tables 3.7 and 3.8), the obtained values were 6.25 Gy for cured tumours and 8 Gy for recurrent tumours. It can be seen that Misonidazole has reduced D_2-D_1 values only by a factor of 1.2 to 1.3, and not by the factor of 2 observed with single dose experiments. This loss in efficiency of Misonidazole in split dose experiments

might be due to the lower dosage of the drug compared with 1 mg/gm used with single doses. The results from Misonidazole also confirm the finding of rapid recovery in this tumour and the smaller D_2-D_1 value for cured tumours.

3.6 The Relationship Between Complete Regression and Local Control Probability:

The incidence of complete regression and the local control probability for X-rays alone are shown in table 3.9. It is clear that the incidence of complete regression is dose dependent. None of the tumours which were exposed to a single dose of 50 Gy or less, showed complete regression. Thereafter, the incidence of complete regression increased with dose, reaching 100 per cent at the 100 per cent curative dose of 80 Gy. Table 3.9 shows also the incidence of complete regression and the local control probability for X-rays, in combination with Misonidazole. Complete regression also appears to be dose dependent, reaching 100 per cent at a single dose of 37.5 Gy.

The data from table 3.9 are shown in figure 3.15, where the local control probability is plotted as a function of the incidence of complete regression. The points for both single doses of X-rays alone and in combination with Misonidazole seem to share a common curve, which is steeper after single doses that produced more than a 20 per cent cure rate or a 60 per cent complete regression. It is interesting to note that the data obtained from split dose experiments for both X-rays alone and in combination with Misonidazole as seen in the same table also share the same curve.

Table 3.9 Incidence of complete regression and local tumour control.

Single doses of X-rays alone

Dose (Gy)	Complete regression		Controlled tumours	
50	0/20	(0)	0/20	(0)
60	10/18	(55)	2/18	(11)
65	11/15	(73)	5/15	(33)
70	20/24	(83)	16/24	(67)
75	10/11	(90)	9/11	(82)
80	19/19	(100)	19/19	(100)

Single doses of X-rays + Misonidazole

Dose (Gy)	Complete regression		Controlled tumours	
15	0/20	(0)	0/20	(0)
20	1/15	(6.6)	0/15	(0)
25	2/17	(11)	1/17	(5.8)
27.5	4/16	(25)	1/16	(6.2)
30	11/13	(84.6)	8/13	(61.5)
35	25/28	(89)	21/28	(75)
37.5	14/14	(100)	14/14	(100)

Split dose experiments of X-rays alone (cumulative results of various intervals)

Initial dose (Gy)	Complete regression		Controlled tumours	
20	28/60	(42)	13/60	(21)
35	56/79	(70)	11/79	(14)
50	38/61	(62)	13/61	(21)

Split dose experiments of X-rays + Misonide (equal fractions of 20 Gy each at 3 hr interval)

Initial dose (Gy)	Complete regression		Controlled tumours	
20	10/10	(100)	7/10	(70)

The numbers in brackets denote percentages.

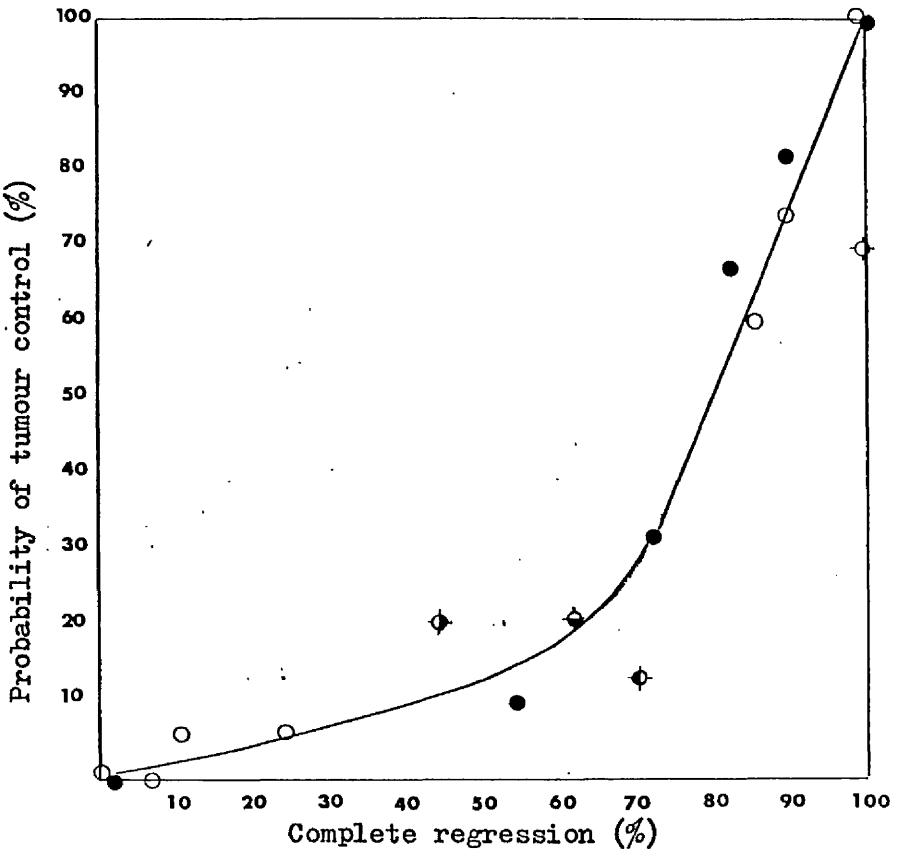


Figure 3.15: The relationship between probability of tumour control and complete regression.

- ● Single doses of X-ray alone
- ○ Single dose of X-rays in combination with Misonidazole
- ⊕⊕⊕ Two dose experiments of X-ray alone 20, 35 and 50 Gy initial dose
- ⊹ Two dose experiment of X-ray in combination with Misonidazole

3.7 The Skin Reactions of Irradiated Tumours:

Visual observations provided the most sensitive estimation of the skin reactions after irradiation, in comparison with serial colour photographs and colorimetric instruments (180). Arbitrary scales for the observed skin reactions have been adopted by several workers (180,251,252,253) and the average skin reaction was obtained from the mean values of several observations over a chosen period of time. Hill et al. (254) in a comparison of the responses of spontaneous mammary tumour and the reactions of the skin over the tumour after irradiation with electrons and neutrons, pointed to the difficulty of identifying meaningful end-points for tumour-normal tissue comparison, and the possibility of disturbance of skin vasculature or blood flow which might occur due to the presence of tumour. Fowler et al. (255) found that the skin reactions over tumours in C_3H mice were difficult to measure and did not vary significantly with dose. They pointed out that no reliable conclusions would be drawn from these observed skin reactions.

In the present tumour system, visual observations and inspection of the skin over and around the tumours were made 3 to 5 days every week, along with measurement of the tumour after irradiation. Although no scoring system was used, the skin observations were in agreement with those of others (254,255), in that the skin reactions over the tumours showed little or no dependence

on the dose. These reactions were similar within the dose ranges which led to tumour regression (section 3.2), i.e. 25 to 50 Gy in the presence of Misonidazole and 50 to 80 Gy in its absence. With smaller X-ray doses, the delay in tumour growth was not enough to allow accurate observations of the skin reactions before sacrificing the animals. Hence, it was difficult to determine if the radiosensitizing effect of Misonidazole had contributed to the skin reactions as reported by Brown (202). This might be the case with the natural existence of some hypoxic cells in the skin of mice (179,204,256); the disturbance of the normal vascularity of the skin due to the presence of the tumour (section 3.11.2) and/or a possible degree of hypoxia induced by the irradiation procedure (section 2.5.4).

The following skin reactions, however, are worthy of mention:-

3.7.1 Alopecia:

Alopecia was noticed to start 8-11 days after irradiation at the summit of irradiated tumours; it then gradually spread in a few days towards the periphery where the two skin layers met as they passed through the slit of the irradiating jig. The time of alopecia marked the time of the start of tumour regression (section 3.2.1).

3.7.2 Erythema and Desquamation:

The epilated skin showed erythema which gradually merged

into a state of moist desquamation. The peak reaction was noticed about 18 to 22 days after irradiation. This was the time when the tumour reached its minimal volume after irradiation. The desquamated skin gradually started to heal underneath the dried scab which fell off in small pieces. The healing process was usually completed about 15 days after the peak reaction i.e. about 35 days after irradiation. The healed skin appeared more whitish than normal skin; leathery and thickened with apparent loss of elasticity. These changes in the skin texture have been reported by other workers (253). The desquamated skin caused some difficulty in measurement of the regressed tumours and the thickened skin during the healing process led to some difficulty in palpating for the presence or absence of residual tumours. However, many tumours have been controlled at 100 days despite the thickened skin, which might suggest that this thickening is not related to the presence of residual tumours. As in the case of alopecia, the maximum degree of skin reaction was noticed at the summit of the irradiated tumours and the scabby areas at that region were last to fall, leaving a longitudinal mark similar to a healed scar.

3.7.3 Appearance of Grey Hair:

Grey hair appeared after about 30 to 35 days at the periphery of the epilated areas and gradually spread in a less dense manner centrally, but in most cases,

permanent alopecia remained at the centre of the epilated areas (plate 3.1).

3.7.4 The Skin Reactions in the Absence of Tumours:

The skin reactions following a single dose of 70 Gy of X-rays alone were essentially similar to those in the presence of tumours. The only difference was the appearance of alopecia 2 to 3 days later than in the presence of tumours.

Plate 3.1: Gray hair with permanent alopecia at the centre of the site of previously cured tumour.

3.8 Transplantation at Sites of Previously Cured Tumours:

10^6 tumour cells injected at these sites produced 100 per cent tumour take rate and a latent period which was not significantly different from that of routine transplanted tumours (figure 3.2). The mean latent periods were 9.3 ± 3.5 and 8.4 ± 3.1 days for routine transplants and cured sites, respectively (table 3.10). The absence of a significant difference ($p > 0.05$) between these means would imply that no relative restriction of growth occurred at the cured sites during the latent period.

The tumours obtained at these sites grew at approximately half the rate of growth of routinely transplanted tumours (table 3.11 and figure 3.16). This was until 5-7 mm mean diameter; the size at which the previously cured tumours were irradiated. Thereafter, many of these tumours showed a period of no or relatively slow growth for 4 to 6 days before resumption of growth at a rate which was not significantly different ($p > 0.05$) from that of routinely transplanted tumours (table 3.12).

There was no difference in the rate and pattern of growth of transplanted tumours at the sites of previous tumours, cured with single doses of 60 Gy or more of X-rays alone and with single doses of 25 Gy or more of X-rays in combination with Misonidazole (table 3.11). This would suggest that the factors affecting tumour growth at these sites were similar, irrespective of the dose level of 25 Gy or more.

Table 3.10 Statistical analysis of the latent periods following transplantation with 10^6 tumour cells.

Tumour Group (20 tumours in each)	Mean latent period (days)	Significance of difference from control *	Comment
Routine transplants (control)	9.3 ± 3.48	-	-
Sites of previously cured tumours	8.4 ± 3.10	$p > 0.05$	Not significant
At sites irradiated with a single dose of 70 Gy	14.4 ± 4.66	$p < 0.01$	Significant
Immunized mice	4.85 ± 2.25	$p < 0.01$	Significant

Errors are $\pm$ 1 standard deviation

* Evaluated by the Wilcoxon (or Mann-Whitney) test (257)

Table 3.11 Statistical analysis of tumour growth rates during the early phase (first 10 days).

Tumour Group	Number of tumours evaluated	Slope of best line * mm/day	Significance of ** difference from control	Comment
Routine transplants (control)	31	0.889 ± 0.164	-	-
Immunized mice	20	1.06 ± 0.282	$p < 0.05$	Border-line significance
Outside cured sites	11	0.827 ± 0.170	$p > 0.05$	Not significant
At sites of previously cured tumours with single doses of X-rays of 60 Gy or more	16	0.493 ± 0.170	$p < 0.001$	Highly significant
At sites of previously cured tumours with single doses of X-rays of 50 Gy or less + Misonidazole	16	0.435 ± 0.170	$p < 0.001$	Highly significant
At sites irradiated with a single dose of 70 Gy	14	0.571 ± 0.122	$p < 0.001$	Highly significant

* Evaluated by the least squares method (appendix II)

** Evaluated by t-test with Bessel's correction

Errors are $\pm$ 1 standard deviation

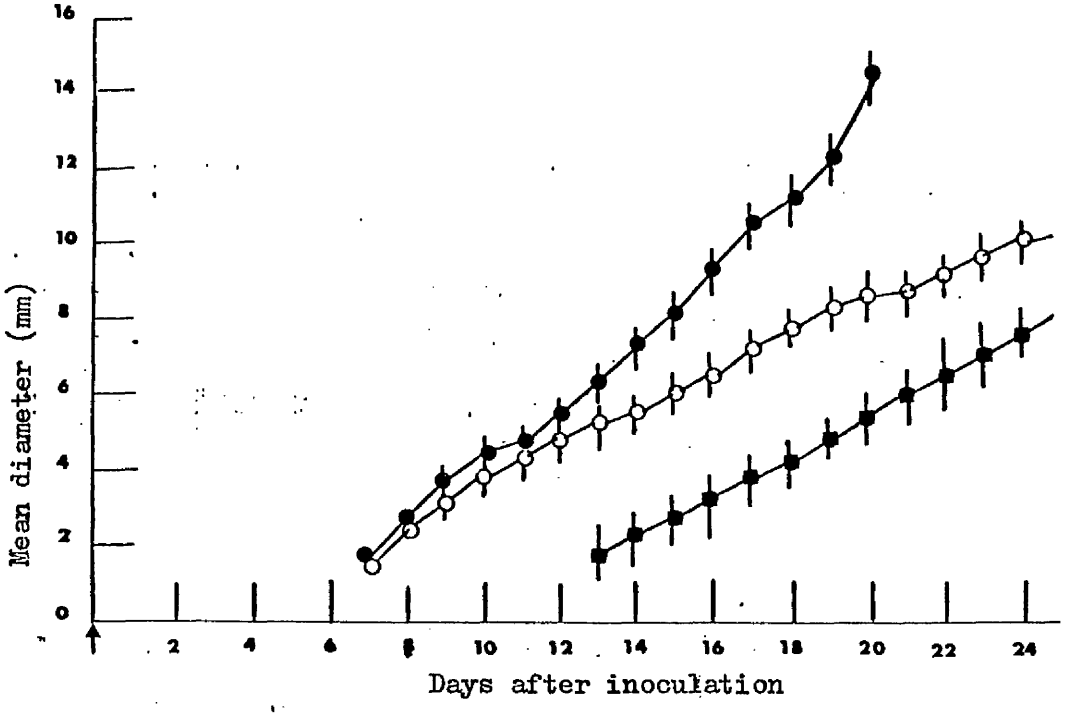


Figure 3.16: Growth of multiple generation C_3H mammary carcinoma.

- ● Routinely transplanted tumours
- ○ Transplants at sites of previously cured tumours
- ■ Transplants at sites irradiated with 70 Gy
- ↑ The day of inoculation

The start of each curve indicates the time at which 50% of the tumours were detectable.
The mean diameter used, does not include skin thickness (1.25 mm).

Table 3.12 Statistical analysis of tumour growth rates during the late phase (last 10 days).

Tumour Group	Number of tumours evaluated	Slope of best line * mm/day	Significance of ** difference from control	Comment
Routine transplants (control) +	31	0.889 ± 0.164	-	-
At sites of previously cured tumours with single doses of 60 Gy or more	16	0.81 ± 0.182	$p > 0.05$	Not significant
At sites irradiated with a single dose of 70 Gy	14	0.73 ± 0.192	$p < 0.01$	Significant
Recurrent tumours after a single dose of 50 Gy	8	0.79 ± 0.109	$p > 0.05$	Not significant
Recurrent tumours after a single dose of 60 Gy	8	0.64 ± 0.134	$p < 0.01$	Significant
Recurrent tumours after a single dose of 65 Gy	7	0.71 ± 0.148	$p < 0.05$	Border-line Significance

+ For control, the same value in table 3.11 was used, as 10 days covered almost the observed period of growth

* Evaluated by the least squares method (appendix II)

** Evaluated by t-test with Bessel's correction

Errors are $\pm$ 1 standard deviation

In a comparative group of mice with cured tumours, transplanted tumours outside the cured sites grew at a rate which was not significantly different from that of routinely transplanted tumours (table 3.11 and figure 3.17). This indicated that the age and general health of the mice had no effect on tumour growth.

3.9 Transplantation at Sites Irradiated with a Single Dose of 70 Gy:

10^6 tumour cells injected at these sites produced 100 per cent tumour take rate. The mean latent periods were 9.3 ± 3.5 and 14.4 ± 4.6 days for routine transplants and irradiated sites, respectively (table 3.10). This five day increase in the mean latent period was statistically significant ($p < 0.01$). It can be seen in figure 3.2 which shows the cummulative incidence of detectable tumours, that 50 per cent of the tumours developed by 13 days following transplantation. This was in contrast with 7.5 days for routine transplants. The rate of growth at the irradiated sites was significantly slower than that of routine transplants, both during the early and late periods of growth (tables 3.11, 3.12). Furthermore, the period of no or slow growth observed at the cured sites when the tumours reached 5–7 mm mean diameter was not obvious at the irradiated sites. The differences revealed in this work between tumour growth at the sites of previously cured tumours and the irradiated sites, suggest that tissues which have been

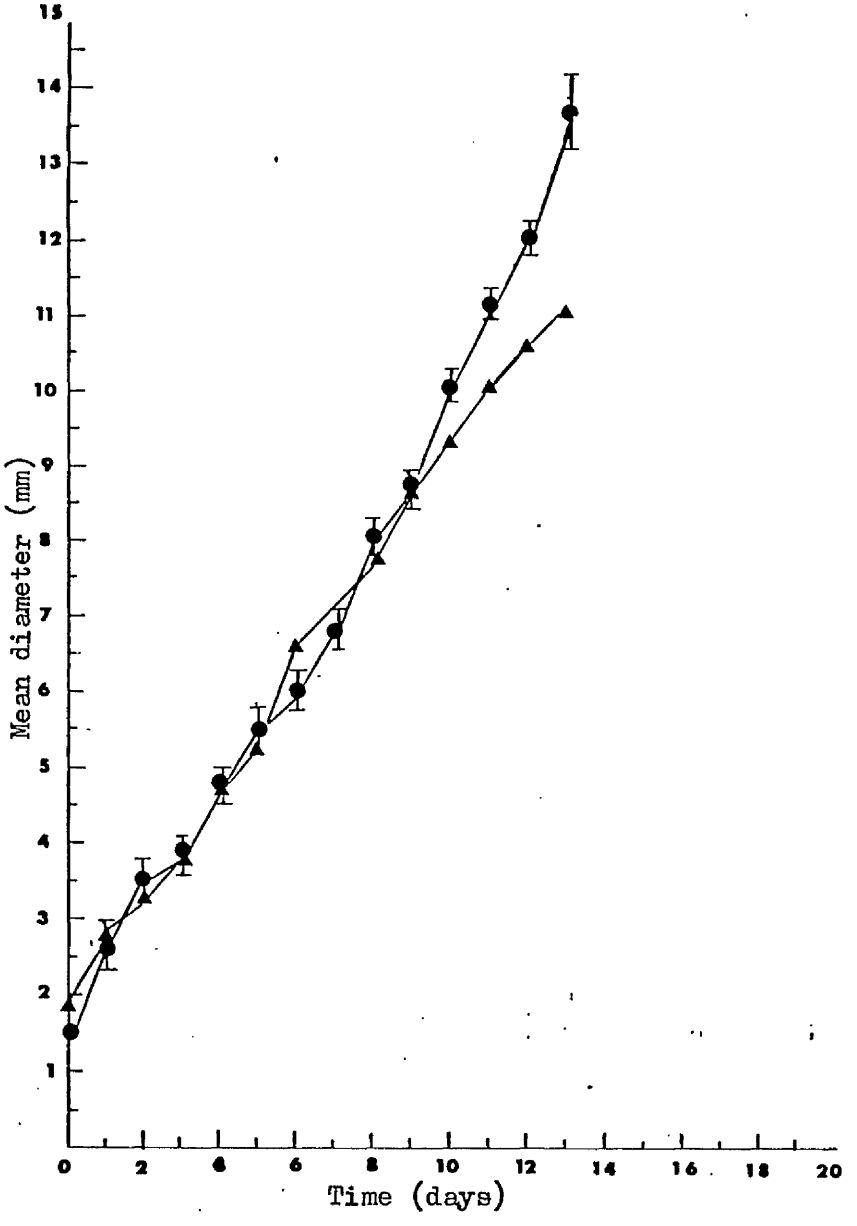


Figure 3.17: The growth curves of the tumour in control and cured mice (outside the cured site).

● = control mice

▲ = transplant outside the cured area

The mean diameter does not include skin thickness (1.25 mm).

stimulated by previous tumours (cured sites) would support the growth of a second tumour, more efficiently than pre-irradiated quiescent normal tissues.

As mentioned in section 3.4, the slow and inconsistent growth of recurrent tumours has led to difficulties in analysis of the rate of growth during the early period. Therefore, the time taken for the tumours to grow from 3 to 10 mm mean diameter was used to compare the rate of growth at the cured and pre-irradiated sites and that of recurrent tumour (table 3.13). Only those tumours which recurred after complete regression were used for such analysis. It appears that recurrent tumours grew at a much slower rate; the time taken for these tumours to grow from 3 to 10 mm was approximately double that of transplanted tumours at the cured and pre-irradiated sites. The absence of the period of no or very slow growth at the pre-irradiated sites, is an apparent cause for the similarity in times at the cured and pre-irradiated sites, despite the slow growth at the latter site during the late period of growth.

Table 3.13 Times for tumours to grow from 3 to 10 mm mean diameter.

Group	Number of tumours evaluated	Time (days)
Routine transplants	50	8.7 ± 0.22
Recurrent tumours after * single doses of 60 Gy or more (X-rays alone)	19	28.4 ± 2.2
Recurrent tumours after * single doses of 25 Gy or more (X-rays + Misonidazole)	10	24.9 ± 3.1
Transplanted tumours at cured sites (single doses of 60 Gy or more)	33	14.3 ± 0.64
Transplanted tumours at cured sites (25 Gy or more)	19	14.6 ± 0.88
Transplanted tumours at irradiated sites with a single dose of 70 Gy	14	13.9 ± 0.54

Errors are ± 1 s.e.m.

* Recurrent tumours after complete regression

3.10 The Immune Status of the Tumour System:

Table 3.14 shows the PHA studies in various groups of mice. The PHA indices of mice bearing either spontaneous or transplanted C_3H mammary tumours were not significantly different from each other but were significantly lower than the mice bearing no tumours. In transplanted tumours, the depression of PHA index occurred when the tumour became just palpable and remained at the same low level thereafter despite the increase in tumour mass. The PHA index of mice cured with radiation rose to a level which was not significantly lower than that of the control mice but was significantly higher than the PHA index of mice with untreated tumours.

This indicated a significant recovery of T-lymphocyte response in cured mice and also confirmed that the depression of PHA indices in mice bearing tumours would be due to the presence of tumours. However, the PHA index of unity for mice with tumours transplanted in irradiated skin folds might suggest some depression of PHA index, resulting from the scattered radiation to the mouse body inside the irradiation jig (section 2.5.3). It was interesting to find that the immunization procedure using lethally irradiated cells, has led to depression of the PHA index in such "immunized" mice before tumour transplantation. The depressed level of PHA index in these mice was similar to that obtained in mice bearing tumours.

Table 3.14

PHA studies of the tumour system.

Group of mice	Number of mice	PHA index	S.E.M.	% Δ control
Control mice	23	4.54	0.58	100 %
Mice with spontaneous tumours	14	1.51	0.23	14 %
Mice with transplanted tumours	32	1.56	0.13	16 %
Immunized mice	8	1.63	1.23	18 %
Mice cured with radiotherapy	17	3.76	0.66	83 %
Mice with tumour transplanted in an irradiated skin fold (70 Gy)	8	1	0	0 %

Significance of difference between the various groups as assessed by "t-test" with Bessel's correction.

Category of difference for assessment		Significance level	Comments
Control	v spontaneous tumours	p 0.001	Highly significant
Control	v transplanted tumours	p 0.001	Highly significant
Control	v immunized mice	p 0.001	Highly significant
Spontaneous tumours	v transplanted tumours	p 0.05	Not significant
Control	v cured mice with radiotherapy	p 0.05	Not significant
Transplanted tumours in irradiated sites	v cured mice with radiotherapy	p 0.001	Highly significant

Immunization of the mice has led to an enhancement of tumour growth during the latent period. 50 per cent of the tumours in the immunized mice were detectable at 3.5 days after transplantation (figure 3.18). This was in contrast with 7.5 days for routine transplants. Statistical analysis of the means of both latent periods (table 3.10) showed that the difference was statistically significant ($p<0.01$).

The enhancement of tumour growth was not limited to the latent period but it continued through the observed period of growth. In figure 3.19 and table 3.11, it can be seen that the tumours in immunized mice grew faster than those of routine transplants. This enhancement of growth was on the border-line of significance ($p<0.05$).

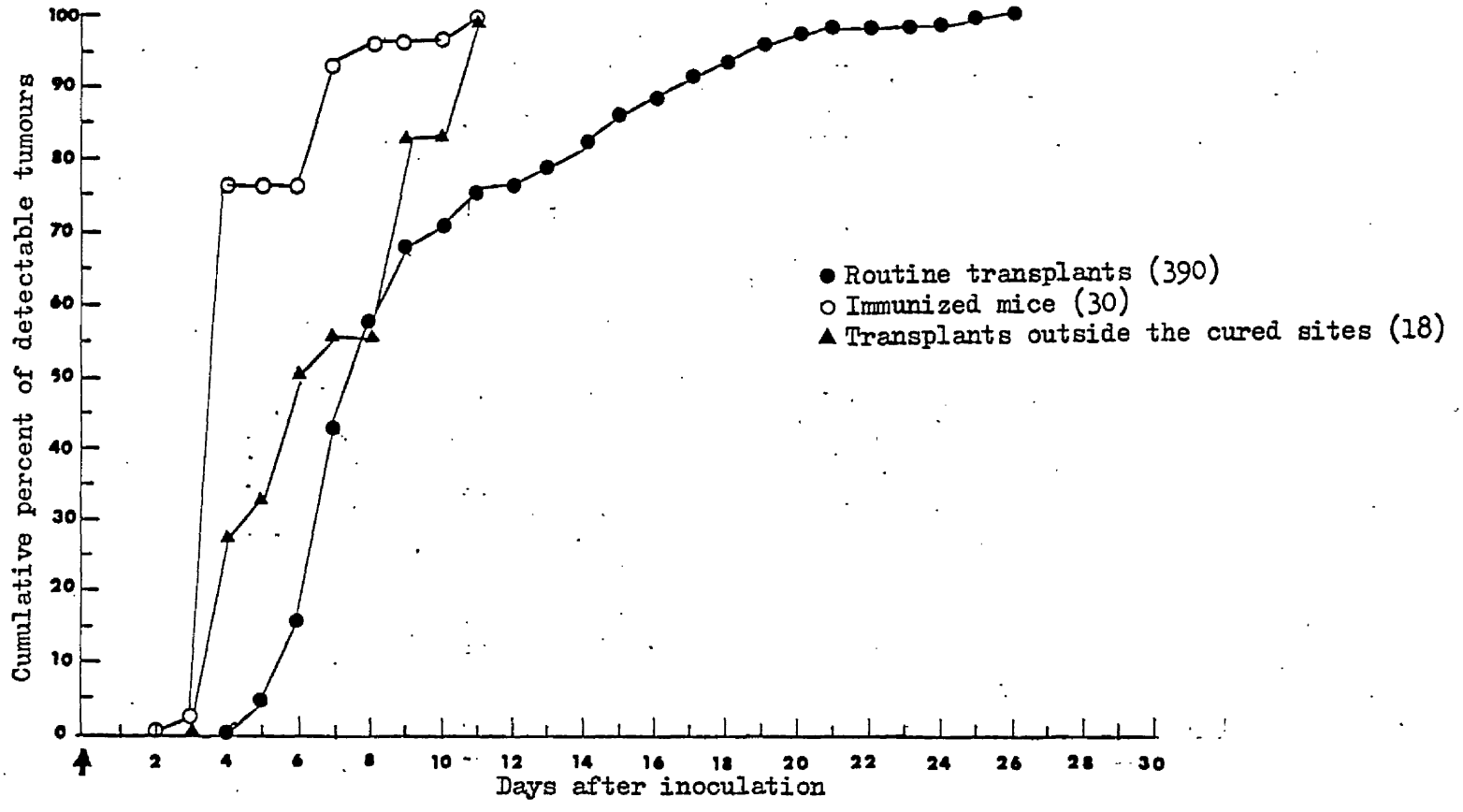


Figure 3.18: The daily cumulative percentage of detectable tumours

(↟) the day of inoculation

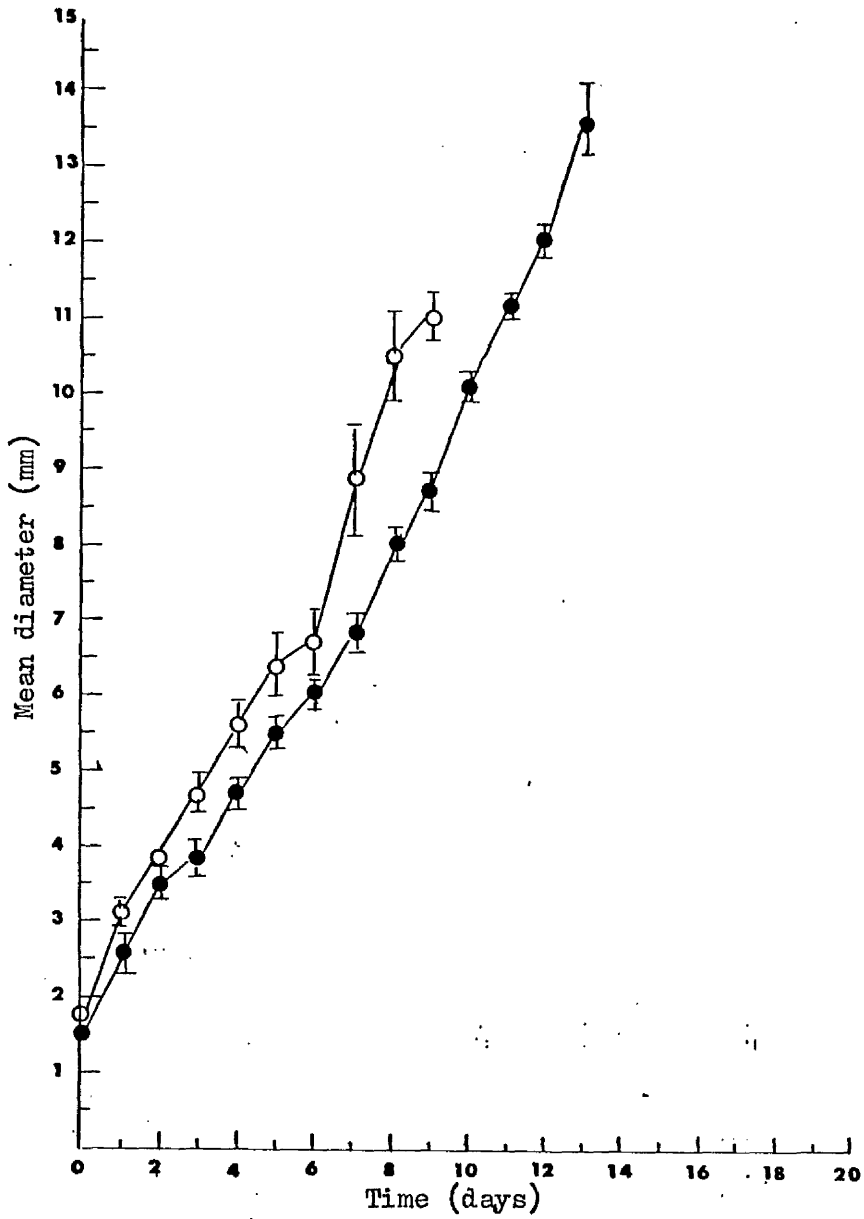


Figure 3.19: The growth curves of the tumours in control and immunized mice.

● = control mice

○ = immunized mice

The mean diameter does not include skin thickness (1.25 mm).

3.11 Losses in Mice with Locally Controlled Tumours Before 100 Days:

During the experimental work, some mice with locally controlled tumours were sacrificed due to sudden sickness or died unexpectedly before the end-point of 100 days. These losses were presumably due to metastases rather than gut or bone marrow death due to the low scatter of radiation to the mouse body during irradiation (less than 1 per cent of the given dose). This has been confirmed in a prospective experiment designed to investigate this phenomenon in 50 mice with their tumours treated with a curative single dose of 80 Gy. One of these tumours recurred at 80 days and was excluded from the analysis. From the remaining 49 mice, 8 died unexpectedly and 7 of them were examined histologically and in all, lung metastases were revealed. Table 3.15 shows the losses in mice with locally controlled tumours, during the whole experimental work. From a total of 283 mice, 33 died unexpectedly or were killed due to sickness, giving an incidence of 12 per cent. It can be seen in figure 3.20 that there is a peak in the incidence of metastases between 25 to 40 days after irradiation. This seems to be the average time needed for the tumour cells to grow in the lung and kill the host. There was no significant difference in the incidence of metastases between the various treatment groups in table 3.15. Furthermore, an inspection of figure 3.21 shows no correlation between the timing of metastases and the dose in each group.

Table 3.15 Incidence of losses in mice with locally controlled tumours before 100 days.

Experimental Group	Number + of mice analyzed	Number of Losses	Days of losses after irradiation	Incidence
Single doses to unclamped tumours	56	5	22, 24, 25, 35, 60	9 %
Single dose of * 80 Gy to unclamped tumours	49	8	19, 26, 26, 31, 38, 39, 59, 73	16 %
Single doses to clamped tumours	37	4	38, 52, 56, 61	11 %
Single doses in combination with Misonidazole	99	11	24, 29, 30, 30, 30, 30, 39, 40, 50, 54, 62	11 %
Split dose experiments of X-rays alone	42	5	35, 36, 40, 44, 45	12 %
Total	283	33		12 %

* Histological examination was performed on all the unexpected deaths except one and all proved to be metastatic deaths

+ All mice with recurrent tumours are excluded

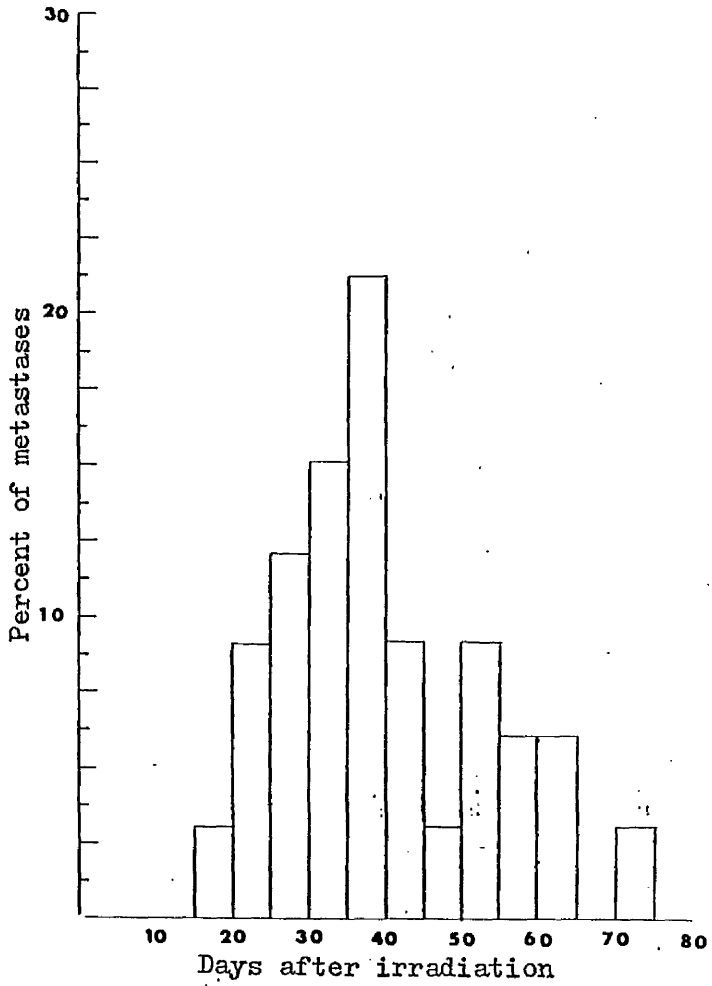


Figure 3.20: The time distribution of unexpected deaths of 33 mice (metastases)

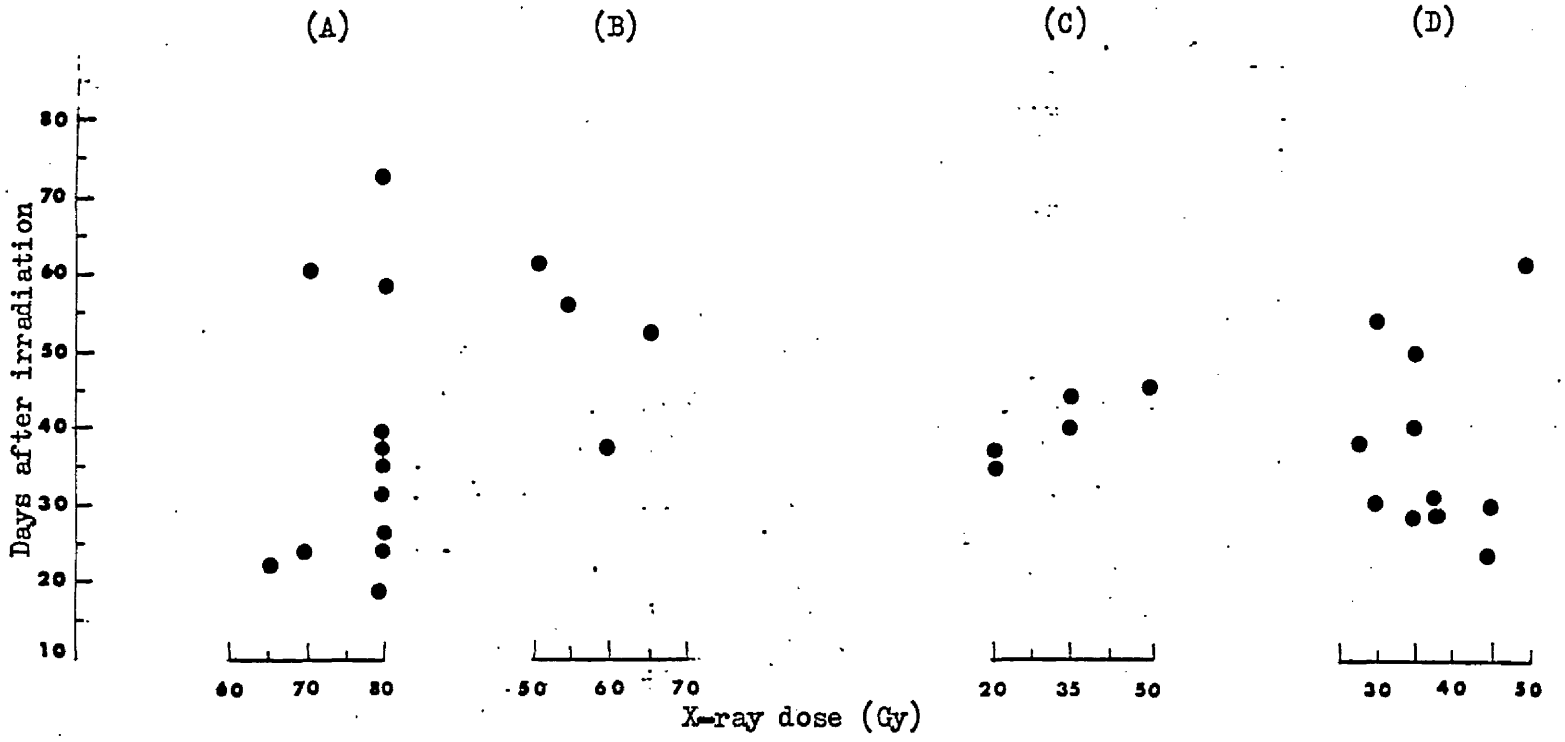


Figure 3.21: Time distribution of unexpected deaths of 33 mice versus doses
(A) unclamped tumours (B) clamped tumours (C) split dose experiments
(D) Misonidazole experiments

These findings suggest an inherent tendency for metastases from the tumour, which is independent of the dose of radiation, the clamping procedure and the use of Misonidazole. However, Walker et al. (258) showed a significant promotion of metastases from the tumour by locally curative hyperthermia.

3.12 Histological Studies:

3.12.1 The Anatomy and Histology of the Skin of the Mouse Back:

Mouse skin is composed of epidermis which is a stratified squamous epithelium and dermis which consists of fibrous connective tissue. A thin layer of stratified muscle fibres; the panniculus carnosus muscle (P.C.) separates the dermis from the subcutaneous loose connective tissue layer. The latter facilitates free movement of the skin over the dorsal muscles and through it, the skin can be stripped off easily with minimal bleeding. Plate 3.2 shows that the blood supply of the skin originates from the axillae and groins. Histologically, the blood capillaries are distributed superficially and deep to the P.C. muscle but mainly in the dermis. This is in contrast with the deeper part of the subcutaneous layer which is nearly avascular. Plate 3.3 shows that the skin with the tumour mass can be stripped off easily from the back of the mouse. It also demonstrates the increased size of the blood supply of the skin and the increased vascular density at the periphery of the tumour. The stimulated vasculature at the site of the inocula can be seen as early as 3 days after transplantation and becomes more obvious as the tumour reaches 3 to 4 mm mean diameter. This vascular density becomes relatively, less obvious in larger tumours, most likely because of displacement and wide separation of the blood vessels by the growing tumour.

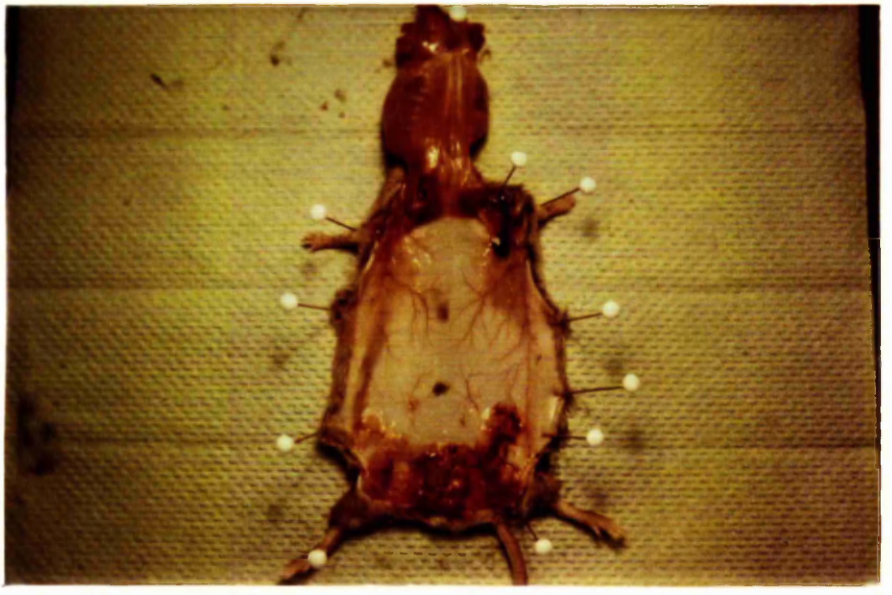


Plate 3.2: The blood supply of the skin.

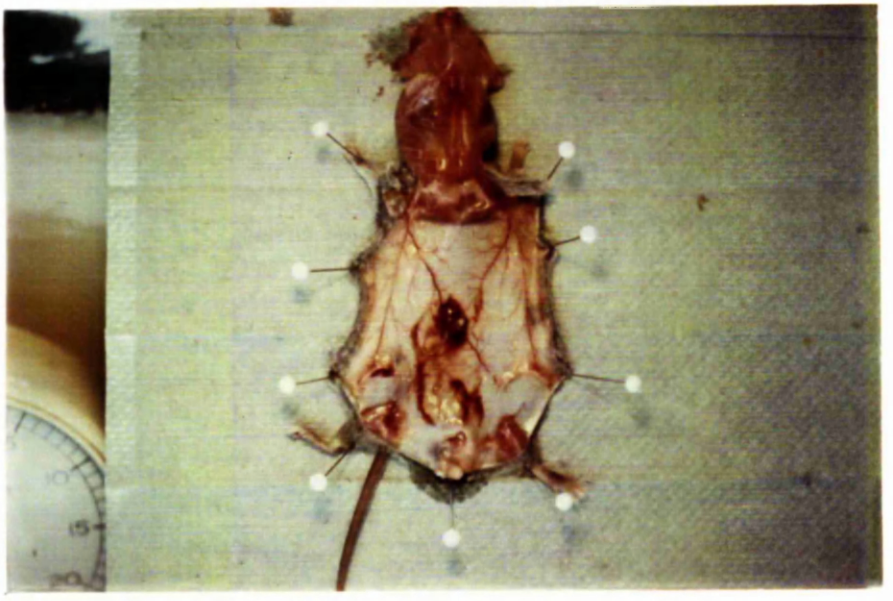


Plate 3.3: The blood supply of the tumour.

Microscopically, this increased vascular density is a reflection of numerous, thin walled, dilated blood vessels in relation to the P.C. muscle.
An interesting observation is the minimal or absent vascularity in the vicinity of those tumours with very slow growth rate or late appearance (> 30 days after transplantation).

3.12.2 Histological Examination of the Tumour:

The early phases of the structural organization of the tumour can be seen in the histological sections of small tumours (2 to 3 mm mean diameter). The tumour appears to grow in the subcutaneous layer from several foci of single cells or clumps of tumour cells. The cells in the vicinity of the P.C. muscle have the opportunity to encircle the numerous blood vessels in that region.
On the other hand, the tumour foci at a deeper level grow in size forming large nodules with a necrotic centre, surrounded by a rim of about 100 μ width of apparently healthy tumour cells. The contact of these nodules with each other and also with the outer part of the tumour, results in necrosis of the inner parts of the rims and hence the formation of a large necrotic zone enclosing in a wavy rim of tumour cells.
Plate 3.4 highlights this type of growth in the absence of nearby stromal blood vessels. The thin wavy rim of this large nodule represents the remnants of several small nodules after necrosis of their adjacent rims.

Plate 3.4: The microscopic features of the tumour in the absence of nearby stromal blood vessels (haematoxylin, eosin X100). The thin wavy rim ($\approx 100\mu$) of apparently viable tumour tissue represents the remnants of several small nodules.

The constant width of this peripheral rim ($\approx$100 μ) is most likely to be due to the death of an inner cell layer for every additional outer cell layer. This is also the histological appearance of some tumours which appeared after a long latent period (> 30 days after transplantation and grew very slowly. The final tumour structure can be seen clearly on histological studies of large tumours (6 and 10 mm mean diameter) plates 3.5 and 3.6:-

In general, the tumour has a signet ring appearance with a thicker dome under the epidermis consisting of several tumour cell layers which taper to a few cell layers or even is defective at the inner part of the tumour. The apparently healthy tumour rim surrounds an eccentric necrotic area present towards the deeper part of the tumour. Numerous tumour cords can be seen inside and at the periphery of the necrotic zone. Each cord consists of about a 100 μ rim of apparently healthy tumour cells encircling a central blood vessel, plate 3.7. These cords become well demarcated with wider central vessels towards the centre of the necrotic zone. However, several of these cords show massive necrotic process, leaving pyknotic tumour nuclei surrounding a large number of red blood cells. The process of necrosis appears to increase with tumour growth. This can be seen in table 3.16 where the relative volume of necrosis is more than doubled as the tumour size increases from 2-3 to 9-10 mm mean diameter.

Plate 3.5:
Photomicrograph of a 6 mm tumour showing an eccentric necrotic area present towards the deeper end of the tumour.
The panniculus muscle can be seen at the periphery, below the epidermis.

Plate 3.6:
Photomicrograph of a 10 mm tumour showing numerous tumour cords inside and at the periphery of the necrotic zones.

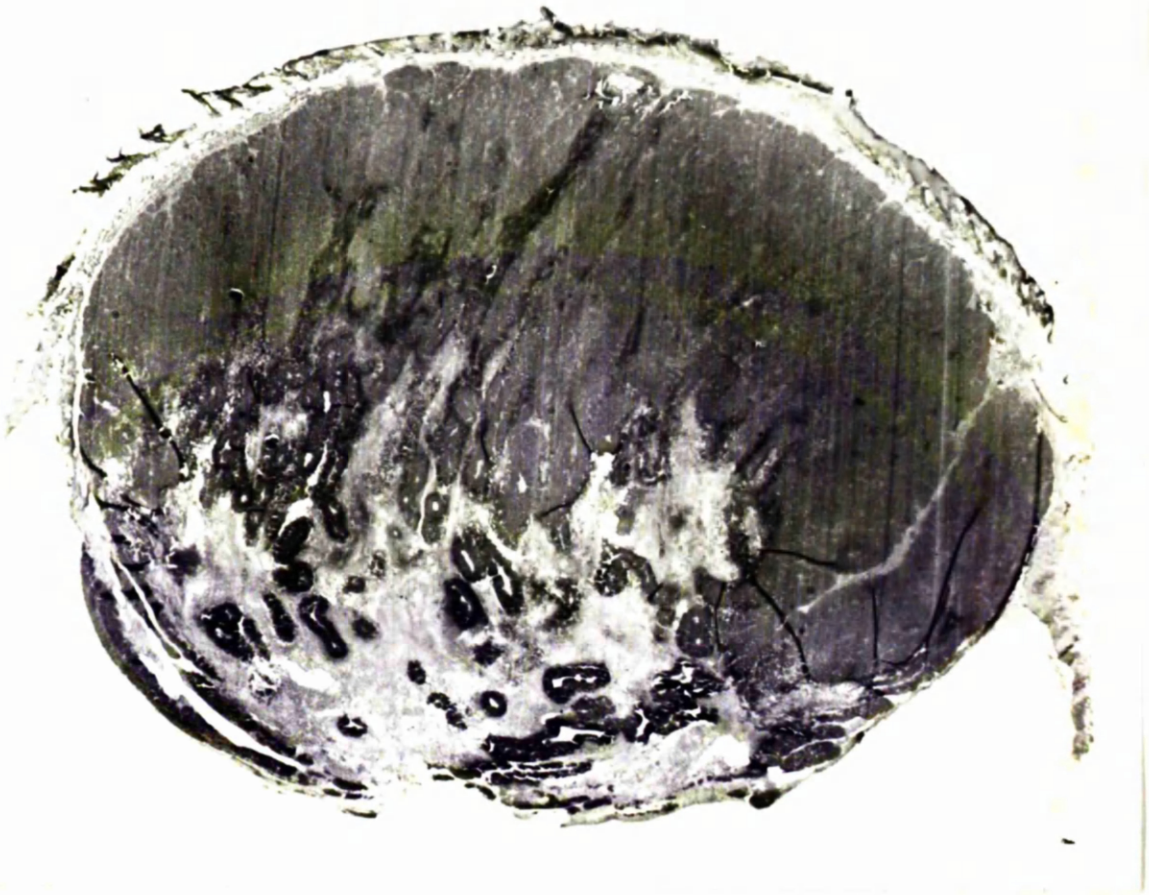


Plate 3.7: Tumour cords in unirradiated tumours (haematoxylin, eosin X100). Each cord consists of about a 100μ rim of apparently healthy tumour cells encircling a central blood vessel. Surrounding the cords, numerous pyknotic nuclei can be seen; these are the result of relatively recent necrosis.

Table 3.16 Necrosis in multiple generation C_3H mammary tumour.

Tumour size (mm)	The relative volume of necrotic tissue (%)		
	Routine Transplants	Recurrent tumours after single dose of 60 Gy	Transplanted tumours at cured sites
2 - 3	16.9 ± 5.8	-	-
5 - 6	21.6 ± 4.9	63 ± 5.44	54 ± 7.15
9 -10	42.2 ± 7.7	54.5 ± 8.19	47 ± 10

Errors are ± 1 standard deviation

The outward displacement of the surrounding tissue by the expanding tumour results in the formation of a fine fibrous capsule around the tumour mass. This capsule is deficient under the epidermis where the thicker outer part of the tumour has destroyed and replaced the P.C. muscle. The presence of tumour cells in the subcutaneous tissue appears to induce a cellular reaction, mainly of fibroblasts and lymphocytes with few macrophages. This reaction is more obvious in small tumours (2 to 3 mm mean diameter) than in large tumours and hence it may indicate a temporary immune reaction at an early phase of growth.

The presence of a tumour capsule and the growth of the tumour by expansion rather than invasion, have facilitated the removal of the tumour intact with careful dissection. The tumour has a fleshy consistency and greyish tinge on cut section. Histologically, the tumour is undifferentiated adenocarcinoma with areas of necrosis seen in the tumour at a very small size. This is in contrast with the original spontaneous C_3H mammary adenocarcinoma, obtained from the Gray Laboratory.

Plate 3.8 shows the histological structure of an early transplant of that tumour; it emphasizes the well differentiated nature, the uniform distribution of the stroma and vascularity and the absent or minimal necrotic areas in the original tumour.

3.12.3 Histology of Irradiated Tumours:

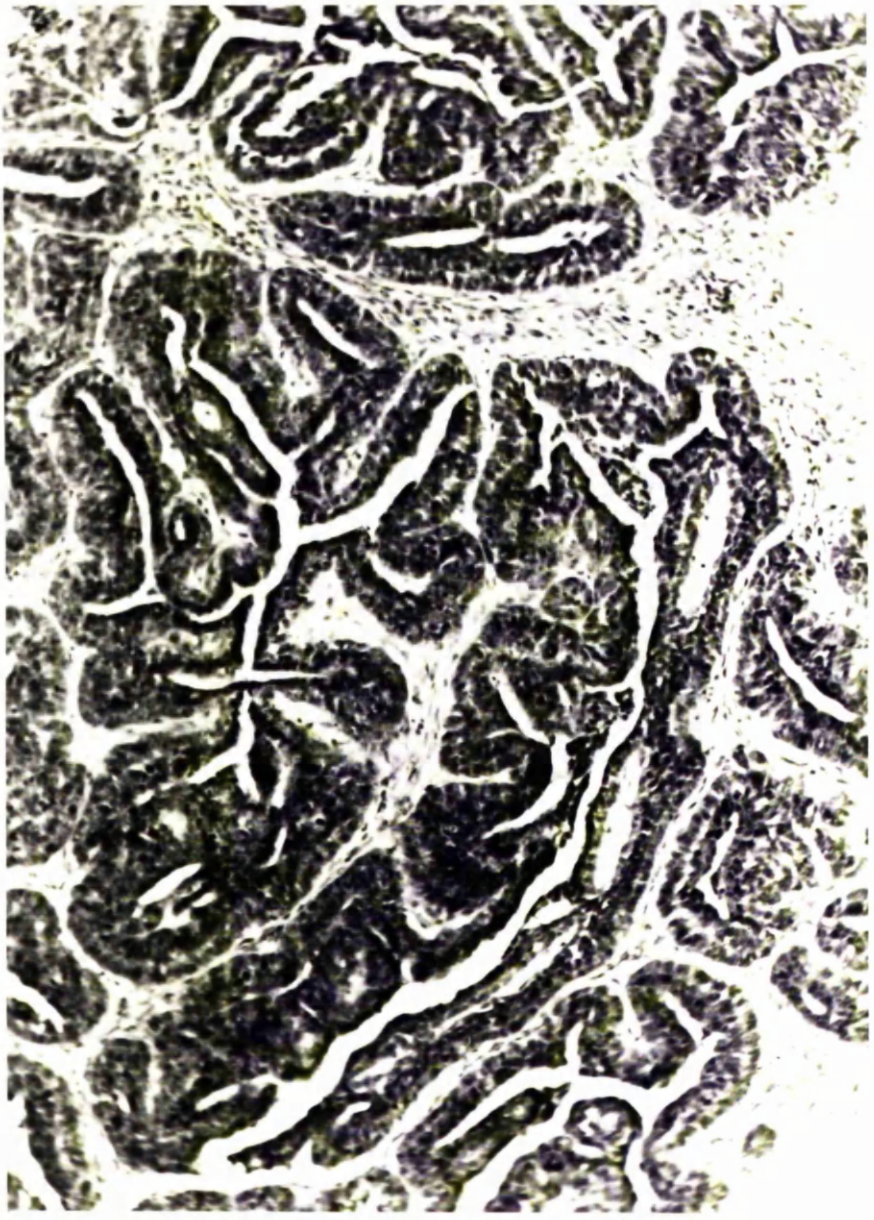


Plate 3.8: Microscopic features of an early transplant of the tumour (haematoxylin, eosin X100). There is acinar formation with uniform distribution of the stroma and vascularity. No necrosis can be seen.

The histological picture during the regression period was similar in all irradiated tumours both with single doses of X-rays alone and in combination with Misonidazole. At 8 to 10 days following irradiation, the tumour mass consisted of a large necrotic centre surrounded by a rim of tumour cells which was usually defective at the deeper part of the tumour (plate 3.9). The apparently viable cells at the periphery of the tumour showed incomplete cell division, forming multinucleated giant cells. The central and deeper part of the tumours suffered most obvious radiation damage with complete disappearance of the tumour cords by 8 to 10 days following irradiation. The histological studies of the regressed tumours at 16 to 18 days revealed a reduction of the relative volume or even complete disappearance of the necrotic areas, leaving a few scattered tumour cells in fibrosclerotic subcutaneous stroma. These histologically intact cells were even present in tumours, irradiated with single curative doses of 80 Gy of X-rays alone and 40 Gy in combination with Misonidazole(plate 3.10). Macrophages in different stages of phagocytosis and neutrophils were seen especially in the regressed tumours 16 to 18 days following irradiation, but no lymphocytes were observed. Vascularity was less marked in the irradiated tumours 8 to 10 days following irradiation; only scattered blood vessels could be seen at the periphery of the tumour mass. However,

Plate 3.9: Microscopic features of an irradiated tumour 10 days, following irradiation (haematoxylin, eosin X100). The tumour mass consists of a large necrotic centre, surrounded with a thin rim of tumour cells. These cells show incomplete cell division, forming multinucleated giant cells.

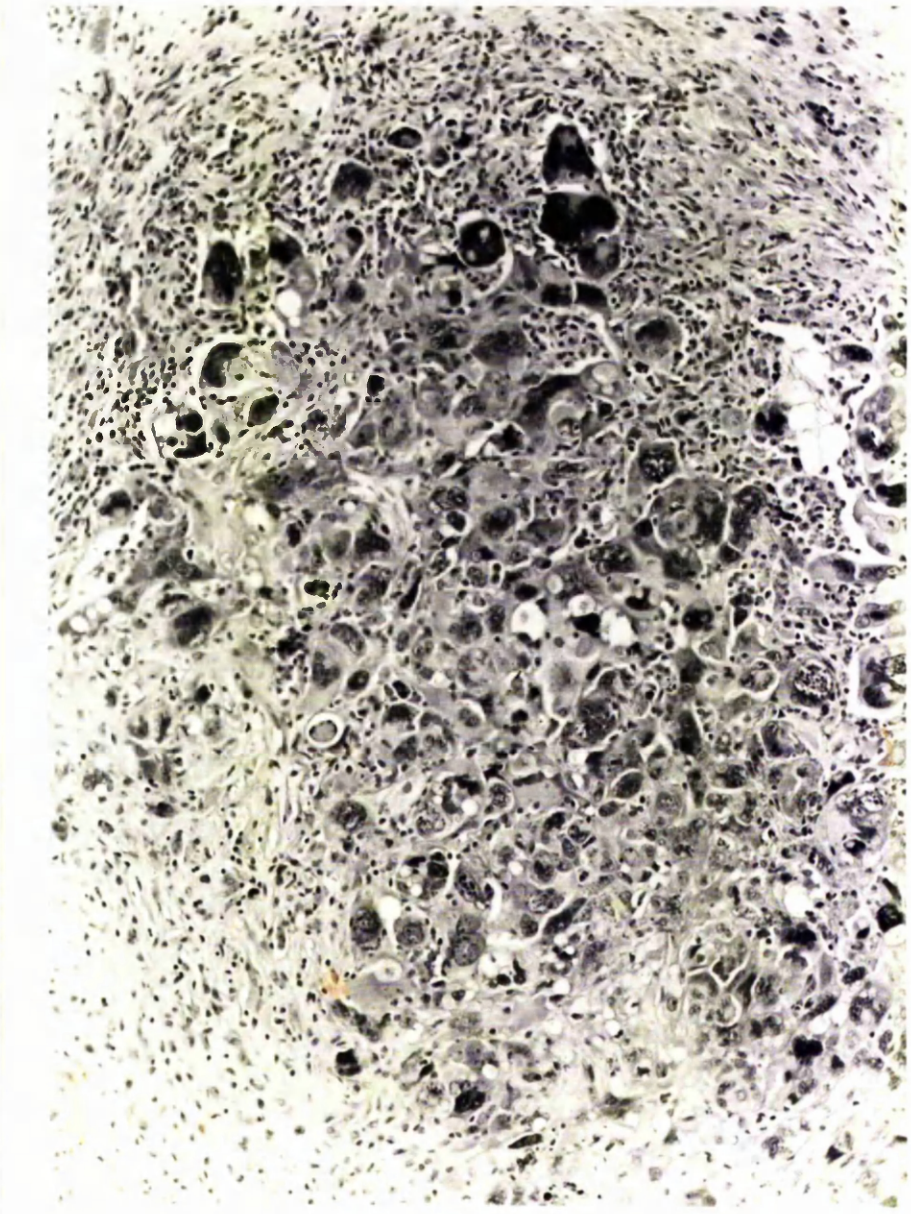


Plate 3.10: Microscopic features of an irradiated tumour 16 days following irradiation with a single dose of 80 Gy (haematoxylin, eosin X200). The necrotic tissue has completely disappeared, leaving scattered tumour cells in fibrosclerotic stroma.

vascularity became relatively more obvious in the regressed tumours 16 to 18 days following irradiation.

3.12.4 Histology of Recurrent Tumours:

The general morphological structure of these tumours was essentially similar to that of unirradiated tumours. However, the relative volume occupied by necrosis was larger (table 3.16) and several necrotic zones extended through the outer tumour dome which appeared thinner than in unirradiated tumours (plate 3.11). The excess necrosis in the recurrent tumours demarcated well and separated widely the tumour cords from each other (plate 3.12). Despite the apparent scatter of the cords within the tumour mass, the width of their rims of healthy tumour cells was still $\approx$ 100 μ. In these tumours, an interesting observation was the sparsity of pyknotic nuclei at the interface between the healthy tumour cells and the necrotic areas, in comparison with unirradiated tumours.

3.12.5 Histology of Transplanted Tumours at the Sites of Previously Cured Tumours:

The histological structure of transplanted tumours at these sites was essentially similar to that of recurrent tumours. Furthermore, the relative volume of necrotic tissue did not differ significantly in the two tumours (table 3.16).

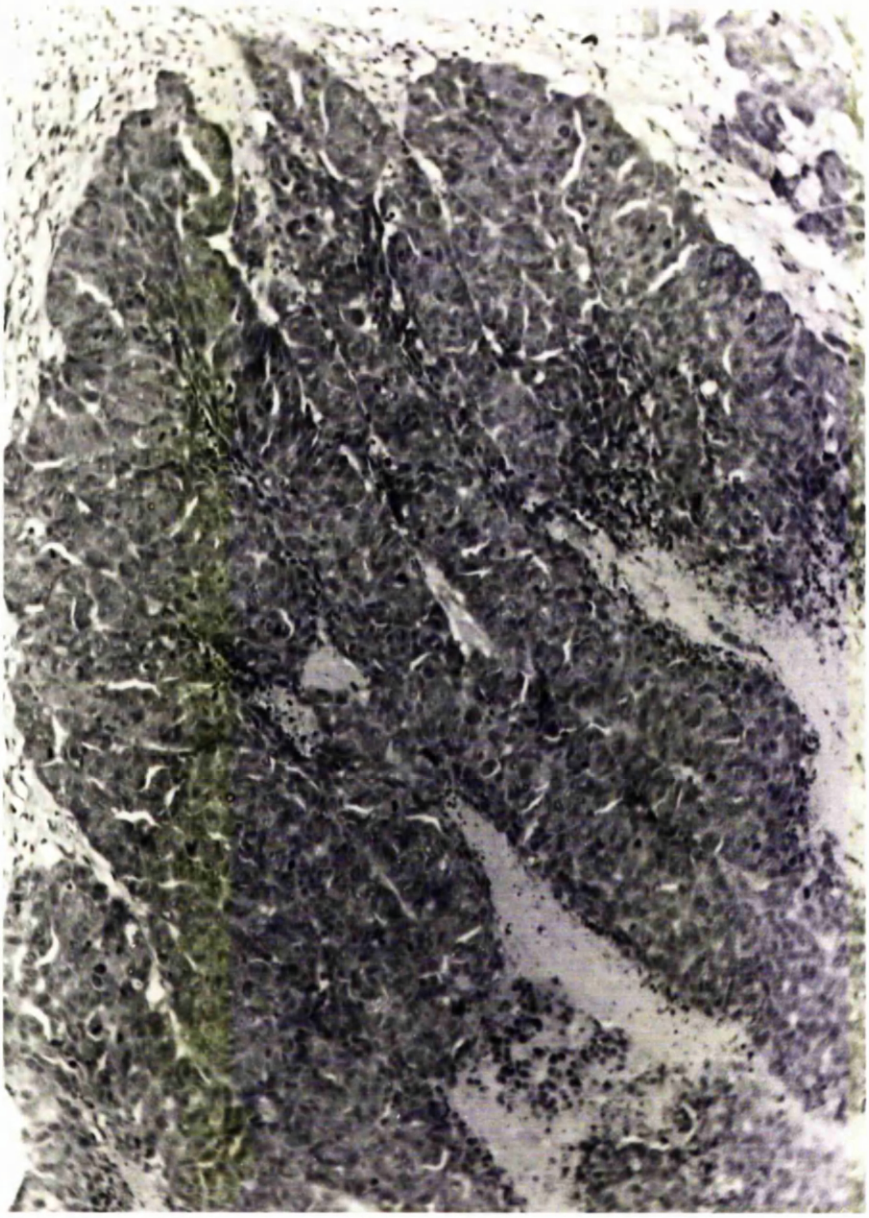


Plate 3.11: Microscopic features of a recurrent tumour after a single X-ray dose of 60 Gy (haematoxylin and eosin X100). The necrotic tissue extends through the outer dome of the tumour which appears thinner than that of unirradiated tumours.

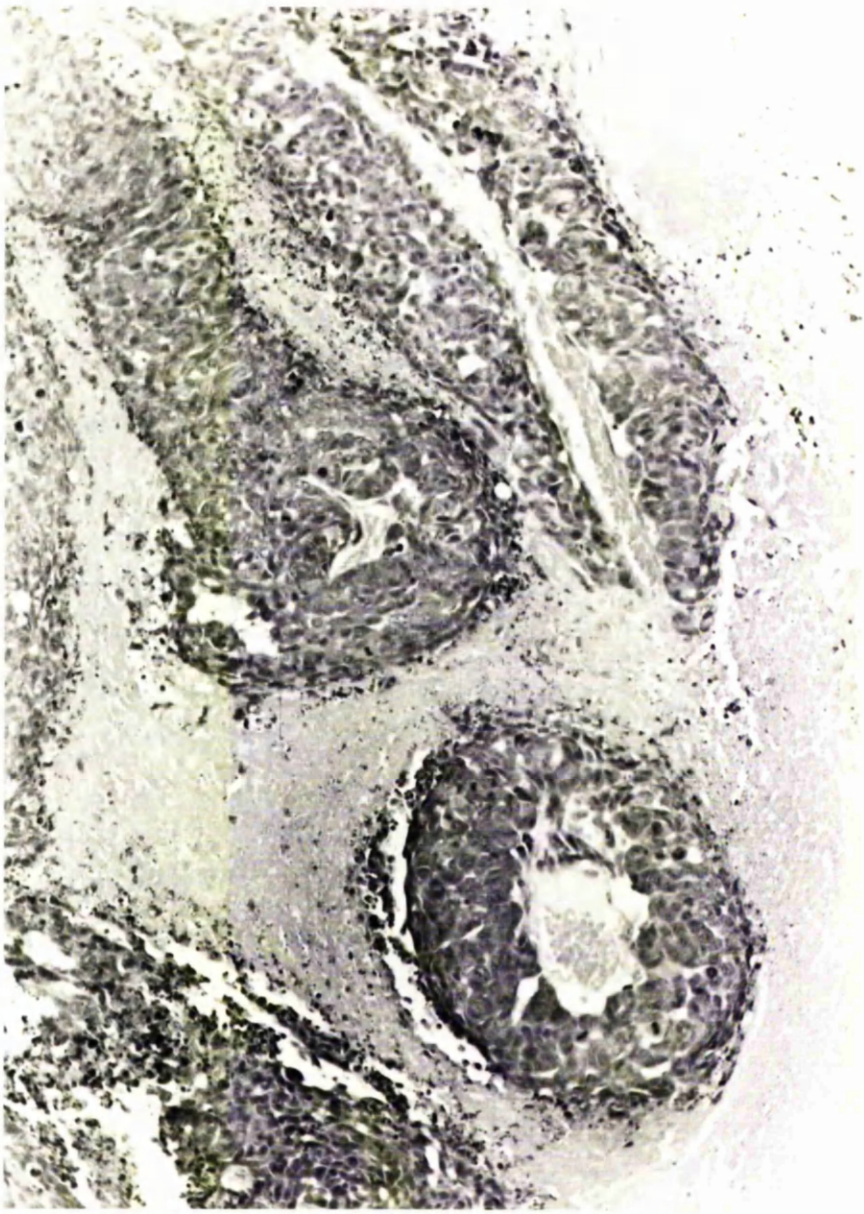


Plate 3.12: Microscopic features of a recurrent tumour after a single X-ray dose of 60 Gy (haematoxylin, eosin X100). The tumour cords are widely separated by necrotic zones.

CHAPTER IV

DISCUSSION

4.1 Tumour Transplantation Using a Cell Suspension:

Many workers transplant small pieces of tumours rather than using a cell suspension. This is to avoid the several problems mentioned in section 3.1 and to obtain suitable tumours for irradiation. In the present work, it was essential to use a cell suspension for transplantation for the following reasons:

1 - The study of the percentage takes and the latent periods for routine transplants and those at pre-irradiated and cured sites;

2 - It would help in the understanding of the structural organization of the tumour at the early phases of growth (section 3.12.2);

3 - During successive transplantation, there would have been a greater possibility of selection of cells other than the tumour cell line, if tumour pieces rather than a tumour cell suspension had been used;

4 - It would permit in vitro explants to be made when necessary and any future comparison between the cell line in vitro and in vivo.

When using a cell suspension for tumour transplantation, the following technical factors would lead to a degree of uncertainty about the exact number of viable cells injected in each mouse:

1 - Variations in cell counting and the inclusion of non-viable but morphologically intact tumour cells;

2 - Loss of some part of the inocula through the needle tracks;

3 - Decrease of the number of tumour cells in successive inocula due to sedimentation and clumping in the syringe during the transplant procedure;

4 - The possible decrease in viability of tumour cells from the time of killing of the tumour bearing mouse and preparation of the cell suspension up to the time of last injection.

These factors were the most likely explanation for the spread in the latent periods in the present tumour system (section 3.1.2).

It was evident from the start of the present work, that skill and extra care were needed for each step of transplantation using a cell suspension. Hence, it was possible to obtain 100 per cent tumour take and enough tumours which were suitable for irradiation.

4.2 Structural Organization of the Multiple Generation C_3H Mammary Tumour:

The distribution of the apparently viable tumour cells appears to follow the distribution of the blood vessels and the diffusion capacity of the nutrients including oxygen. Furthermore, it has confirmed the importance of oxygen and its diffusion range (section 1.5). The structural arrangement of the skin and the distribution of the blood vessels in relation to the panniculus carnosus muscle point to well vascularized and well oxygenated zone in the skin along the muscle layer.

The signet ring appearance of the tumour described in section 3.12.2 would suggest that the thicker outer part of the tumour rim is utilizing the rich vascularity at the site of the destroyed panniculus muscle. The deeper part of the rim, on the other hand, appears to depend mainly on diffusion from the surrounding tissues. However, diffusion seems to be less efficient towards the inner pole of the tumour due to its separation from the well oxygenated zone along the muscle layer by the growing tumour mass.

The naked eye and histological examinations of the tumour have shown an increased vascularity at the transplantation site and intratumour blood vessels. The growth of the tumour from several foci suggests that these inner blood vessels are more likely to be pre-existing vessels encircled by the tumour cells, rather than penetrating vessels from the periphery.

The relative volume of apparently viable tumour tissue decreases from $83 \pm 5.8\%$ to $58 \pm 7.7\%$ as the tumour size increases from 3 mm to 10 mm mean diameter, respectively (table 3.16). This most likely reflects the progressive inadequacy of the blood supply to support the growing tumour mass and might also explain the slower rate of growth with increased tumour size. It is interesting that the accumulation of the necrotic material within the tumour, follows the direction of oxygen diffusion, i.e. necrosis occurs at the inner surface of the peripheral rim and at the outer surface of the tumour

cords. Both the tumour capsule and the peripheral rim enclose this material with progressive increase in its relative volume as the tumour grows, although some of this necrotic material might be absorbed naturally from the tumour. When using a cell suspension for transplantation, some tumour cells are deposited at the deeper avascular part of the subcutaneous layer. Their division and growth seem to depend solely on diffusion from the surrounding tissues, in the absence of nearby blood vessels. The tumour mass, in this case, consists of a necrotic centre surrounded by a thin rim of apparently viable tumour cells (plate 3.4). Furthermore, the growth rate is slow and represents mainly an increase in the size of the necrotic centre and the death of an inner cell layer for every additional outer cell layer. The histological studies of the tumour suggest that the structural arrangement of the skin plays an important role in the structural organization of the tumour. These studies also point to the hypoxic nature of the tumour and the capability of the tumour cells to survive on diffusion in the absence of blood vessels in the vicinity of these cells.

4.3 The Characteristic Pattern and Rate of Tumour Regression: In the present tumour system, a characteristic pattern and rate of regression exist after irradiation even at a relatively low single dose of 25 Gy as revealed by Misonidazole. The magnitude of cell killing with doses

less than 20 Gy is not enough to show if this pattern still exists at such lower doses. This is of interest, since the tumour bed effect seems to plateau at doses of about 20 Gy (section 1.7). Below this dose level, damage to the tumour vasculature, lymphatics and the cell loss mechanisms would be less and hence a faster rate of regression might occur.

The similar pattern of regression that emerges following non-curative and curative doses of X-rays alone or in combination with Misonidazole, strongly suggests that the rate of regression is a poor indicator of radiation curability. Once repopulation becomes less effective as a result of depopulation of the tumour to a low level or when all tumour cells have been killed, the pattern and the rate of regression would be similar. This would be the result only of the rate of removal of the doomed tumour cells, cell debris and the efficiency of the mechanisms responsible for clearance, after irradiation. This is confirmed by the histological picture of irradiated tumours during the regression period (section 3.11.3). The tumour mass consisted mainly of a large necrotic centre surrounded with an incomplete rim of tumour cells which showed incomplete cell division. A reduction or even complete disappearance of the necrotic centre was observed near the end of the regression period. The tumour cells forming the peripheral rim seem to differ from the rest in an unknown way which maintains cell division for a limited period in the

majority of cells (mitotic death) but indefinitely in one or more cells. The latter would be responsible for tumour recurrences. It is possible that these cells have survived the treatment due to hypoxia at the time of irradiation. However, the persistence of a relatively better oxygenation status immediately after treatment, at the periphery of the tumour in comparison with the deeper parts, allowed the cells at the periphery to recover from sublethal damage and to continue cell division. This is despite the possibility of reduced recovery from potentially lethal damage in this case (section 1.8).

The rate and pattern of regression following irradiation appear to be a characteristic feature of each type of tumour (section 1.10). This has also been stressed by Trott et al. (259) and Kovacs et al. (260). Therefore, one can not expect a faster rate of regression, however high a dose is given; this would be so whether or not all the tumour cells are rendered sensitive to radiation killing by oxygen or hypoxic cell radiosensitizers. Other modalities of treatment such as chemotherapy or hyperthermia may produce a different response. In this work, the presence of hypoxic cells in the tumours can be assumed to explain the difference in the observed gross response between tumours irradiated with X-rays alone and those irradiated in combination with Misonidazole after the same dose of radiation (figures 3.5, 3.6). This may explain the observed heterogeneity in response to similar

doses of radiation between tumours of the same type; both human and animals. If this is the case, the characteristic pattern of regression of the tumour type, will be revealed either by an increase in dose or radiosensitization of the cells by oxygen or hypoxic cell radiosensitizers. The difference in the regression features between tumours of different types, both human and animals, might be related to the histological structure of the tumour, especially its vascular architecture; the mode of cell death (mitotic or interphase death and/or the efficiency of the mechanisms responsible for digestion and removal of the doomed cells and the cell debris after irradiation. The first of the aforementioned is believed, by several workers (25,239,260), to be the most important factor.
The process of reoxygenation appears to occur early when the tumour shrinks rapidly following irradiation (section 1.9). In the present tumour, however, regression only became evident about twelve days from the day of irradiation and single doses below 50 Gy in the absence of Misonidazole did not produce any regression at all. It is tempting to say that reoxygenation will not be an important factor in this tumour system and other slowly regressing tumours. Therefore, other means designed to overcome the radioresistant hypoxic cells should be used in these tumours, where the beneficial effect of reoxygenation with fractionated irradiation is not expected.

4.4 The Relation Between Tumour Regression and the Probability of Local Control:

There has been much controversy about the existence of a relationship between tumour regression and local control in both human and animal tumours. The establishment of a consistent relationship would permit selection of optimum radiation doses and any further treatment necessary to achieve cure, if the outcome of the treatment is known in advance. Suit et al. (150) did not find any prognostic significance of tumour regression after large single doses in a spontaneous C_3H mammary tumour and in 72 patients with squamous cell carcinoma of the head and neck treated with convential fractionated radiotherapy. Trott et al. (259) did not find any correlation between the rate and pattern of tumour regression and the radiation curability of 3 different tumours, adenocarcinoma 284, fibrosarcoma SSK and Harding Passey melanoma. They concluded that the tumour gross response is a very misleading judge of the radiosensitivity of the tumour type. Friedman et al. (261) in a study of 123 patients with Hodgkin's disease found a mixed pattern of tumour shrinkage in one half of these patients and concluded that the rate of shrinkage, could not be used for clinical or radiobiological purposes. On the other hand, Marcial and Bosch (262), and Grossman et al. (263), in studies of patients with carcinoma of the uterine cervix, concluded that complete tumour regression, early or late is associated with

exellent survival. Denekamp (151) in a retrospective analysis of the first generation C_3H mammary tumour found a correlation between shrinkage and the ultimate cure probability after single or fractionated irradiation.

Suit and Shalek (173,264) and Suit and Gallagher (265), found morphologically intact and normally stained tumour cells in the residual thickening or nodularity which often persisted at the site of regressed C_3H mammary tumours, even at times greater than 110 days. These residual nodularities were not followed by local recurrence and tumour cells from them failed to grow tumours on transplantation. This indicated that the persistence of histologically intact cells in irradiated tissue, in no way related to regrowth of tumours.

In the present study, any tumour which showed minimal or partial regression eventually grew to a large size, irrespective of the dose; while those that regressed completely, followed the same pattern and rate of regression after non-curative and curative doses of X-rays alone and in combination with Misonidazole. The histological appearance of these regressed tumours was also similar during the period of regression. Certainly, it was impossible to predict the ultimate fate of the tumours from both the characteristic pattern of tumour regression and the histological appearance. On the other hand, the complete regression status following irradiation was associated with local control of the

tumours, but within a certain relationship which suggested a high probability of local control with any treatment, once an incidence of complete regression of more than 60 per cent is reached (section 3.6).
As this relationship can predict the ultimate local control of the tumours, it can be used as an early end point for the local control experiments, instead of 100 days (section 3.3.1). This would shorten the period of experimental work.
If other tumours including human ones, follow a similar relationship, the outcome of X-ray treatment can be predicted at an early time with respect to the observed incidences of complete tumour regression following such treatment.

4.5 The Delay in Growth Method:

This method has been used extensively in radiobiology for the studies of the oxygen effect (8,88); neutron radiation (126,266,267,268), recovery phenomenon (147, 170,171) and the hypoxic cell radiosensitizers (131,133, 135,269,174,201). It has the following advantages:-

1 - The response is being measured in situ, without removing the cells to a different environment as in vivo-vitro studies (133,141,269). The immediate post-irradiation environment seems to play an important role in the fate of irradiated tumour cells in vivo.

2 - It is a suitable method to study the tumour response

at low doses of radiation and tumours recurring after high doses of radiation.

3 - It requires a reasonably short observation period, with relatively rapidly obtainable results. This is of special importance for the study of metastasizing tumours which shorten the life span of the animals before local control (cure) of the tumours by irradiation.

4 - It is relevant to the clinical situations, i.e. palliative radiotherapy where the local control of the tumours is not intended and in the study of new methods of cancer treatment as is the case with the hypoxic cell radiosensitizers (section 1.12.5).

Various methods have been used for the construction and the mathematical analysis of the dose-response curves for the delay in growth. Thomlinson and Craddock (88) plotted the absolute time of delay in growth against the dose. Howlett et al. (268) used the "relative delay" defined as the ratio of times for irradiated and non-irradiated (control) tumours to grow to an arbitary size. Denekamp and Harris (201) expressed the delay in growth in terms of cell doubling needed to restore the tumour to its original size at irradiation. Barendsen and Broerse (141) substracted the doubling time of non-irradiated tumours from the time interval for tumours to regrow to double the volume at irradiation.

In a variety of experimental tumours, the curve obtained for X-rays, under air breathing conditions shows a

biphasic pattern in a similar way to the survival curves of a mixed population of oxic and anoxic cells (108,109, 110). This is suggestive of the presence of a significant proportion of hypoxic cells in the tumour, which dominate the response after the break point, leading to a smooth and less steep upper part of the curve. This biphasic nature of the curve disappears with hyperbaric oxygen (88); hypoxic cell radiosensitizers (131); neutron radiation (110,171,266, 267) and fractionated irradiation (170,171), due to reduced effect of hypoxia in the tumours as evidenced by steepening of the shallower upper part of the curve. The biphasic nature disappears also with irradiation under anoxic condition, where the lower dose region of the curve becomes as shallow as the upper part of the higher dose region, due to the uniform hypoxic state of all tumour cells.

For estimation of cell kill and the proportion of the hypoxic cells from these curves, it is usually assumed that the delay in growth, is a consequence, mainly of cell killing and the fraction of surviving clonogenic cells (88,201). However, several workers (88,131,270, 271) have drawn attention to the possibility of incorrect conclusions based on such assumption, and ignoring the other factors which may drastically modify the growth of recurrent tumours. This has been confirmed in the present work (see below).

4.6 The Growth of Recurrent Tumours:

The time distribution and growth of recurrent tumours would depend, not only on the proliferation kinetics of the surviving tumour cells but also on several other factors as those mentioned for the growth of non-irradiated tumours (section 1.2). However, they would be modified and altered by irradiation; a situation which confuses even further our limited knowledge concerning the growth kinetics of recurrent tumours. The rate of growth of these recurrences, in a variety of animal tumours, varied from a rate similar to that of non-irradiated tumours to a slower rate, even after the same dose of radiation (85,88,141,148,173). Several in vitro and vivo studies have suggested changes in the proliferation kinetics and possibly intrinsic alterations in the surviving tumour cells following irradiation (21,149,272,273,274). Radiation also produces histopatholgical changes in the normal tissues (especially vascular stroma) in which the tumour is growing (sections 1.6, 1.7). Most workers believe that these cellular and tumour bed changes would delay the appearance and restrain the growth of recurrences, more than would be expected on the basis of the decreasing number of surviving cells.

In the present work, there was a definite growth restraint of recurrent tumours. Most of this growth retardation occurred during the early period of growth, until the tumours grew up and beyond the size at

irradiation (6 ± 1 mm mean diameter). Thereafter, growth gradually steepened towards that of non-irradiated tumours, an observation similar to that of Thomlinson for fibrosarcoma $R1B_5$ (8).

The normal growth of tumours obtained by transplantation of a cell suspension from recurrent tumours (section 3.4) has suggested a tumour bed effect, rather than a radiation induced intrinsic cellular defect, as a cause for the observed growth restraint.

The magnitude of this tumour bed effect was difficult to measure from the growth studies of recurrent tumours for the following reasons:-

1 - It was possible, only, to measure the rate of growth during the late and more predictable period of growth. However, this period might not represent the maximum tumour bed effect due to the possibility of revascularization from the less damaged periphery of the tumour bed. The rate of growth during this period was not significantly different from that of non-irradiated tumours after a single dose of 50 Gy, while it was still significantly slower after higher doses. This might be due to the reduction in the relative volume of viable tumour tissue, seen on histological studies (section 3.12.4) and possibly a decreased rate of cell division. The latter was suggested by the narrow zones of recent necrosis in recurrent tumours, in comparison with non-irradiated tumours.

2 - The growth of recurrent tumours after complete or incomplete regression was very slow and inconsistant. This led to difficulties in performing the growth analysis (used in table 3.11) for the early period of growth and in identifying the second wave of delay in growth observed by some workers in other tumour systems (88,130).

3 - The existence of a tumour bed effect during the regression and the subclinical periods of irradiated tumours could not be deduced from the growth studies of recurrent tumours.

In an attempt to identify and measure the tumour bed effect for recurrent tumours, transplantation was performed at the sites of previously cured tumours and at pre-irradiated sites. At the cured sites, retardation of growth was evident, only during a limited period of growth, namely, the early period of growth. Neither the latent period, nor the late period of growth were significantly different from those of non-irradiated tumours (tables 3.10, 3.12). On the other hand, tumour growth at the pre-irradiated sites was significantly restrained during the latent period and both the early and late periods of growth.

The discrepancies between the results at the cured and pre-irradiated sites would suggest structural and/or functional differences at these sites. These might be differences in the radiation response of

vascularity already existing at the time of tumour irradiation and that of non-stimulated vascularity at the pre-irradiated sites.

Tumour growth at the cured sites was expected to be similar in many aspects to that of recurrent tumours. However, the rate of growth of tumours recurring after complete regression was much slower than that of tumours transplanted at the cured sites. The time taken by the latter to grow from 3 to 10 mm was nearly half that of recurrent tumours (table 3.13).

The present studies have confirmed the existence of a tumour bed effect; the magnitude of which at various periods of growth can be measured for both the cured and the pre-irradiated sites. However, the results obtained with their discrepancies point to the need for further studies before a possible extrapolation to the situation of recurrent tumours. Histological studies of recurrent tumours, smaller than those examined in this work, might reveal a structural organization similar to that in plate 3.4. This would be the case if there is delay and/or impairment of revascularization of these tumours. A growth similar to that of recurrent tumours might have been obtained at the cured sites, if fewer tumour cells (less than 10^6 cells) were used for transplantation. This would need to be confirmed by a dilution assay, using various concentrations of tumour cell suspension for transplantation.

It is interesting to note that the rate of growth during the early period at both the cured and pre-irradiated sites was approximately half that of non-irradiated tumours (table 3.11), a similar finding to that of Hewitt and Black (95). Furthermore, the present work has also confirmed these workers view that the tumour bed effect is not an important factor for eradication of tumours. Heavily irradiated tissue seems capable of supporting the growth of transplanted tumours and would also support the growth of recurrent tumours, though tumour growth would be slow, a beneficial effect for palliative radiotherapy.

4.7 Recovery from Sublethal Damage:

Two fraction experiments were designed in this work, in an attempt to study the recovery capacity from sublethal damage of the few, probably hypoxic clonogenic tumour cells which determine recurrence or cure of irradiated tumours. For such an attempt, the total dose and the expected equivalent doses should be on the exponential part of the dose response curve. Furthermore, these doses and the initial fractions should induce the maximum tumour bed effect (section 1.7). This would hopefully, minimize any influence of variations in magnitude of the tumour bed effect on the results of delay in growth. A 70 Gy total dose and initial fractions not less than 20 Gy seemed to be suitable for such an attempt.

The analysis of the results of these experiments (section 3.5) showed large D_2-D_1 values and an apparent Elkind pattern (peaks and troughs), using the data of delay in growth. This was in contrast to small D_2-D_1 values and an absence of Elkind pattern, using the data of local control. The reasons for these discrepancies are not clear, but two explanations are put forward:-

1 - Recurrent Tumours Differ in Some Way from the Cured Ones:

It is possible that the cells which survived the initial doses are critically hypoxic, out of cycle and have a relatively low recovery capacity from sublethal damage. Such cells do exist in tumours (section 1.8). In cured tumours, these cells might remain in a hypoxic state in the intervals between fractions, hence the low D_2-D_1 value and the absence of Elkind pattern. On the other hand, an immediate improvement of the oxygenation status of these cells in recurrent tumours would improve their recovery from sublethal damage and stimulate cell division, hence a high D_2-D_1 value and Elkind pattern can be expected. However, such reoxygenation would reduce recovery from potentially lethal damage which is more efficient in hypoxic, non-cyclic cells (section 1.8).

This explanation is similar to the suggestion put forward by Hall (166) for split dose experiments in tumours and the explanation mentioned in section

4.3 for the histological appearance of irradiated tumours.

2 - The Data of Delay in Growth Reflect Mainly Recovery of Normal Tissues (Tumour Bed) and Not That of Tumour Cells:

The fluctuations in D_2-D_1 values would in this case, reflect the magnitude of residual damage of the tumour bed, especially that of the blood vessels. Recurrent tumours would grow faster with less vascular damage, hence a larger D_2-D_1 value is obtained and vice versa with greater vascular damage. Accepting such explanation, the data of local control and not that of delay in growth should be used for estimation of recovery in tumours. This means that all the published reports using the data of delay in growth for such recovery may be ambiguous. Furthermore, variations in the magnitude of the tumour bed effect can be expected up to 70 Gy and not about 20 Gy as mentioned in Section 1.7.

Further studies are needed to confirm or dispute the above explanations, using tumour systems where the analysis of both the tumour local control and the delay in growth can be compared.

As mentioned in section 1.11, accurate estimation of recovery in tumours is difficult and complicated due to the interaction of various radiobiological processes during the intervals between fractions. This was also the case in the present tumour, especially with the

discrepancies found between the data of local tumour control and those of delay in growth.

The pattern and rate of tumour regression were similar for both single and split dose experiments, with no regression during the interval between fractions. These and the positive D_2-D_1 values suggest that reoxygenation plays a minimal role in the present tumour (see section 1.11). The apparently hypoxic status of the tumour is suggested by the single dose experiments (section 3.3.3) and the histological structure of the tumour (section 3.12.2).

Both the apparently hypoxic nature of the tumour and the minimal role of reoxygenation would suggest that the D_2-D_1 values obtained in the split dose experiments are values for hypoxic tumour cells. When comparing the results obtained in this work, with those for other tumours, it is important to correct for the OER of hypoxic cells; the latter is not known for certain in the present tumour. However, if a correction for OER is applicable to these recovery data, then assuming a value of 2.8, as that used by Phillips (175), a D_2-D_1 between 3 and 4 Gy would be obtained which is similar to the range of 2 to 4 Gy quoted by Phillips for two tumour types, sarcomas and mammary carcinomas.

4.8 The Hypoxic Cell Radiosensitizer, Misonidazole:

Local failure is unfortunately, still common in radiation treatment of malignant disease. Substantial numbers of

patients who die annually of cancer, do so with uncontrolled primary tumour. The relatively radioresistant hypoxic cells (section 1.8) believed to exist in tumours are regarded as an important reason for local failure after radiotherapy. In the past few years, appreciable sensitization of these cells has been demonstrated in many types of animal solid tumours, treated by Misonidazole prior to irradiation (section 1.12.5). This has also been confirmed in the present tumour system where an enhancement ratio as high as two was achieved. In the presence of Misonidazole, only half the radiation dose was required to produce the same radiobiological effect (local tumour control and delay in growth) as that produced in the absence of the drug. Potentially, therefore, Misonidazole is a valuable drug and offers an improvement in both curative and palliative radiotherapy. The drug, however, was used in the present work, mainly to understand the gross response of the tumours following irradiation. It was possible in the presence of Misonidazole to depopulate the tumours by relatively low doses of radiation and to reveal the existence of the characteristic pattern of tumour regression at such doses. Furthermore, the sites of previously cured tumours by X-rays in the presence and absence of Misonidazole were used to study the possibility of growth restraint over a wide range of radiation doses.

4.9 The Immune Status of the Tumour System:

Although host resistance against tumours is complex and far from clear, cell mediated immunity is generally believed to be an important factor in suppression of tumour growth (275,276). In the present tumour, temporary cellular reaction, mainly of fibroblasts and lymphocytes was observed on histological studies of small tumours (section 3.12.2). This might indicate a temporary immune reaction at an early phase of growth when the number of tumour cells is relatively small. However, such immunity seems to be negligible for the following reasons:-

1 - The variations in percentage tumour take during the transplantation period can be traced to technical difficulties.

2 - Constant pattern of biological behaviour of the tumour since the start of the radiobiological studies in 1975.

3 - No regression of established tumours has been seen at any time

and 4 - The use of MTA positive C_3H mice for transplantation where immunity is unlikely to occur (section 1.4).

While tumour immunogenicity seemed improbable, the depressed response of lymphocytes to phytohaemagglutinin has suggested suppression of the immune response. This appeared to be secondary to the presence of the tumours rather than a primary event; a situation described

in human malignancy, particularly in advanced disease (277,278). This has also been confirmed by the recovery of the lymphocyte response in the cured mice to a level, not significantly different from that of mice bearing no tumours.

The impaired reactivity of lymphocytes and the promotion of tumour growth in immunized mice are disturbing observations due to the possibility of them occurring in cancer patients when immunization is attempted. Furthermore, it poses the question of whether immunodepression is as important as immunostimulation in modifying tumour growth and possibly its curability. There is, however, a possibility that the mechanisms behind the inhibition of lymphocyte response might differ from those responsible for promotion of tumour growth. This warrants further studies in non-immunized mice, where the impaired reactivity of lymphocytes was observed only when the tumours became palpable and fell to the same low level for mice bearing spontaneous C_3H mammary tumours and those immunized with lethally irradiated cells.

4.10 Differences Between First and Multiple Generation C_3H Mammary Carcinoma:

First generation transplants of spontaneous C_3H mouse mammary carcinoma have been used extensively for radiobiological investigations at the Gray Laboratory (1). The multiple generation counterpart of this tumour,

described in this thesis, was originally obtained from a spontaneous tumour bearing C_3H mouse from the Gray Laboratory. The biological properties of this tumour have departed from those of the original one, due to serial transplantation. Hence, it was possible to compare several of the properties of both tumours, despite differences in the techniques of transplantation and irradiation.

1 - Differences in Growth and Morphology:

First generation transplants have a wide range of doubling times (two to fourteen days near the size at irradiation (6 ± 1 mm mean diameter) (1). Most of these tumours are well differentiated adenocarcinoma, in which considerable volume is occupied by glandular ducts and also, frequently, large blood filled or empty sinusoids (174). The percentage of non-tumour cell material in 6 and 10 mm tumours are $42 \pm 13\%$ and $35 \pm 19\%$ respectively. These histological findings are similar to those of early generation transplants of the present tumour (plate 3.8). After nearly a year of serial transplantation, both the rate of growth and the histological structure have departed from those described for first generation tumours. The rate of growth of multiple generation tumours is more predictable with a doubling time of about one day near the size at irradiation (6 ± 1 mm mean diameter). Histologically, the tumours are

undifferentiated adenocarcinoma with no uniform intratumour stromal tissues nor glandular ducts. Another feature is the extensive necrosis observed in multiple generation tumours; this appears to be absent or minimal in first generation.

2 – Differences in Tumour Regression Following Irradiation:

After a single dose of 1.5 Gy, first generation transplants regressed rapidly within one day reaching approximately 50% of the initial volume at seven days (130). This pattern of regression is different from that observed for multiple generation tumours; no tumour regression was noticed except with single doses of 50 Gy or more of X-rays alone and 25 Gy or more of X-rays in combination with Misonidazole and then not until twelve days after irradiation. These differences in tumour regression induced by serial transplantation may be due to the changes observed in the histological structure, mode of cell death and/or the efficiency of the mechanisms responsible for removal of the dead cells or cell debris.

3 – Differences in the Oxygenation Status of Both Tumours Following Irradiation:

The process of reoxygenation appears to occur early, when the tumour shrinks rapidly following irradiation (section 1.9). Fowler et al. (1,136) showed that reoxygenation was the most important factor in

determining the response of first generation C_3H mammary carcinoma to fractionated irradiation. In that tumour, reoxygenation coincided with the reduction of the tumour volume in 2 to 3 days after a single dose of 1.5 Gy (129). In the multiple generation transplants, used in the present work, rapid regression did not occur as in first generation tumours. This might suggest that reoxygenation is not an important factor in the present tumour. However, this would need to be investigated by an experiment similar to that described by Howes (129) and Fowler et al. (1).

4 - Differences in the Recovery Capacity from Sublethal Damage in Both Tumours:

Fowler et al. (1) estimated this recovery in first generation tumours from the TCD_{50}s of clamped off tumours after single doses and two equal doses of X-rays given at an interval of 24 hours. The recovery increment was found to be 12.8 Gy in these hypoxic tumours, yielding an oxic recovery increment of 4.3 to 5.1 Gy, depending on whether an OER of 3.0 or 2.5 is assumed. In the present multiple generation transplants, a recovery increment of 8.75 Gy is estimated from the cure data for a single dose of 70 Gy and two equal doses of 35 Gy each, at an interval of 24 hours (section 3.5). As reoxygenation appears to be absent or inefficient in the present tumour, an oxic recovery increment of 2.9 to 3.5 Gy

would be expected, assuming the same OER

Apparently, recovery from sublethal damage seems to be lower in multiple generation than first generation transplants. This might be due to the appearance of hypoxic cells in the multiple generation tumours by serial transplantation as evident on histological studies (section 3.12.2). These hypoxic cells are less efficient in repairing the sublethal damage; hence a low recovery increment would be expected (section 1.8).

5 - Differences in the TCD_{50}s of Both Tumours:

When considering the effect of serial transplantation on the probability of tumour control, the results of irradiated tumours under hypoxic conditions (clamped) have been chosen. This is to avoid the possible differences in the oxygenation status of the two tumours at the time of irradiation and also the differences in the techniques of irradiation. The average TCD_{50} at 150 days (hypoxic) for first generation transplants was found to be 52.9 Gy (1). This is in contrast with 65 Gy TCD_{50} at 100 days (hypoxic) for multiple generation tumours, used in this work. That is to say, serial transplantation has increased the TCD_{50} by 12.1 Gy, rendering the multiple generation tumours less readily curable than their first generation counterparts. Recovery from sublethal damage can not be responsible for such an increase in the TCD_{50} as

such recovery seems less efficient in multiple generation tumours. However, differences in antigenicity, recovery from potentially lethal damage and/or the intrinsic radiosensitivity of the tumour clonogenic cells can not be excluded. Further exploration of these possibilities is needed; this might provide insight into the curability of various tumours.

6 - Serial Transplantation and Metastases:

Weiss (279) and Mellgren (280) pointed out the practical difficulties in quantitative estimation of the size and incidence of metastases in both human and animal tumours. Weiss (279) in his overview of the basic mechanisms and factors responsible for tumour dissemination, concluded that every step of the metastatic cascade seems to be a highly selective process. Thus a very small proportion of the total cells in the primary tumour is expected to be released and survive the trauma of dissemination, arrest and interactions with the host tissues. This low incidence of dissemination has been stressed in animal tumours by Wallace (281) and Kim (282). Wexler et al. (45) in studies of 3 chemically induced and 2 spontaneous tumours found that each tumour has its own malignant pattern as determined by its growth rate and distant metastases and that serial transplantation has no uniform effect on the tendency to form metastases. Weiss (279) pointed out that the

apparent relation between the high growth rate and the risk of metastases, in both human and animal tumours, might merely indicate that metastases from rapidly growing tumours would also grow rapidly and thus be recognized earlier.

Sheldon et al. (283) in extensive studies of lung metastases from first generation transplants of spontaneous mammary carcinoma in C_3H mice, found a 10.9% (23/211) incidence of lung metastases after curative radiotherapy. These investigators suggested that the peak in incidence of lung metastases observed between 60 to 90 days after treatment, would represent the average time from seeding of the cells to growth into a visible lung nodule.

The incidence of lung metastases from the multiple generation C_3H mammary tumour, used in the present work was 12% (section 3.11), not significantly different from that of the first generation transplants used by Sheldon et al. (283). However, the peak in incidence in the present tumour occurred between 25 to 40 days, i.e. less than half the time for the peak in the first generation transplants. These findings would suggest that serial tumour transplantation did not increase the tendency for lung metastases from C_3H mammary tumour. However, it led to an earlier appearance of metastases, most probably due to the increased rate of growth of the

tumour and hence its metastases during successive transplantation.

4.11 Conclusions and Possible Future Studies:

The aim of this thesis was to investigate the basic biological and radiobiological characteristics of the multiple generation C_3H mammary adenocarcinoma. This was done to determine the feasibility of establishing a suitable solid tumour system for further radiobiological and other research studies.

So far, the work presented describes the growth of the tumour after subcutaneous transplantation using a cell suspension of 10^6 tumour cells and its response to single and two fraction X-ray irradiation. The hypoxic cell radiosensitizer 'Misonidazole' was used mainly to understand this response.

Summary of Principal Results:

1 - The multiple generation C_3H mammary carcinoma is a suitable tumour system for radiobiological studies for the following reasons:-

i - It is apparently free from immune stimulating activity.

ii - Its growth is stable and uniform with neither erratic behaviour nor spontaneous regression.

iii - Its response to radiation treatment can be assayed in a reasonable experimental period (100 days) by both delay in growth and local

control methods.

iv – Its low rate of metastasis permits studies of metastasis as a phenomenon and also allows the radiobiological studies without excess early losses.

v – It is an easily quantifiable model of hypoxic nature and a high TCD_{50} (67 Gy), hence it is suitable for the study of modalities designed to overcome the problem of hypoxia i.e. neutron; radiosensitizers and hyperthermia. Furthermore, the studies of such a tumour would add to the understanding of similar less radioresponsive tumours in human.

vi – The host strain (C_3H mouse) is robust and fertile and the transplantation site at the dorsum of the mouse is both humane and highly suitable for radiation studies without anaesthesia and significant dosage to the rest of the animal.

2 – The multiple generation C_3H mammary carcinoma is a fast growing tumour with a successful take rate of 100 per cent and a mean latency of about 9 days. As growth progresses, the doubling time increases from 0.5 days at 2 mm mean diameter to about 3 days at 10 mm mean diameter and the relative volume of necrotic tissue increases from 16.9 ± 5.8 to 42.2 ± 7.7 per cent over the same size range. These increases, most likely reflect a progressive

inadequacy of the blood supply to support the growing tumour mass.

3 - In mouse skin, the blood capillaries are distributed superficial and deep to the panniculus carnosus muscle. The deeper part of the subcutaneous layer is loose and avascular connective tissue. This structural arrangement and its disorganization by the growing tumour mass seems to determine the processes of diffusion and perfusion at different regions of the tumour, hence its structural organization (signet ring appearance).

4 - The multiple generation C_3H mammary carcinoma is apparently free from immune stimulating activity and there is even suppression of the immune response in in tumour bearing mice.

The impaired reactivity of lymphocytes to phytohaemagglutinin and the promotion of tumour growth in immunized mice are disturbing observations due to the possibility of them occurring in cancer patients when immunization is attempted.

5 - The present tumour shows a characteristic pattern and rate of regression, once sufficient depopulation is achieved with X-ray irradiation, alone or in combination with Misonidazole. The tumours continue to increase in size for about 2 days, then return slowly to the pre-irradiated size in about 12 days. This is followed by a relatively faster regression towards a minimal size in about 20 days after

irradiation. The similar pattern of regression that emerged following non-curative and curative doses of radiation strongly suggests that the rate of regression is a poor indicator of radiation curability. However, in completely regressed tumours, a high probability of local control is observed once an incidence of complete regression of more than 60 per cent is attained.

6 - Following irradiation, massive necrosis occurs towards the central part of the tumour, leaving only a thin rim of tumour cells at the periphery, in a state of incomplete cell division. Gradual reduction and complete disappearance of this necrotic material is observed near the end of the regression period. This histological appearance, certainly suggests that the rate of regression of the present tumour reflects mainly the rate of removal of the doomed cells and cell debris. Though clonogenic cells cannot be identified on exclusively histological grounds, nevertheless the histological appearance of irradiated tumours is suggestive of a biological difference between the tumour cells at the periphery and those elsewhere in the tumour. This difference appears to maintain cell division for a limited period in the tumour cells forming the peripheral rim. However, the possibility of indefinite cell division in one or more cells cannot be ruled out, hence tumour recurrence. It is

tempting to speculate that tumour curability is determined by certain biological characteristics of such cells at the tumour periphery.

7 - In the present work, there is a definite growth restraint of recurrent tumours as a result of radiation damage to the tumour bed rather than an intrinsic cellular defect in the surviving tumour cells. This tumour bed effect would delay the appearance and restrain the growth of recurrent more than would be expected on the basis of the decreasing number of surviving cells and clearly, it should be considered in any analysis based on the growth delay. The magnitude of this effect, however was difficult to assess from the growth studies of recurrent tumours. Furthermore, growth studies of transplanted tumours at the sites of previously cured tumours and at the pre-irradiated sites offered only limited help in this assessment. The results obtained at these sites with their discrepancies pointed to the need for further studies to determine the magnitude of the tumour bed effect at the various periods of growth of recurrent tumours. This might also help in understanding the differences in the results of the two fraction experiments when using the data of delay in growth and those of local tumour control.

In the present studies, there is no evidence that the tumour bed effect is an important factor for

irradication of tumours. Heavily irradiated tissue seems capable of supporting the growth of transplanted tumours and would also support the growth of recurrent tumours, though tumour growth would be slow, a beneficial effect for palliative radiotherapy.

8 - The appreciable sensitization of the hypoxic cells by Misonidazole has been confirmed in the present tumour system. The TCD_{50} of non-clamped tumours is reduced from 67 Gy for X-rays alone to 31 Gy for X-rays in combination with Misonidazole, giving an enhancement ratio as high as two with single doses of radiation. Misonidazole and other chemical radiosensitizers seem to be valuable drugs and offer an improvement in both curative and palliative radiation treatment.

9 - The recovery capacity from sublethal damage (SLD) appears to be similar to other tumour systems. However, the values obtained using the cure data differed from those using the delay in growth data. The latter also showed the Elkind pattern (peaks and troughs) characteristic of the split dose experiments. These observations indicate the caution needed in the interpretation of the results using either set of data. This is especially true when comparing the recovery from SLD of various tumours.

10 - Comparative analysis of the biological characteristics of the present tumour and its first generation

counterpart, clearly shows that serial transplantation has led to several morphological and biological changes. Progression towards characters of greater virulence is evident, reflected by the acceleration of growth and the early losses of animals, presumably due to rapidly growing metastasis. In addition, there is an increase in the degree of anaplasia; the proportion of necrosis and the TCD_{50}. These are associated with a relatively slower rate of tumour regression following irradiation, in comparison with that of the first generation C_3H mammary carcinoma. The mechanisms by which these changes arose, still remain uncertain and require further exploration. This would also help in understanding the complexity and the variety of processes influencing tumour growth.

Suggestions for Further Experiments:

A – Tumour Growth Studies:

Several interesting observations deserve mention and further studies, these include:

1 – Growth deceleration as tumour size increases;

2 – Acceleration of tumour growth in immunized mice;

and 3 – Slow rate of growth and different histological appearance of those few tumours implanted accidentally at the deeper avascular part of the subcutaneous layer of the skin.

The following investigations are suggested to elucidate the complex host–tumour interactions influencing tumour growth and structural organization:–

i – Estimation of the TD_{50}, latent period and the pattern of growth during latency using the dilution assay technique.

ii – Cell population kinetic studies using tritiated thymidine and autoradiographic techniques to measure the lenth of the cell cycle and its phases; the growth fraction and the extent of cell loss.

iii – Thorough anatomical studies of tumour vascularity with serial histological sections stained with Luxol fast blue, periodic acid–schiff for better visualization of the blood vessels.

and iv – Functional studies of the tumour vascularity using more reliable radioisotope techniques such as ^{86}Rb uptake and ^{125}I–HSA.

These investigations should be carried out in both non-immunized and immunized mice during the various phases of tumour growth and also for tumours transplanted at tissues of varying structural arrangement and vascularity other than skin.

B – Tumour Regression Studies:

There is need for exploration of the basic mechanisms responsible for removal of the doomed cells and cell debris and their efficiency after irradiation and other treatment modalities. This would help in understanding tumour regression and the mechanisms underlying different rates of regression of various tumours.

The following investigations are suggested at various periods of tumour regression:

i – Thorough anatomical and functional studies of tumour vascularity;

ii – Exploring the changes in the cell population kinetics;

iii – Exploring the possible biological difference between tumour cells at the periphery and those elsewhere in the tumour and also testing the clonogenic ability of these cells by transplantation and in vitro clone formation assays.

and iv – Investigation of the autolytic digestion of the dead cells and the phagocytic activity of the host scavenger cells.

C – Growth Restraint Studies of Recurrent Tumours:

The following investigations are suggested for further exploration of the mechanism and the magnitude of growth restraint during the various phases of growth of recurrences and transplanted tumours at cured and pre-irradiated sites:-

i – Thorough anatomical and functional studies of tumour vascularity;

ii – Cell population kinetic studies;

iii – Estimation of the TD_{50}, latent period and the pattern of growth during latency using dilution assay techniques with a cell suspension from recurrent tumours injected at both routine and cured sites.

and iv – Estimation of the TD_{50}, latent period and the pattern of growth during latency for routine tumours transplanted at both cured and pre-irradiated sites of various dose levels.

These investigations should be carried out with a large number of animals for each study.

D – Studies of Recovery from Sublethal Damage:

It is desirable to confirm the findings reported in this thesis regarding the recovery capacity from SLD using a large number of animals and both TCD_{50} and delay in growth assays. Future studies should be directed towards exploring ways and means for selective inhibition of the recovery phenomena in the tumours to achieve better cure rates with fractionated radiation treatment.

E – Other Studies:

i – Investigations of the proportion of hypoxic cells in the tumours and the possibility of their reoxygenation with fractionated irradiation.

ii – Exploring regression, cure and recurrence of the tumour after fractionated irradiation alone and in combination of Misonidazole.

Future Prospects:

The development of radiation therapy has, of necessity, been extensively based on empiricism and the major contribution of radiobiology to radiotherapy at present is the discovery of mechanisms influencing tumour response to ionizing radiation. However currently available radiobiological data are still inadequate to explain the complex interacting phenomena occurring in the biological systems. So far these data can be related to the observed phenomena only by means of hypothesis and extrapolation.

More recently, however, experimental radiobiological studies have indicated lines of research along which intensification of efforts would be particularly desirable and it seems increasingly probable that the radiobiological findings will indeed prove to be applicable to clinical problems. For example, the identification of the oxygen effect as a possible mechanism of tumour resistance to radiation treatment, has led to the exploration of the clinical use of hyperbaric oxygen, high LET radiation, radiosensitizers and hyperthermia. Further experimental studies including those along the line suggested in the present work may lead to the design of a practical and sound approach to clinical radiotherapy and to additional possibilities for the eradication of human cancer.

APPENDICES

APPENDIX I

Method of Local Tumour Control 'Cure' Analysis:

The radiation TCD_{50} is a useful parameter for comparing radiation sensitivities of different tumours under various conditions of irradiation. It is preferable to any other end-point because it may be estimated with the greatest accuracy for a given number of irradiated tumours.

The TCD_{50} is defined as the radiation dose necessary to obtain local control or 'cure' of one half (i.e. 50 per cent) of the tumours at some stated time. The curve of 'cures' versus the dose plotted on a linear grid is usually a steeply rising sigmoid. It is difficult to estimate the TCD_{50} accurately from such a curve, as it falls on a very steep part of the curve. Several methods have been used to obtain an accurate estimation of the TCD_{50}; namely: The probit analysis (284); The Logit method (285) and the single-cell kinetics method of Porter and Peters (136).

In the present work, the log-log transformation analysis has been used for the TCD_{50} calculations using a computer program provided by T.E. Wheldon. This analysis is best regarded as a quick and easy way of getting a reasonable estimate of the TCD_{50} with only less than 5% difference from the estimated values using the other methods mentioned above. It generates a straight line, from which the TCD_{50} may be read directly.

The log-log transformation analysis is based on the following assumption:-

a) Tumour cure requires elimination of all clonogenic cells;

b) There exists a unique species of clonogen (possibly a

sub-population of the total) of uniform radiosensitivity which dominates radiocurability;

c) All tumours contain equal numbers of dominant clonogens;

d) The radiation survival curve of the dominant clonogens follows the single-hit multi-target equation.

e) The killing of cells by radiation is a random process conforming to Poisson statistics. Hence, the survival curve equation gives the mean fraction surviving a particular radiation dose. The probability that a particular fraction survives in any single case is then given by a Poisson distribution.

Thus, the probability that (say) X clonogens survive dose d is given by

$$P(X) = \frac{(SN)^X}{X!} \cdot \bar{e}^{SN}$$

where S is the mean fraction of cells surviving dose d and N the number of clonogens originally present. If cure requires elimination of all clonogens, then the probability of cure P_c is the probability P(0) that no clonogens survive. Hence,

$$P_c = \bar{e}^{SN}$$

Adopting the familiar 'off the shoulder' approximation to the multi-target survival expression we have

$$S \approx n\, e^{-d/D_o}$$

where d is the dose, n the extrapolation number and D_o the reciprocal of the survival curve slope beyond the shoulder. Hence,

$$P_c = -e^{nN\, e^{-d/D_o}}$$

which can be rewritten in the form

$$\ln(-\ln P_c) = \ln(nN) - (1/D_o) \cdot d$$

to show that a plot of $\ln(-\ln P_c)$ against d should (if the assumptions made are correct) yield a straight line of slope $(-1/D_o)$ and intercept $\ln(nN)$ on the y-axis.

For calculation of TCD_{50} simply note that $\ln(-\ln(0.5)) = -0.367$ and by drawing a horizontal line from -0.367 on the $\ln(-\ln P_c)$ axis to intersect the line through the cure data and dropping a vertical line from the point of intersection to the dose axis, TCD_{50} can be estimated.

APPENDIX II

Mathematical Description of Tumour Growth:

Most experimental tumours grow more slowly when large than small and recognition of this almost universal feature has led to the use of 'decelerating' growth curves such as the Gompertz curve (section 1.2) as a convient description of tumour growth.

In general, however, if a purely empirical description of tumour growth is sought, any mathematical function with the appropriate decelerating property will usually suffice. Since many tumours are (nearly) spherical, a linear increase in diameter with time implies a corresponding cubic-increase in volume, giving a growth curve which may be fitted to the Gompertz equation. The advantage of the diameter versus time description is that it stays closer to tumour measurements and, as only straight-line fitting is required, extensive computational facilities are unnecessary. In the present study, a best straight-line was fitted by the method of unweighted least squares for each individual tumour growth curve using a II-58 programmable calculator. The practice of fitting each growth curve individually was adopted because, although linearity of increase in diameter with time was usually excellent (with correlation coefficients typically in excess of 0.95), there occurred in the treated groups occasional tumours whose growth pattern departed markedly from linearity (e.g. correlation coefficient 0.30) and for which a simple linear model was deemed inappropriate. Such tumours were excluded from the analysis.

Non-excluded tumours in each group then provided a statistical population of growth rates (i.e. slope of linear increase in diameter with time) which, in practice showed no great departure from a normal distribution, and allowed populations in different groups to be compared by standard parametric tests, such as Student's t-test.

REFERENCES

1. FOWLER, J.F., SHELDON, P.W., BEGG, A.C., HILL, S.A. & SMITH, A.M. Biological properties and response to X-rays of first generation transplants of spontaneous mammary carcinoma in C_3H mice. International Journal of Radiation Biology and Related Studies in Physics, Chemistry & Medicine, 27:463-480, 1975.

2. MADDEN, R.E. & BURK, D. Production of viable single cell suspensions from solid tumours. Journal of the National Cancer Institute, 27:841-861, 1961.

3. REINHOLD, H.S. A cell dispersion technique for use in quantitative transplantation studies with solid tumours. European Journal of Cancer, 1:67-71, 1965.

4. SILOBRCIC, V. & SUIT, H.D. Tumor-specific antigen(s) in a spontaneous mammary carcinoma of C_3H mice. I. Quantitative cell transplants into mammary-tumor-agent-positive and mammary-tumor-agent-free mice. Journal of the National Cancer Institute, 39:1113-1119, 1967.

5. KALLMAN, R.F., SILINI, G. & VAN PUTTEN, L.M. Factors influencing the quantitative estimation of the in vivo survival of cells from solid tumours. Journal of the National Cancer Institute, 39:539-549, 1967.

6. HEWITT, H.B., CHAN, D.P-S. & BLAKE, E.R. Survival curves for clonogenic cells of a murine keratinizing squamous carcinoma irradiated in vivo or under hypoxic conditions. International Journal of Radiation Biology and Related Studies in Physics, Chemistry & Medicine, 12:535-549, 1967.

7. HEWITT, H.B., BLAKE, E.R. & PORTER, E.H. The effect of lethally irradiated cells on the transplantability of murine tumours. British Journal of Cancer, 28:123-135, 1973.

8. THOMLINSON, R.H. An experimental method for comparing treatments of intact malignant tumours in animals and its application to the use of oxygen in radiotherapy. British Journal of Cancer, 14:555-576, 1960.

9. SUIT, H.D. & MAEDA, M. Hyperbaric oxygen and radiobiology of a C_3H mouse mammary carcinoma. Journal of the National Cancer Institute, 39:639-652, 1967.

10. PORTER, E.H., HEWITT, H.B. & BLAKE, E.R. The transplantation kinetics of tumour cells. British Journal of Cancer, 27:55-62, 1973.

11. HEWITT, H.B., BLAKE, E.R. & WALDER, A.S. A critique of the evidence for active host defence against cancer, based on personal studies of 27 murine tumours of spontaneous origin. British Journal of Cancer, 33:241-

259, 1976.

12. PETERS, L.J., HEWITT, H.B. The influence of fibrin formation on the transplantability of murine tumour cells: Implications for the mechanism of the Revesz effect. British Journal of Cancer, 29:279-291, 1974.

13. RÉVÉSZ, L. Effect of lethally damaged tumour cells upon the development of admixed viable cells. Journal of the National Cancer Institute, 20:1157-1186, 1958.

14. DEWYS, W.D. A quantitative model for the study of the growth and treatment of a tumour and its metastases with correlation between proliferative state and sensitivity to Cyclophosphamide. Cancer Research, 32: 367-373, 1972.

15. VAN DEN BRENK, H.A.S., CROWE, M.C. & STONE, M.G. Reactions of the tumour bed to lethaly irradiated tumour cells and the Révész effect. British Journal of Cancer, 36:94-104, 1977.

16. McCREADIE, J.A., INCH, W.R., KRUUV, J. & WATSON, T.A. The rate of tumour growth in animals. Growth, 29:331-347, 1965.

17. LAIRD, A.K. Dynamics of Embryonic growth. Growth, 30: 263-275, 1966.

18. LAIRD, A.K. Postnatal growth of birds and mammals. Growth, 30:349-363, 1966.

19. LAIRD, A.K. Dynamics of tumour growth: Comparisons of growth rates and extrapolation of growth curve to one cell. British Journal of Cancer, 19:278-291, 1965.

20. LAIRD, A.K. Dynamics of tumour growth. British Journal of Cancer, 18:490-502, 1964.

21. HERMENS, A.F. & BARENDSEN, G.W. Changes of cell proliferation characteristics in a rat rhabdomyosarcoma before and after X irradiation. European Journal of Cancer, 5:173-189, 1969.

22. FRINDEL, E., MALAISE, E., ALPEN, E. & TUBIANA, M. Kinetics of cell proliferation of an experimental tumour. Cancer Research, 27:1122-1131, 1967.

23. TUBIANA, M. The kinetics of tumour cell proliferation and radiotherapy. Review article. British Journal of Radiology, 44:325-347, 1971.

24. DENEKAMP, J. Cellular proliferation kinetics of animal tumours. Cancer Research, 30:393-400, 1970.

25. DENEKAMP, J. The relationship between the "cell loss factor" and the immediate response to radiation in animal tumours. European Journal of Cancer, 8:335-340, 1972.

26. McCREADIE, J.A., INCH, W.R. & SUTHERLAND, R.M. Differences in growth and morphology between the spontaneous C3H mammary carcinoma in the mouse and its syngeneic transplants. Cancer, 27:635-642, 1971.

27. BURTON, A.C. Rate of growth of solid tumours as a problem of diffusion. Growth, 30:157-176, 1966.

28. SUMMERS, W.C. A model of tumour growth in irradiated hosts. Nature, 205:414, 1965.

29. SUMMERS, W.C. Dynamics of tumour growth: A mathematical model. Growth, 30:333-338, 1966.

30. BRUNTON, G.F. & WHELDON, T.E. Characteristic species dependent growth patterns of mammalian neoplasms. Cell & Tissue Kinetics, 11:161-175, 1978.

31. NORTON, L., SIMON, S., BRETERTON, H.D. & BOGDEN, A.E. Predicting the cause of Compertzian growth. Nature, 264:542-545, 1976.

32. BURNET, M. Cancer - A Biological approach I. The processes of control. British Medical Journal, I: 779-786, 1957.

33. DECOSSE, J. & GELFANT, S. Non-cycling tumour cells: mitogenic response to antilymphocytic serum. Science, 162:698-699, 1968.

34. DEWYS, W., PORIES, W.J., RICHTER, M.C. & STRAIN, W.H. Inhibition of Walker 256 Carcinosarcoma Growth by Dietary Zinc Deficiency. Proceedings of the Society for Experimental Biology and Medicine, 135:17-22, 1970.

35. McQUITTY, J.T., DEWYS, W.D., MONACO, L., STRAIN, W.H., ROB, C.G., APGAR, J. & PORIES, W.J. Inhibition of tumour growth by Dietary Zinc deficiency. Cancer Research, 30:1387-1390, 1970.

36. REGAN, J.D., VODOPICK, H., TAKEDA, S., LEE, W.H. & FAULCON, F.M. Serine requirement in Leukaemic and normal blood cells. Science, 163:1452-1453, 1969.

37. WEIR, D.R. & MORNINGSTAR, W.A. The effect of Pyridoxine deficiency induced by Desoxypyridoxine on Acute Lymphatic Leukaemia of Adults. Blood, 9:173-182, 1954.

38. GULLINO, P.M., GRANTHAM, F.H., SMITH, S.H. & HAGGERTY,

A.C. Modification of the acid base status of the Internal Milieu of tumours. Journal of the National Cancer Institute, 34:857-869, 1965.

39. BIDEL, P. Tumor growth inhibiting effect on JB-1 ascitic fluid, 1. An in vivo investigation. European Journal of Cancer, 6:291-296, 1970.

40. SYLVÉN, B. & HOLMBERG, B. On the structure and biological effects of a newly discovered cytotoxic Polypeptide in tumor fluid. European Journal of Cancer, 1:199-202, 1965.

41. TOPIO RYTÖMAA. The Chalone Concept. International review of experimental pathology, 16: 155-206,1976.

42. ABERCROMBIE, M. & HEAYSMAN, J.E.M. Observations on the social behaviour of cells in tissue culture I. speed of movement of chick heart fibroblasts in relation to their mutual contacts. Experimental Cell Research, 5:111-131, 1953.

43. ABERCROMBIE, M., HEAYSMAN, J.E.M. & KARTHAUSER, H.M. Social behaviour of cells in tissue culture III Mutual influence of sarcoma cells and fibroblasts. Experimental Cell Research, 13:276-291, 1957.

44. ABERCROMBIE, M. & AMBROSE, E.J. Interference microscope studies of cell contacts in tissue culture. Experimental Cell Research, 15:332-345, 1958.

45. WEXLER, H., ORME, S.K. & KETCHAM, A.S. Biological behaviour through successive transplant generations of transplantable tumours derived originally from primary chemically induced and spontaneous sources in mice. Journal of the National Cancer Institute, 40:513-523, 1968.

46. STEEL, G.G., ADAMS, K., HODGETT, J. & JANIK, P. Cell population kinetics of a spontaneous rat tumour during serial transplantation. British Journal of Cancer, 25: 802-811, 1971.

47. WOODRUFF, M.F.A. & SYMES, M.O. The significance of splenomegaly in tumour-bearing mice. British Journal of Cancer, 16:120-130, 1962.

48. WOODRUFF, M.F.A. & SYMES, M.O. Evidence of loss of tumour-specific antigen on repeatedly transplanting a tumour in the strain of origin. British Journal of Cancer, 16:484-488, 1962.

49. SYMES, M.O. Further observations on the growth of mouse mammary carcinoma in the strain of origin: The

influence of the hosts immunological resistance on the deletion of tumour specific antigens. British Journal of Cancer, 19:181-188, 1965.

50. SYMES, M.O. The relation between the degree of tumour specific antigenicity and malignancy. British Journal of Cancer, 19:189-194, 1965.

51. ROCKWELL, S.C., KALLMAN, R.F. & FAJARDO, L.F. Characteristics of a serially transplanted mouse mammary tumour and its tissue culture adapted derivative. Journal of the National Cancer Institute, 49:735-749, 1972.

52. BEGG, A.C. Kinetic and histological changes of a serially transplanted mouse tumour. Cell & Tissue Kinetics, 4:401-411, 1971.

53. GOLDFEDER, A. & NAGASAKI, F. Spontaneous transformation from carcinomatous to sarcomatous-like growth. Cancer Research, 14:267-270, 1954.

54. SANFORD, K.K., DUNN, T.B., WESTFALL, B.B., CARALESKI, A.B., DUPREE, L.T. & EARLE, W.R. Sarcomatous changes and maintenance of differentiation in long-term cultures of mouse mammary carcinoma. Journal of the National Cancer Institute, 26:1139-1183, 1961.

55. KLEIN, G., SJÖGREN, H.O. & KLEIN, E. Demonstration of host resistance against isotransplantation of lymphomas induced by the Gross agent. Cancer Research, 22:955-961, 1962.

56. KLEIN, G., SJÖGREN, H.O., KLEIN, E. & HELLSTRÖM, K.E. Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary autochthonous host. Cancer Research, 20:1561-1572, 1960.

57. RIGGINS, R.S. & PILCH, Y.H. Immunity to spontaneous and methylcholanthrene-induced tumors in inbred mice. Cancer Research, 24:1994-1996, 1964.

58. WEISS, D.W., FAULKIN, L.J. & DE ORME, K.B. Acquisition of heightened resistance and susceptibility to spontaneous mouse mammary carcinomas in the original host. Cancer Research, 24:732-741, 1964.

59. FOLEY, E.J. Attempts to induce immunity against mammary adenocarcinoma in inbred mice. Cancer Research, 13:578-580, 1953.

60. FOLEY, E.J. Antigenic properties of methylcholanthrene-induced tumours in mice of the strain of origin. Cancer Research, 13:835-837, 1953.

61. SUIT, H.D. & SILOBRCIC, V. Tumor-specific antigen(s) in a spontaneous mammary carcinoma of C3H mice. II. Active immunization of mammary-tumor-agent-free mice. Journal of the National Cancer Institute, 39: 1121-1128, 1967.

62. VAAGE, J. Non virus-associated antigens in virus-induced mouse mammary tumors. Cancer Research, 28: 2477-2483, 1968.

63. VAAGE, J. & MEDINA, D. Virus oncogenesis and tumour immunogenicity in the mouse mammary tumour system. Cancer Research, 34: 1319-1324, 1974.

64. SUIT, H.D. & KASTELAN, A. Tumor control by irradiation: A C_3H/He mouse mammary carcinoma in mammary-tumor-agent-positive and mammary-tumor-agent-free mice. Journal of the National Cancer Institute, 40:945-950, 1968.

65. RUBIN, P. & CASARETT, G. Part I. Microcirculation of tumors. Anatomy, Function and Necrosis. Clinical Radiology, 17:220-229, 1966.

66. RUBIN, P. & CASARETT, G. Part II. Microcirculation of tumors: the supervascularized state of irradiated regressing tumors. Clinical Radiology, 17:346-355, 1966.

67. REINHOLD, H.S. Improved microcirculation in irradiated tumours. European Journal of Cancer, 7:273-280, 1971.

68. KLIGERMAN, M.M. & HENEL, D.K. Some aspects of the microcirculation of a transplantable experimental tumor. Radiology, 76:810-817, 1961.

69. ALGIRE, G.H. & CHALKEY, H.W. Vascular reactions of normal and malignant tissues in vivo. I. Vascular reactions of mice to wounds and to normal and neoplastic transplants. Journal of the National Cancer Institute, 6:73-85, 1945.

70. FOLKMAN, J., MERLER, E., ABERNATHY, C. & WILLIAMS, G. Isolation of a tumor factor responsible for angiogenesis. Journal of Experimental Medicine, 133:275-288, 1971.

71. FOLKMAN, J. & COTRAN, R. Relation of vascular proliferation to tumor growth. International Review of Experimental Pathology, 16:207-248, 1976.

72. KNIGHTON, D., AUSPRUNK, D., TAPPER, D. & FOLKMAN, J. Avascular and vascular phases of tumour growth in the chick embryo. British Journal of Cancer, 35:347-356, 1977.

73. THOMLINSON, R.H. & GRAY, L.H. The histological

structure of some human lung cancers and the possible implications for radiotherapy. British Journal of Cancer, 9:539-549, 1955.

74. CATER, D.B., GRIGSON, C.M.B. & WATKINSON, D.A. Changes of oxygen tension in tumours induced by vasoconstrictor and vasodilator drugs. Acta Radiologica, 48:401-434, 1962.

75. KRUUV, J.A., INCH, W.R. & McCREDIE, J.A. Blood flow and oxygenation of tumours in mice I. Effect of vasodilator drugs. Cancer, 20:60-65, 1967.

76. TANNOCK, I.F. The relation between cell proliferation and the vascular system in a transplanted mouse mammary tumour. British Journal of Cancer, 22:258-273, 1968.

77. TANNOCK, I.F. Population kinetics of carcinoma cells, capillary endothelial cells, and fibroblasts in a transplanted mouse mammary tumor. Cancer Research, 30: 2470-2476, 1970.

78. TANNOCK, I.F. & HAYASHI, S. The proliferation of capillary endothelial cells. Cancer Research, 32:77-82, 1972.

79. GREENE, H.S.N. Heterologous transplantation of mammalian tumors. Journal of Experimental Medicine, 73:461-486, 1941.

80. FOLKMAN, J., COLE, P. & ZIMMERMAN, S. Tumour behaviour in isolated perfused organs: in vitro growth and metastases of biopsy material in rabbit thyroid and canine intestinal segment. Annals of Surgery, 164:491-502, 1966.

81. FOLKMAN, J. & HOCHBERG, M. Self-regulation of growth in three dimensions. Journal of Experimental Medicine, 138:745-753, 1973.

82. JOLLES, B., REMINGTON, M. & SIMON-REUSS, I. Indirect radiation effects and diffusible factors in irradiated tissues (Stromatex). Acta Radiologica, 56:57-64, 1961.

83. MOUNT, D. & BRUCE, W.R. Local plasma volume and vascular permeability of rabbit skin after irradiation. Radiation Research, 23:430-445, 1964.

84. THOMLINSON, R.H. Radiation and the vascularity of tumours. British Medical Bulletin, 29:29-32, 1973.

85. SONG, C.W. & LEVITT, S.H. Vascular changes in Walker 256 carcinoma of rats following X-irradiation. Radiology, 100:397-407, 1971.

86. SONG, C.W., PAYNE, T. & LEVITT, S.H. Vascularity and blood flow in X-irradiated Walker carcinoma 256 of rats. Radiology, 104:693-697, 1972.

87. MERWIN, R., ALGIRE, G.H. & KAPLAN, H.S. Transplanted-chamber observations on the response of a transplantable mouse mammary tumor to local roentgen irradiation. Journal of the National Cancer Institute, 11:593-623, 1950.

88. THOMLINSON, R.H. & CRADDOCK, E.A. The gross response of an experimental tumour to single doses of X-rays. British Journal of Cancer, 21:108-123, 1967.

89. RUBIN, P. Reoxygenation versus deoxygenation. In time and dose relationships in radiation biology as applied to radiotherapy. NCI-AEC Conference, Carmel, California, 1969. BNL 50203(C-57) 227-231, 1970, Brookhaven National Laboratory, Upton, New York.

90. BRECHER, G. & TESSMER, C.F. Late effects on vascular tissue. In time and dose relationships in radiation biology as applied to radiotherapy. NCI-AEC Conference, Carmel, California, 1969. BNL 50203(C-57) 186-191, 1970, Brookhaven National Laboratory, Upton, New York.

91. FAJARDO, L.F. & STEWART, J.R. Capillary injury preceding radiation-induced myocardial fibrosis. Radiology, 101:429-433, 1971.

92. SAEKI, Y., SHIMAZAKI, S. & URANO, M. Radiation effect on the vascularization of a C3H mouse mammary carcinoma: microangiographic studies of the tumor in preirradiated tissue and of the recurrent tumor. Radiology, 101:175-180, 1971.

93. McALISTER, W.H. & MARGULIS, A.R. Angiography of malignant tumours in mice following irradiation. Radiology, 81:664-674, 1963.

94. RUBIN, P. Discussion. In time and dose relationships in radiation biology as applied to radiotherapy. NCI-AEC Conference, Carmel, California, 1969. BNL 50203 (C-57) 253-254, 1970, Brookhaven National Laboratory, Upton, New York.

95. HEWITT, H.B. & BLAKE, E.R. The growth of transplanted murine tumours in preirradiated sites. British Journal of Cancer, 22:808-824, 1968.

96. SUMMERS, W.C., CLIFTON, K.H. & VERMUND, H. X-irradiation of the tumor bed. I. A study of the indirect actions of radiation on transplantable tumours. Radiology, 82:691-703, 1964.

97. KERMUND, H., STENSTROM, K.W., MOSSER, D.G. & JOHNSON, E.A. Effects of roentgen irradiation on the tumor bed. Part II. Radiation Research, 5:354-364, 1956.

98. URANO, M. & SUIT, H.D. Experimental evaluation of tumor bed effect of C_3H mouse mammary carcinoma and for C_3H mouse fibrosarcoma. Radiation Research, 45: 41-49, 1971.

99. CLIFTON, K.H. & JIRTLE, R. Mammary carcinoma cell population growth in preirradiated and unirradiated transplant sites. Radiology, 117:459-465, 1975.

100. STENATROM, K.W. VERMUND, H., MOSSER, D.G. & MARVIN, J.F. Effects of roentgen irradiation on the tumor-bed. Part I. Radiation Research, 2:180-191, 1955.

101. JIRTLE, R., RANKIN, J.H.G. & CLIFTON, K.H. Effects of X-irradiation of tumour bed on tumour blood flow and vascular response to drugs. British Journal of Cancer, 37:1033-1038, 1978.

102. GRAY, L.H. Cellular radiobiology. Radiation Research Supplement, 1:73-101, 1959.

103. DEWEY, D.L. Effect of oxygen and nitric oxide on the radiosensitivity of human cells in tissue culture. Nature, 186:780-782, 1960.

104. GRAY, L.H., CONGER, A.D., EBERT, M., HORNSEY, S. & SCOTT, O.C.A. The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. British Journal of Radiology, 26:638-648, 1953.

105. DEWEY, D.L. & BOAG, J.W. Modification of the oxygen effect when bacteria are given large pulses of radiation. Nature, 183:1450-1451, 1959.

106. HOWARD, A. Intracellular oxygen and radiation sensitivity. Nature, 207:776-777, 1965.

107. GRAY, L.H. Radiobiologic basis of oxygen as a modifying factor in radiation therapy. American Journal of Roentgenology, Radium Therapy and Nuclear Medicine, 85:803-815, 1961.

108. POWER, W.E. & TOLMACH, L.J. A multi-component X-ray survival curve for mouse lymphosarcoma cells irradiated in vivo. Nature, 197:710-711, 1963.

109. POWERS, W.E. & TOLMACH, L.J. Demonstration of an anoxic component in a mouse tumour cell population by in vivo assay of survival following irradiation. Radiology, 83:328-336, 1964.

110. HEWITT, H.B. & WILSON, C.W. The effect of tissue oxygen tension on the radiosensitivity of leukaemia cells irradiated in situ in the livers of leukaemic mice. British Journal of Cancer, 13:675-684, 1959.

111. BELLI, J.A. & ANDREW, J.R. Relationship between tumour growth and radiosensitivity. Journal of the National Cancer Institutes, 31:689-703, 1963.

112. KOCH, C.J. & KRUUV, J. & FREY, H.E. The effect of hypoxia on the generation time of mammalian cells. Radiation Research, 53:43-48, 1973.

113. BEDFORD, J.S. & MITCHELL, J.B. The effect of hypoxia on the growth and radiation response of mammalian cells in culture. British Journal of Radiology, 47:687-696, 1974.

114. BORN, R., HUG, O. & TROTT, K.R. The effect of prolonged hypoxia on growth and viability of Chinese hamster cells. International Journal of Radiation, Oncology, Biology, Physics, 1:687-697, 1976.

115. PHILLIPS, T.L. & HANKS, G.E. Apparent absence of recovery in endogenous colony-forming cells after irradiation under hypoxic conditions. Radiation Research, 33:517-532, 1968.

116. LITTBRAND, B. & RÉVÉSZ, L. The effect of oxygen on cellular survival and recovery after radiation. British Journal of Radiation, 42:914-924, 1969.

117. NIAS, A.H.W., SWALLOW, A.J., KEENE, J.P. & HODGSON, B.W. Absence of a fractionation effect in irradiated HeLa cells. International Journal of Radiation Biology and Related Studies in Physics, Chemistry & Medicine, 23:559-569, 1973.

118. SUIT, H. & URANO, M. Repair of sublethal radiation injury in hypoxic cells of a C_3H mouse mammary carcinoma. Radiation Research, 37:423-434, 1969.

119. HAHN, G.M., BASHAW, M.A., EVANS, R.G. & GORDON, L.F. Repair of potentially lethal lesions in X-irradiated density-inhibited Chinese hamster cells; metabolic effects and hypoxia. Radiation Research, 55:280-290, 1973.

120. LITTLE, J.B. Factors influencing the repair of potentially lethal radiation damage in growth-inhibited human cells. Radiation Research, 56:320-333, 1973.

121. HAHN, G.M. & LITTLE, J.B. Plateau-phase cultures of mammalian cells; an in vitro model for human cancer. Current Topics in Radiation Research Quarterly, 8:

39-83, 1972.

122. KALLMAN, R.F. The phenomenon of reoxygenation and its implications for fractionated radiotherapy. Radiology, 105:135-142, 1972.

123. SUIT, H.D. Radiation biology: A basis for radiotherapy. In text book of radiotherapy (G.H. Fletcher, Ed.) 2nd ed., pp. 75-121. Lea & Febiger, Philadelphia, 1973.

124. SAMBROOK, D.K. Theoretical aspects of dose-time factors in radiotherapy technique Part I - Dose factors. Clinical Radiology, 14:290-297, 1963.

125. THOMLINSON, R.H. Oxygen therapy - biological considerations. In modern trends in Radiotherapy, eds. T.J. Deeley & C.A.P. Wood, pp. 52-72 (Butterworths, London), 1967.

126. THOMLINSON, R.H. The oxygen effect and radiotherapy with fast neutrons. European Journal of Cancer, 7: 139-144, 1971.

127. FIELD, S.B. An historical survey of radiobiology and radiotherapy with fast neutrons. Current Topics in Radiation Research Quarterly, Volume II p.1-86, 1976.

128. FOWLER, J.F., MORGAN, R.L. & WOOD, C.A.P. The biological and physical advantages and problems of neutron therapy. British Journal of Radiology, 36: 77-80, 1963.

129. HOWES, A.E. An estimation of changes in the proportions and absolute numbers of hypoxic cells after irradiation of transplanted C3H mouse mammary tumours. British Journal of Radiology, 42:441-447, 1969.

130. HOWES, A.E., PAGE, A. & FOWLER, J.F. The effect of single and fractionated doses of X-rays on the effective proportion of hypoxic cells in C3H mouse mammary tumors. British Journal of Radiology, 45:250-254, 1972.

131. HILL, S.A. & FOWLER, J.F. Radiosensitizing and cytocidal effects on hypoxic cells of RO-07-0582, and repair of X-ray injury, in an experimental mouse tumour. British Journal of Cancer, 35:461-469, 1977.

132. McNALLY, N.J. A comparison of the effects of radiation on tumour growth delay and cell survival. The effect of oxygen. British Journal of Radiology, 46:450-455, 1973.

133. McNALLY, N.J. The effect of an hypoxic cell sensitizer on tumour growth delay and cell survival: Implications for cell survival in situ and in vitro. British Journal

of Cancer, 32:610-618, 1975.

134. McNALLY, N.J. A comparison of the effect of radiation on tumour growth delay and cell survival. The effect of radiation quality. British Journal of Radiology, 48:141-145, 1975.

135. McNALLY, N.J. & SHELDON, P.W. The effect of radiation on tumour growth delay, cell survival and cure of the animal using a single tumour system. British Journal of Radiology, 50:321-328, 1977.

136. FOWLER, J.F., DENEKAMP, J., SHELDON, P.W., SMITH, A.M., BEGG, A.C., HARRIS, S.R. & PAGE, A.L. Optimum fractionation in X-ray treatment of C_3H mouse mammary tumors. British Journal of Radiology, 47:781-789, 1974.

137. SUIT, H.D. & SUCHATO, C. Hyperbaric oxygen and radiotherapy of a fibrosarcoma and of a squamous-cell carcinoma of C_3H mice. Radiology, 89:713-719, 1967.

138. SUIT, H.D., LINDBERG, R., SUCHATO, C. & OZENNE, A. Radiation dose fractionation and high pressure oxygen in radiotherapy of the DBA mouse mammary carcinoma. American Journal of Roentgenology, Radium Therapy & Nuclear Medicine, 99:895-899, 1967.

139. SUIT, H.D. & SCHIAVONE, J.V. Effect of a single dose of radiation on proportion of hypoxic cells in a C_3H mouse mammary carcinoma. Radiology, 90:325-328, 1968.

140. DU SAULT, L.A. Reoxygenation of tumours during fractionated radiotherapy. Radiology, 92:626-628, 1969.

141. BARENDSEN, G.W. & BROERSE, J.J. Experimental radiotherapy of a rat rhabdomyosarcoma with 15 MeV neutrons and 300 kV X-rays. 1. Effects of single exposure. European Journal of Cancer, 5:373-391, 1969.

142. CHESHIRE, P.J. & LINDOP, P.J. The influence of intracellular recovery and hypoxic cells on the radiation response of mammary tumours and skin in C_3H mice. British Journal of Radiology, 42:215-223, 1969.

143. VAN PUTTEN, L.M. & KALLMAN, R.F. Oxygenation status of a transplantable tumor during fractionated radiation therapy. Journal of the National Cancer Institute, 40: 441-451, 1968.

144. VAN PUTTEN, L.M. Tumor reoxygenation during fractionated radiotherapy: Studies with a transplantable osteosarcoma. European Journal of Cancer, 4:173-182, 1968.

145. DU SAULT, L.A. Discussion. In time and dose

relationships in radiation biology as applied to radiotherapy. NCI-AEC Conference, Carmel, California, 1969. BNL 50203 (247-248, 1970, Brookhaven National Laboratory, Upton, New York.

146. FOWLER, J.F., SHELDON, P.W., HARRIS, S.R., HILL, S.A. & AYRES, S.G. Relative effectiveness of 12-hourly fraction and a non-uniform X-ray schedule in the optimum fractionation of C3H mouse mammary tumors. British Journal of Radiology, 48:581-589, 1975.

147. HAWKES, M.J., HILL, R.P., LINDOP, P.J., ELLIS, R.E., & ROTBLAT, J. The response of C_3H mammary tumours to irradiation in single and fractionated doses. British Journal of Radiology, 41:134-141, 1968.

148. DENEKAMP, J. & THOMLINSON, R.H. The cell proliferation kinetics of four experimental tumours after acute X-irradiation. Cancer Research, 31:1279-1284, 1971.

149. SZCZEPANSKI, L.V. & TROTT, K.R. Post-irradiation proliferation kinetics of a serially transplanted murine adenocarcinoma. British Journal of Radiology, 48:200-208, 1975.

150. SUIT, H.D., LINDBERG, R.L. & FLETCHER, G.H. Prognostic significance of extent of tumor regression at completion of radiation therapy. Radiology, 84:1100-1107, 1965.

151. DENEKAMP, J. Tumour regression as a guide to prognosis: a study with experimental animals. British Journal of Radiology, 50:271-279, 1977.

152. THOMLINSON, R.H. Reoxygenation as a function of tumour size and histopathological type. In time and dose relationships in radiation biology as applied to radiotherapy. NCI-AEC Conference, Carmel, California, 1969. BNL 50203 (C-57) 242-247, 1970, Brookhaven National Laboratory, Upton, New York.

153. BROWN, J.M. The effect of acute X-irradiation on the cell proliferation kinetics of induced carcinomas and their normal counterpart. Radiation Research, 43:627-653, 1970.

154. BARENDSEN, G.W. & BROERSE, J.J. Experimental radiotherapy of a rat rhabdomyosarcoma with 15 MeV neutrons and 300 kV X-rays. 11. Effects of fractionated treatments, applied five times a week for several weeks. European Journal of Cancer, 6:89-109, 1970.

155. SHELDON, P.W. & HILL, S.A. Hypoxic cell radiosensitizers and local control by X-ray of a

transplanted tumour in mice. British Journal of Cancer, 35:795–808, 1977.

156. ELKIND, M.M. & SUTTON, H. X-ray damage and recovery in mammalian cells in culture. Nature, 184:1293–1295, 1959.

157. ELKIND, M.M. & SUTTON, H. Radiation response of mammalian cells grown in culture. I. Repair of X-ray damage in surviving Chinese hamster cells. Radiation Research, 13:556–593, 1960.

158. SINCLAIR, W.K. Cyclic X-ray responses in mammalian cells in vitro. Radiation Research, 33:620–643, 1968.

159. TERASIMA, T. & TOLMACH, L.J. Variations in several responses of HeLa cells to X-irradiation during the division cycle. Biophysical Journal, 3:11–33, 1963.

160. DEWEY, W.C. & HYMPHRY, R.M. Relative radiosensitivity of different phases in the life-cycle of L–P59 mouse fibroblasts and ascites tumor cells. Radiation Research, 16:503–530, 1962.

161. YU, C.K. & SINCLAIR, W.K. Mitotic delay and chromosomal aberrations induced by X-rays in synchronized Chinese hamster cells in vitro. Journal of the National Cancer Institute, 39:619–632, 1967.

162. PHILLIPS, R.A. & TOLMACH, L.J. Repair of potentially lethal damage in X-irradiated HeLa cells. Radiation Research, 29:413–432, 1966.

163. WEISS, B.G. & TOLMACH, L.J. Modification of X-ray induced killing of HeLa S3 cells by inhibitors of DNA synthesis. Biophysical Journal, 7:779–795, 1967.

164. YOUNG, J.M. & FOWLER, J.F. The effects of X-ray induced synchrony on two-dose cell survivalexperiments. Cell & Tissue Kinetics, 2:95–110, 1969.

165. TANNOCK, I.F. Oxygen diffusion and the distribution of cellular radiosensitivity in tumours. British Journal of Radiology, 45:515–524, 1972.

166. HALL, E.J. The effect of hypoxia on the repair of sublethal radiation damage in cultured mammalian cells. Radiation Research, 49:405–415, 1972.

167. KOCH, C.J. & KRUUV, J. The effect of extreme hypoxia on recovery after radiation by synchronized mammalian cells. Radiation Research, 48:74–85, 1971.

168. FOSTER, C.J., MALONE, J., ORR, J.S. & MacFARLANE, D.E. The recovery of the survival curve shoulder after

protracted hypoxia. British Journal of Radiology, 44: 540-545, 1971.

169. HALL, E.J., BEDFORD, J.S. & OLIVER, R. Extreme hypoxia; its effects on the survival of mammalian cells irradiated at high and low dose-rates. British Journal of Radiology, 39:302-307, 1966.

170. DENEKAMP, J. & HARRIS, S.R. Studies of the processes occurring between two fractions in experimental mouse tumours. International Journal of Radiation Oncology, Biology, Physics, 1:421-430, 1976.

171. FIELD, S.B., JONES, T. & THOMLINSON, R.H. The relative effects of fast neutrons and X-rays on tumours and normal tissue in the rat. 11. Fractionation: recovery and reoxygenation. British Journal of Radiology, 41: 597-607, 1968.

172. DENEKAMP, J. & HARRIS, S.R. The response of a transplantable tumour to fractionated irradiationl 1. X-rays and the hypoxic cell radiosensitizer RO-07-0582. Radiation Research, 66:66-75, 1976.

173. SUIT, H.D. & SHALEK, R.J. Response of anoxic C_3H mouse mammary carcinoma isotransplants (1-25 mm^3) to X-irradiation. Journal of the National Cancer Institute, 31:479-495, 1963.

174. BEGG, A.C. Studies of cell removal from irradiated and unirradiated murine tumours using ^{125}Iododeoxyuridine and tritiated thymidine. A Ph.D. thesis - University of London, 1975.

175. PHILLIPS, T.L. Split-dose recovery in euoxic and hypoxic normal and tumour cells. Radiology, 105:127-134, 1972.

176. WITHERS, H.R. Capacity for repair in cells of normal and malignant tissues. In time and dose relationships in radiation biology as applied to radiotherapy. NCI-AEC Conference, Carmel, California, 1969. BNL 50203 (C-57) 54-65, 1970, Brookhaven National Laboratory, Upton, New York.

177. HAHN, G.M., ROCKWELL, S., KALLMAN, R.F., GORDON, L.F. & FRINDEL, E. Repair of potentially lethal damage in vivo in solid tumour cells after X-irradiation. Cancer Research, 34:351-354, 1974.

178. LITTLE, J.B., HAHN, G.M., FRINDEL, E. & TUBIANA, M. Repair of potentially lethal radiation damage in vitro and in vivo. Radiology, 106:689-694, 1973.

179. EMERY, E.W., DENEKAMP, J. & BALL, M.M. & FIELD, S.B.

Survival of mouse skin epithelial cells following single and divided doses of X-rays. Radiation Research, 41:450-466, 1970.

180. FOWLER, J.F., MORGAN, R.L., SILVESTER, J.A., BEWLEY, D.K. & TURNER, B.A. Experiments with fractionated X-ray treatment of the skin of pigs. 1. Fractionation up to 28 days. British Journal of Radiology, 36:188-196, 1963.

181. WITHERS, H.R. Recovery and repopulation in vivo by mouse skin epithelial cells during fractionated irradiation. Radiation Research, 32:227-239, 1967.

182. DENEKAMP, J., BALL, M.M. & FOWLER, J.F. Recovery and repopulation in mouse skin as a function of time after X-irradiation. Radiation Research, 37:361-370, 1969.

183. SUIT, H.D., HOWES, A.E. & HUNTER, N. Dependence of response of a C_3H mammary carcinoma to fractionated irradiation on fractionation number and intertreatment interval. Radiation Research, 72:440-454, 1977.

184. ADAMS, G.W. Chemical radiosensitisation of hypoxic cells. British Medical Bulletin, 29:48-53, 1973.

185. ADAMS, G.E., FLOCKHART, I.R., SMITHEN, C.E., STRATFORD, I.J., WARDMAN, P., WATTS, M.E. Electron-affinic sensitization - VII. A correlation between structures one-electron reduction potentials, and efficiencies of nitroimidazoles as hypoxic cell sensitizers. Radiation Research, 67:9-20, 1976.

186. CHAPMAN, J.D., WEBB, R.G. & BORSA, J. Radiosensitisation of mammalian cells by p-nitroacetophenone. I. Characterisation in asynchronous and synchronous populations. International Journal of Radiation Biology & Related Studies in Physics, Chemistry & Medicine, 19:561-573, 1971.

187. ADAMS, G.E., ASQUITH, J.C., DEWEY, D.L., FOSTER, J.L., MICHAEL, B.D. & WILLSON, R.L. Electron affinic sensitisation. II. p-nitroacetophenone: a radiosensitiser for anoxic bacterial and mammalian cells. International Journal of Radiation Biology & Related Studies in Physics, Chemistry & Medicine, 19:575-585, 1971.

188. RALEIGH, J.A., CHAPMAN, J.D., BORSA, J., KREMERS, W. & REUVERS, A.P. Radiosensitisation of mammalian cells by p-nitroacetophenone. III. Effectiveness of nitrobenzene analogues. International Journal of Radiation Biology & Related Studies in Physics, Chemistry & Medicine, 23: 377-387, 1973.

189. ADAMS, G.E., ASQUITH, J.C., WATTS, M.E. & SMITHEN, C.E. Radiosensitization of hypoxic cells in vitro: a water soluble derivation of paranitroacetophenone. Nature, New Biology, 239:23-24, 1972.

190. WHITMORE, G.F., GULYAS, S. & VARGHESE, A.J. Studies on the radiation-sensitizing action of NDPP. A sensitizer of hypoxic cells. Radiation Research, 61: 325-341, 1975.

191. DENEKAMP, J. & MICHAEL, B.D. Preferential sensitization of hypoxic cells to radiation in vivo. Nature, New Biology, 239:21-23, 1972.

192. SHELDON, P.W. & SMITH, A.M. Modest radiosensitization of solid tumours in C_3H mice by the hypoxic cell radiosensitizer NDPP. British Journal of Cancer, 31: 81-88, 1975.

193. CHAPMAN, J.D., REUVERS, A.P., BORSA, J., PETKAU, A. & McCALLA, D.R. Nirofurans as radiosensitizers of hypoxic mammalian cells. Cancer Research, 32:2616-2624, 1972.

194. CHAPMAN, J.D., REUVERS, A.P. & BORSA, J. Effectiveness of nitrofuran derivatives in sensitising hypoxic mammalian cells to X-rays. British Journal of Radiology, 46:623-630, 1973.

195. ASQUITH, J.C., WATTS, M.E., PATEL, K., SMITHEN, C.E. & ADAMS, G.E. Electron affinic sensitization v. radiosensitization of hypoxic bacteria and mammalian cells in vitro by some nitro-imidazoles and nitro-pyrazoles. Radiation Research, 60:108-118, 1974.

196. BEAMAN, A.G., TAUTZ, W. & DUSCHINSKY, R. Studies in the nitroimidazole series: III: 2-nitroimidazole derivatives substituted in the 1-Position. American Society for Microbiology, 520-530, 1968. In Antimicrobial Agents and Chemotherapy, 1967, G.L. Hobby, ed.

197. MOORE, B.A., PALCIC, B., SKARSGARD, L.D. Radiosensitizing and toxic effects of the 2-nitroimidazole RO-07-0582 in hypoxic mammalian cells. Radiation Research, 67:459-473, 1976.

198. STRATFORD, I.J. & ADAMS, G.E. The effect of the hypoxic cell radiosensitizer RO-07-0582 on mammalian cells in vitro. British Journal of Cancer, 35:309-313, 1977.

199. HALL, E.J., ROIZIN., TOWLE, L. Hypoxic sensitizers: Radiobiological studies at the cellular level. Radiology, 117: 453-457, 1975.

200. DENEKAMP, J., MICHAEL, B.D. & HARRIS, S.R. Hypoxic cell radiosensitizers: Comparative tests of some electron affinic compounds using epidermal cell survival in vivo. Radiation Research, 60:119-132, 1974.

201. DENEKAMP, J. & HARRIS, S.R. Test of two electron-affinic radiosensitizers in vivo using regrowth in an experimental carcinoma. Radiation Research, 61:191-203, 1975.

202. BROWN, J.M. Selective radiosensitization of hypoxic cells of mouse tumours with the nitroimidazoles metronidazole and RO7-0582. Radiation Research, 64: 633-647, 1975.

203. SHELDON, P.W., FOSTER, J.L., FOWLER, J.F. Radiosensitization of C_3H mouse mammary tumors by a 2-nitroimidazole drug. British Journal of Cancer, 30: 560-565, 1974.

204. STONE, H.B., WITHERS, H.R. Tumor and normal tissue response to metronidazole and irradiation in mice. Radiology, 113:441-444, 1974.

205. FOWLER, J.F. & ADAMS, G.E. Radiosensitization of hypoxic cells in solid tumours in mice. British Journal of Radiology, 48:77-78, 1975.

206. SHELDON, P.W., HILL, S.A., FOSTER, J.L. & FOWLER, J.F. Radiosensitization of C_3H mouse mammary tumours using fractionated doses of X-rays with the drug RO-07-0582. British Journal of Radiology, 49:76-80, 1976.

207. HALL, E.J. & BIAGLOW, J.E. RO-07-0582 as a radiosensitizer and cytotoxic agent. International Journal of Radiation, Oncology, Biology, Physics, 2:521-530, 1977.

208. ASQUITH, J.C., FOSTER, J.L., WILLSON, R.L., INGS, R. & McFADZEAN, J.A. Metronidazole ("Flagyl") a radiosensitizer of hypoxic cells. British Journal of Radiology, 47:474-481, 1974.

209. WHITMORE, G.F., GULYAS, S. & VARGHESE. Sensitizing and toxicity properties of Misonidazole and its derivatives. British Journal of Cancer, 37, suppl.III, 115-119, 1978.

210. SRIDHAR, R., KOCH, C. & SUTHERLAND, R. Cytotoxicity of two nitroimidazole radiosensitizers in an in vitro tumor model. International Journal of Radiation Oncology, Biology, Physics, 1:1149-1157, 1976.

211. HALL, E.J., ASTOR, M., GEARD, C. & BIAGLOW, J. Cytotoxicity of RO-07-0582; enhancement by hypothermia and protection by cysteamine. British Journal of

Cancer, 35:809-815, 1977.

212. SRIDHAR, R. & SUTHERLAND, R. Hyperthermic potentiation of cytotoxicity of RO-07-0582 in multicell spheroids. International Journal of Radiation Oncology, Biology, Physics, 2:531-535, 1977.

213. BROWN, J.M. Cytotoxic effects of the hypoxic cell radiosensitizer RO-07-0582 to tumor cells in vivo. Radiation Research, 72:469-486, 1977.

214. DENEKAMP, J. Cytotoxicity and radiosensitization in mouse and man. British Journal of Radiology, 51:636-637, 1978.

215. BLEEHEN, N.M., HONESS, D.J. & MORGAN, J.E. Interaction of hyperthermia and the hypoxic cell sensitizer RO-07-0582 on the EMT6 mouse tumor. British Journal of Cancer, 35:299-306, 1977.

216. GRAY, A.J., DISCHE, S., ADAMS, G.E., FLOCKHART, I.R. & FOSTER, J.L. Clinical testing of the radiosensitiser RO-07-0582. I. Dose tolerance, serum and tumour concentrations. Clinical Radiology, 27:151-157, 1976.

217. FLOCKHART, I.R., LARGE, P. & TROUP, D., MALCOLM, S.L. & MARTEN, T.R. Pharmacokinetic and metabolic studies of the hypoxic cell radiosensitizer misonidazole. Xenobiotica, 8:97-105, 1978.

218. FOWLER, J.F., ADAMS, G.E., DENEKAMP, J. Radiosensitizers of hypoxic cells in solid tumors. Cancer Treatment Revision, 3:227-256, 1976.

219. STONE, H.B., WITHERS, H.R. Enhancement of the radioresponse of a murine tumour by a nitroimidazole. British Journal of Radiology, 48:411-412, 1975.

220. SHELDON, P.W. & HILL, S.A. Hypoxic cell radiosensitizers and local control by X-ray of a transplanted tumour in mice. British Journal of Cancer, 35:795-806, 1977.

221. FOWLER, J.F., SHELDON, P.W. & DENEKAMP, J. Optimum fractionation of the C_3H mouse mammary carcinoma using X-rays; the hypoxic cell radiosensitizer RO-07-0582, or fast neutrons. International Journal of Radiation Oncology, Biology, Physics, 1:579-592, 1976.

222. JOHNSON, R., GONER, C., AMBRUS, J., PEARCE, J. & BOYLE, D. An investigation of the pharmacological and radiosensitizing effects of the 2-nitroimidazole RO-07-0582 in primates. British Journal of Radiology, 49:294-295, 1976.

223. JOHNSON, R., GONER, C., PEARCE, J. An investigation of

the radiosensitizing effects of RO-07-0582 on hypoxic skin in primates. International Journal of Radiation Oncology, Biology, Physics, 1:593-599, 1976.

224. DISCHE, S., SAUNDERS, M.I., LEE, M.E., ADAMS, G.G. & FLOCKHART, I.R. Clinical testing of the radiosensitizer RO-07-0582: experience with multiple doses. British Journal of Cancer, 35:567-579, 1977.

225. THOMLINSON, R.H., DISCHE, S., GRAY, A.J. & ERRINGTON, L.M. Clinical testing of the radiosensitiser RO-07-0582. III. Response of tumours. Clinical Radiology, 27: 167-174, 1976.

226. SCHARER, K. Selective alterations of Purkinje cells in the dog after oral administration of high doses of nitroimidazole derivatives. Verhandlungen der Deutschen Gesellschaft für Pathologie, 56:407-410, 1972.

227. DEUTSCH, G., FOSTER, J.L., McFADZEAN, J.A. & PARNELL, M. Human studies with "high dose" metronidazole: a non-toxic radiosensitiser of hypoxic cells. British Journal of Cancer, 31:75-80, 1975.

228. URTASUN, R., BAND, P., CHAPMAN, J.D., FELDSTEIN, M.L., MIELKE, B., FRYER, C. Radiation and high dose metronidazole in supratentorial glioblastomas. New England Journal of Medicine, 294:1364-1367, 1976.

229. COXON, A. & PALLIS, A. Metronidazole neuropathy. Journal of Neurology, Neurosurgery & Psychiatry, 39:403-405, 1976.

230. SAUNDERS, M.I., DISCHE, S., ANDERSON, P. & FLOCKHART, I.R. The neurotoxicity of Misonidazole and its relationship to dose, half-life and concentration in the serum. British Journal of Cancer, 37: Suppl. III, 268-270, 1978.

231. URTASUN, R.C., CHAPMAN, J.D., FELDSTEIN, M.L., BAND, R.P., RABIN, H.R., WILSON, A.F., MARYNOWSKI, B., STARREVELD, E. & SHNITKA, T. Peripheral neuropathy related to misonidazole: Incidence and pathology. British Journal of Cancer, 37: Suppl. III, 271-275, 1978.

232. WILTSHIRE, C.R., WORKMAN, P., WATSON, J.V. & BLEEHEN, N.M. Clinical studies with Misonidazole. British Journal of Cancer, 37: Suppl. III, 286-289, 1978.

233. DISCHE, S., SAUNDERS, M.L. & FLOCKHART, I.R. The optimum regime for the administration of misonidazole and the establishment of multi-centre clinical trials. British Journal of Cancer, 37: Suppl. III, 318-321, 1978.

234. ZANELLI, G.D. & LUCAS, P.B. Effect of stress on blood perfusion and vascular space in transplanted mouse tumours. British Journal of Radiology, 49:382-383, 1976 (Correspondence).

235. ZANELLI, G.D., LUCAS, P.B. & FOWLER, J.F. The effect of anaesthetics on blood perfusion in transplanted mouse tumours. British Journal of Cancer, 32:380-390, 1975.

236. JOHNSON, R., FOWLER, J.F. & ZANELLI, G.D. Changes in mouse blood pressure, tumor blood flow, core and tumor temperatures following nembutal or urethane anesthesia. Radiology, 118:697-703, 1976.

237. KALLMAN, R.F., DE NARDO, G.L. & STASCH, M.J. Blood flow in irradiated mouse sarcoma as determined by the clearance of Xenon-133. Cancer Research, 32:483-490, 1972.

238. MÄNTYTÄ, M., KUIKKA, J. & REKONEN, A. Regional blood flow in human tumours with special reference to the effect of radiotherapy. British Journal of Radiology, 49:335-338, 1976.

239. BIERMAN, H.R., KELLY, K.H., DOD, K.S. & BYRON, R.L. Studies on the blood supply of tumours in man. I. Fluorescence of cutaneous lesions. Journal of the National Cancer Institute, 11; 877-889, 1951.

240. GOLDACRE, R.J. & SYLVÉN, B. A rapid method for studying tumour blood supplies with systemic dyes. Nature, 184: 63-64, 1959.

241. OWEN, L.N. A rapid method for studying tumour blood supply using Lissamine green. Nature, 187:795-796, 1960.

242. CARTER, D.B. & SILVER, I.A. Quantitative measurements of oxygen tension in normal tissues and in the tumour of patients before and after radiotherapy. Acta Radiologica, 53:233-256, 1960.

243. JOHNSON, R. A thermodynamic method for investigation of radiation induced changes in the microcirculation of human tumours. International Journal of Radiation Oncology, Biology, Physics, 1:659-670, 1976.

244. GULLINO, P.M. & GRANTHAM, F.H. Studies on the exchange of fluids between host and tumour. II. The blood flow of hepatoma and other tumours in rats and mice. Journal of the National Cancer Institute, 27:1465-1491, 1961.

245. ZANELLI, G.D. & FOWLER, J.F. The measurement of blood perfusion in experimental tumours by uptake of ^{86}Rb.

Cancer Research, 34:1451-1456, 1974.

246. GUMP, F.E. & WHITE, R.L. Determination of regional tumour blood flow by Krypton-85. Cancer, 21:871-875, 1968.

247. GILLESPIE, F.C. Some factors influencing the interpretation of regional blood flow measurements using inert gas clearance techniques. In: Blood flow through organs and tissues. Eds. Bain & Harper, Livingstone, Edinburgh, 1968.

248. STRANG, R. The measurement of choroidal blood flow using Krypton-85. Ph.D. thesis, University of Glasgow, 1975.

249. STEEL, G.G., ADAMS, K. & BARRETT, J.C. Analysis of the cell population kinetics of transplanted tumours of widely-differing growth rate. British Journal of Cancer, 20:784-800, 1966.

250. DETHLEFSEN, L.A., PREWITT, J.M.S. & MENDELSOHN, M.L. Analysis of tumour growth curves. Journal of the National Cancer Institute, 40:389-405, 1968.

251. DENEKAMP, J. & FOWLER, J.F. Further investigations of the response of irradiated mouse skin. International Journal of Radiation Biology and Related Studies in Physics, Chemistry and Medicine, 10:435-441, 1966.

252. DENEKAMP, J. Changes in the rate of repopulation during multifraction irradiation of mouse skin. British Journal of Radiology, 46:381-387, 1973.

253. BURLIN, T.E., CHALLONER, A.V.J., HUTTON, W.C., MAGNUS, I.A., RANU, H.S. & SPITTLE, M. Effects of radiation on the visual appearance and mechanical properties of mouse skin. British Journal of Radiology, 50:123-128, 1977.

254. HILL, R.P., CHESHIRE, P.J., LINDOP, P.J. & FIELD, S.B. A comparison of the response of the tumour and normal tissue in the mouse exposed to single doses of fast neutrons or electrons. British Journal of Radiology, 43:894-897, 1970.

255. FOWLER, J.F., DENEKAMP, J., PAGE, A.L., BEGG, A.C., FIELD, S.B. & BUTLER, K. Fractionation with X-rays and neutrons in mice: response of skin and C_3H mammary tumours. British Journal of Radiology, 45:237-249, 1972.

256. FOWLER, J.F., KRAGT, K., ELLIS, R.E., LINDOP, P.J. & BERRY, R.J. The effect of divided doses of 15-MeV electrons on the skin of mice. International Journal of Radiation Biology and Related Studies in Physics,

Chemistry and Medicine, 9:241-252, 1965.

257. COLQUHOUN, D. Two sample randomization test on ranks. The Wilcoxon (or Mann-Whitney) test. In Lectures on Biostatistics: An introduction with applications in Biology and Medicine, pp.143-148. Clarendon Press, Oxford, 1971.

258. WALKER, A., McCALLUM, H.M., WHELDON, T.E., NIAS, A.H.W. & ABDELAAL, A.S. Promotion of metastasis of C_3H mouse mammary carcinoma by local hyperthermia. British Journal of Cancer, 38:561-563, 1978.

259. TROTT, K.R., SZCZEPANSKI, L.V., KUMMERMEHR, J. & HUG, O. Tumour control probability and tumour regression rate after fractionated radiotherapy of two mouse tumours. In Proceedings of the International Symposium on Radiobiological Research Needed for the Improvement of Radiotherapy, Vienna, 22-26 Nov., 1976. I.A.E.A. Vol I, pp.29-42, 1977.

260. KOVACS, C.J., EVANS, M.J., WAKEFIELD, J.A. & LOONEY, W.B. A comparative study of the response to radiation by experimental tumours with markedly different growth characteristics. Radiation Research, 72:455-468, 1977.

261. FRIEDMAN, M., PEARLMAN, A.W. & TURGEON, L. Tumor lethal dose and iso-effect recovery curve. American Journal of Roentgenology, 99:843-851, 1967.

262. MARCIAL, V.A. & BOSCH, A. Radiation induced tumor regression in carcinoma of the uterine cervix: Prognostic Significance. American Journal of Roentgenology, 108:113-123, 1970.

263. GROSSMAN, I., KUROHARA, S., WEBSTER, J.H. & GEORGE, F.W. The prognostic significance of tumor response during radiotherapy in cervical carcinoma. Radiology, 107: 411-415, 1973.

264. SUIT, H.D. & SHALEK, R.J. Response of spontaneous mammary carcinoma of the C_3H mouse to X-irradiation given under conditions of local tissue anoxia. Journal of the National Cancer Institute, 31:497-509, 1963.

265. SUIT, H.D. & GALLAGHER, H.S. Intact tumour cells in irradiated tissue. Archives of Pathology, 78:648-651, 1964.

266. FIELD, S.B., JONES, T. & THOMLINSON, R.H. The relative effects of fast neutrons and X-rays on tumour and normal tissue in the rat. I - Single doses. British Journal of Radiology, 40:834-842, 1967.

267. DENEKAMP, J., HARRIS, S.R., MORRIS, C. & FIELD, S.B.

The response of a transplantable tumor to fractionated irradiation. II - Fast neutrons. Radiation Research, 68:93-103, 1976.

268. HOWLETT, J.F., THOMLINSON, R.H. & ALPER, T. A marked dependence of the comparative effectiveness of neutrons on tumour line, and its implications for clinical trials. British Journal of Radiology, 48:40-47, 1975.

269. ALPER, T. Aspects of neutron therapy based on an analysis of relationships between RBE and dose. British Journal of Radiology, 45:39-47, 1972.

270. BROWN, J.M. & HOWES, A.E. Comparison of tumour growth delay with cell survival. British Journal of Radiology, 47:509-510, 1974 (Correspondence).

271. SUIT, H.D., SEDLACEK, R., FAGUDES, L., GOITEIN, M. & ROTHMAN, J. Time distributions of recurrences of immunogenic and non-immunogenic tumours following local irradiation. Radiation Research, 73:251-266, 1978.

272. SUIT, H.D. Response to X-irradiation of a tumour recurring after a TCD_{95} radiation dose. Nature, 221: 996-997, 1966.

273. PEARSON, A.E.G. Serial irradiation of mouse tumours: Some changes in histology and cytology. British Journal of Cancer, 14:200-205, 1960.

274. SINCLAIR, W.K. X-ray induced heritable damage (small-colony formation) in cultured mammalian cells. Radiation Research, 21:584-611, 1964.

275. HERBERMAN, R.B. & HOLDEN, H.T. Natural cell-mediated immunity. Advances in Cancer Research, 27:305-377, 1978.

276. HERBERMAN, R.B. Cell-mediated immunity to tumour cells. Advances in Cancer Research, 19:207-263, 1974.

277. HERSH, E.M. & OPPENHEIM, J.J. Impaired in vitro lymphocyte transformation in Hodgkin's Disease. New England Journal of Medicine, 273:1006-1012, 1965.

278. WHITTAKER, M.G. & CLARK, C.G. Depressed lymphocyte function in carcinoma of the breast. British Journal of Surgery, 58:717-720, 1971.

279. WEISS, L. A pathobiologic overview of metastasis. Seminars in Oncology, 4:5-17, 1977.

280. MELLGREN, J. Quantitation of metastasis in experimental animals: In Fundamental Aspects of Metastasis. Ed. Weiss, L., North Holland, Amsterdam, pp.243-252, 1976.

281. WALLACE, A.C. The occurrence of metastasis in a group of related rat tumours. British Journal of Cancer, 10: 724-732, 1956.

282. KIM, U. Metastasizing mammary carcinoma in rats: Induction and study of their immunogenicity. Science, 167:72-74, 1971.

283. SHELDON, P.W., BEGG, A.C., FOWLER, J.F. & LANSLEY, I.F. The incidence of lung metastasis in C_3H mice after treatment of implanted solid tumours with X-rays or surgery. British Journal of Cancer, 30:342-348, 1974.

284. FINNEY, D.J. Statistical Method in Biological Assay, 2nd edn. Griffin, London, 1964.

285. SUIT, H.D., SHALEK, R.J. & WETTE, R. Radiation response of C_3H mouse mammary carcinoma evaluated in terms of cellular radiation sensitivity. In Cellular Radiation Biology, pp.514-530. Williams and Wilkins Co., Baltimore, 1965.