A CELLULAR ANALYSIS OF RESIDUAL INJURY IN SKIN

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in the Faculty of Medicine

by

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EDUCATION AND RESEARCH EXPERIENCE

I graduated in June, 1972 from Chung-Tai Medical Technology College. After 2 years service in the army hospital, I entered the University of Chinese Culture from where I graduated in June, 1977, with the degree of Bachelor of Science. During August, 1977 and August, 1978, I had a year's experience in the Radiology Department of the Cathay General Hospital. I studied in the Department of Medical Biophysics in the University of Dundee from October, 1978 to September, 1979, with the degree of Master of Science in Radiation Biophysics. After a year as a Research Assistant in the Veteran's General Hospital and two years as Junior Lecturer in the National Yang-Ming Medical College, I undertook research work at the Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester, from June, 1983 to August, 1986, under the supervision of Dr J H Hendry. It is on that work that this thesis is based.

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ABSTRACT

The effect of residual injury in the skin was investigated using skin reaction, macrocolony, skin healing and a new developed microcolony technique. The main studies included: (1) the development of the microcolony technique; (2) to study the effect of field size using single and fractionated doses; (3) the residual injury under investigation was (a) the threshold dose, (b) the effect of fractionated and repeated doses on residual injury, (c) the causes of the residual injury e.g. change in cell number or in radiosensitivity or both. The main findings were:

(1). The skin reaction (i.e. erythema or severe erythema) was found to be a good indicator of the time to detect microcolony growth in irradiated skin.

(2). A new microcolony technique has been developed using both mitotic arrest and labelling techniques.

(3). In terms of the Do value, there was a good correlation between microcolony (2.7±0.1 Gy) and healing (2.6±0.3 Gy) data. The correlation for the macrocolony data was less good because of the coalescence of microcolonies at lower doses. About 3% of the basal cells were clonogenic, and all microcolonies (> 32 cells) in epidermis developed into macrocolonies.

(4). There was a significant field-size effect when 1.3 cm^2 (2 cm tail) was compared with 3.2 cm^2 (4 cm tail) or 4.2 cm^2 (6 cm tail). This effect in terms of gross skin response was shown for the first time to be reflected by similar changes in the survival of colony-forming cells. Also, the field size effect may be caused by a difference in sensitivity of the cells. When the total dose was fractionated into small doses per fraction the field-size effect diminished.

(5). The threshold dose for residual injury was 16.25 Gy single dose, or 34 Gy in 12 fractionated doses. The combination of 15 mg/kg adriamycin with radiation did not enhance the degrees of residual injury.

(6). Using iso-effective first treatments with various fractionated doses there was a trend toward a higher survival after a test dose for groups which had a higher number of priming fractions. The difference in response between aged controls and the pretreated skin was due to a change in sensitivity.

(7). After three repeated tolerance doses a maximum of 10%, 22% and 27% of residual injury was observed respectively using microcolony, macrocolony and healing techniques. The sensitivity between the aged control skin and skin receiving 3 previous tolerance doses was not significantly different. There was a significant reduction of the numbers of microcolony-forming cells per unit area in epidermis which had recovered following priming doses.

SECTION ONE

INTRODUCTION

1.1. RADIATION EFFECTS ON CELLS

Proliferating cells can be killed by inhibiting their ability to divide. This failure to proliferate is termed 'reproductive death', which is defined as "the loss of a cells ability to undergo unlimited cell division". Consequently, cells capable of a limited number of post-irradiation divisions that produce sterile progeny are defined as killed even though morphologically, physiologically and biochemically they may appear normal (Coggle, 1983).

After very high doses (e.g. hundreds of gray), rapid cessation of cellular metabolism and cellular disintegration may occur. This also occurs after low doses in a few tissue types e.g. lymphocytes and salivary glands. This type of death is often termed 'interphase death'.

After irradiation some cells exhibit a change in appearance that involves rapid condensation of both nucleus and cytoplasm, cellular budding with membrane sealing to produce multiple fragments in which organelle integrity is maintained, and phagocytic engulfment of the fragments by neighbouring cells. This was called 'apoptosis' by Kerr et al (1972). In contrast to classical cell necrosis, apoptosis is not accompanied by an inflammatory response. Apoptosis occurs with a low frequency in untreated normal and tumour tissue and is augmented by various cytotoxic drugs (Searle et al, 1975). What is known of its mechanism (Wyllie et al, 1980) suggests a process of controlled (programmed) self-destruction.

Other types of damage after irradiation involve the 'slow growth' of cells, due to chromosome fragment loss which was found to relate

closely to impaired (slow growth) or inhibited colony-forming ability (Revell, 1983).

1.1.1. Cell Survival

(i). Methods of Measuring Survival

Over the years, many assay techniques have been developed for assessing the colony-forming ability (reproductive integrity) of cells *in vitro* and *in vivo*. These techniques have been described in detail and collated recently (Potten and Hendry, 1985). The principle in all of these is to achieve a sparse distribution of colony-forming cells, which are stimulated to divide many times to form discrete micro- or macroscopic foci of daughter cells. The foci are counted and the number reflects the original number of colony-forming cells. In practice the foci are more easily counted on a surface. This applies with colonies growing in culture dishes or flasks, with colonies growing on the surface of the spleen, lung, skin or intestine in mice, or with sections taken of intestine, growing cartilage, testis, kidney or tumours. The stimulus to divide is achieved *in vitro* by the supply of nutrients and the lack of any growth inhibitor.

Apart from techniques available with established cell lines of normal and malignant origins, many techniques are now being developed for growing colonies of normal cells and epidermal fibroblasts and keratinocytes (Potten and Hendry, 1985b). *In vivo*, cell depletion achieved by resection (e.g. partial hepatectomy) or by the action of a cytotoxic agent, induces proliferation in surviving cells. With several techniques *in vivo*, macrocolonies are produced which contain 10^6 or more cells and these can be counted by eye. This applies to colonies

produced on the skin, intestine and the spleen in mice. Estimates of the changes in the clonogenic fraction of these cells with increasing dose can be obtained from the number of colonies per unit area. With some microcolony techniques in vivo, whole structures are either ablated or regenerated, such as intestinal crypts or spermatogenic tubules. In this case the average number of cells (m) surviving per structure is calculated from the proportion of structures ablated (the mortality fraction M) using Poisson statistics, so that $m = \exp(-M)$.

(ii). Cell Survival Curves

The dose-dependence of cells to survive irradiation and form a colony of descendants, is described by a cell survival curve and associated mathematical formalisms (reviewed recently by Hendry (1985)). The basis of this is that lethal events in cells produced by irradiation occur at random. In this case a Poisson (exponential) distribution can be used to describe the range of numbers of lethal events per cell after a given dose, and the increase in the average number of lethal events per cell with increasing dose. At high doses, equal increments of dose produce approximately equal increases in the average number of lethal events per cell. Hence it is common practice to plot cell survival on a logarithmic scale versus dose on a linear scale. This transforms the sigmoid dose response curve using a linear plot (top panel, Figure 1.1) into a curve with a shoulder region and a terminal portion which either asymptotes to an exponential line or continues bending in this plot. Simplified versions of common mathematical equations which describe the shape of the curves in the middle and bottom panels in Figure 1.1 are as follows:

(a) $S = n. \exp(-D/D_0)$ (middle panel, Figure 1.1).

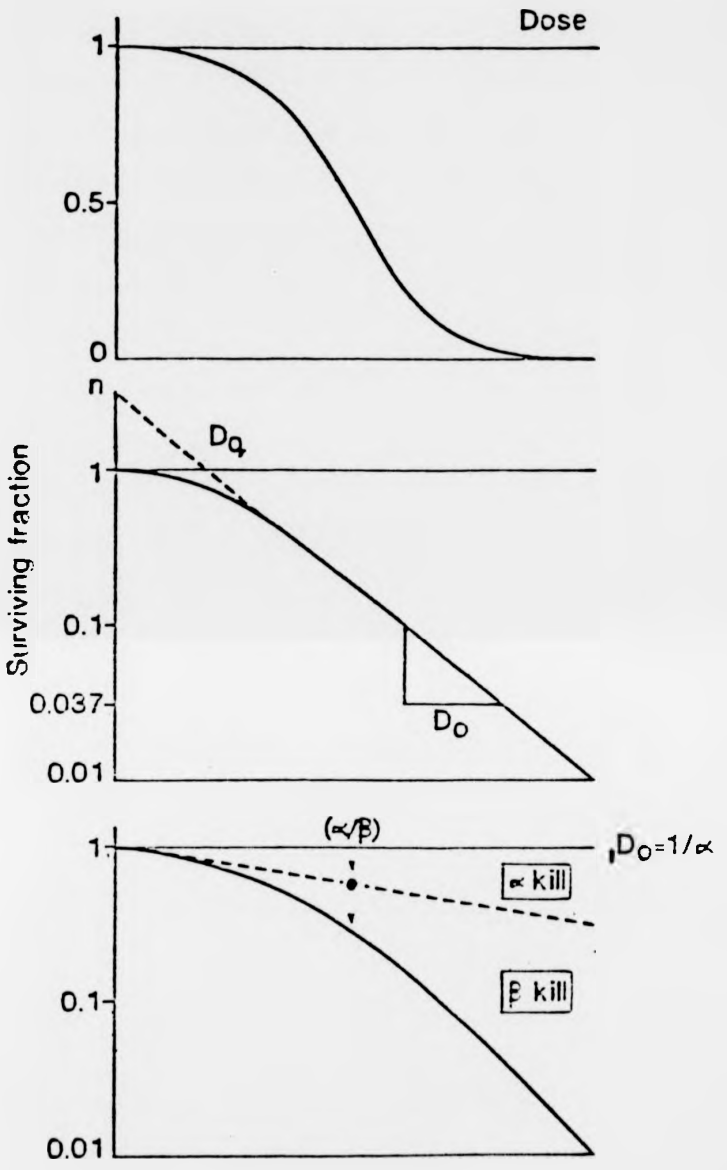


Figure 1.1: Presentation of survival curves (see text).

Where S = the surviving fraction of cells.

D_0 = the mean lethal dose. This is the dose which will just sterilise a typical cell which has already survived a high dose delivered immediately preceding the D_0 dose. In practical terms D_0 is the dose which will reduce survival to 37% of its preceding value on the exponential portion of the survival curve.

n = the extrapolation number. This is the point of extrapolation of the exponential portion of the survival curve on the survival axis. Another parameter associated with the above equation is the quasi-threshold dose Dq which equals (D_0.ln n).

(b) $S = \exp - (\alpha D + \beta D^2)$ (bottom panel, Figure 1.1).
This is shown as the linear quadratic equation for survival. The constant α describes sensitivity to low doses i.e. ($1/_1 D_0$) and β indicates that survival decreases with increasing dose more rapidly than exponentially. The ratio (α/β) is the dose at which equal cell depletion is caused by both α and β components.

Cell survival curves described by the above parameters have been obtained for many cell types, by plating cells *in vitro* or by *in situ* or transplantation methods *in vivo*.

1.2. FACTORS INFLUENCING THE RADIOBIOLOGICAL EFFECT

1.2.1. Cellular and Subcellular Repair

(a). Repair of Sublethal Damage

Cell survival curves for mammalian cells following sparsely ionizing radiation, rarely show a simple exponential relationship, but usually possess an initial shouldered region. The presence of this shoulder

implies that damage must be accumulated before a lethal effect is produced. A number of models have been devised to explain the shoulder region. Some models propose that there is one or a number of critical sites which must be damaged within a cell before it loses its reproductive capacity (e.g. Lea, 1946; Kellerer and Rossi, 1972; Chadwick and Leenhouts, 1973). Other models propose that as damage is produced it is repaired by various mechanisms which become gradually saturated with increasing dose (Powers, 1962; Alper, 1979).

If a cell is not damaged in all of its critical sites, or alternatively has not had its repair mechanisms saturated, then it is said to have sub-lethal damage (SLD); that is, it is damaged but not killed. Given time the cell may be able to repair the effects of SLD and completely recover. The SLD repair phenomenon is a time dependent process and is completed within about 2 hours *in vitro* (Elkind *et al*, 1965) and about 4 hours *in vivo* (e.g. Chen and Withers, 1972; Hendry and Potten, 1974).

The evidence for the repair of sublethal damage has come from "split-dose" experiments pioneered by Elkind and Sutton (1960) and is referred to as "Elkind repair". Figure 1.2 shows a typical result that might be expected from such an experiment. The recovery of the cells is indicated by the reappearance of the shoulder of the survival curve between the first and second doses. This situation, though, is somewhat idealised, and is only seen if the cells exhibit to the second dose the same sensitivity as seen after the first dose. In a cell population, the phase of the cell cycle at the time of irradiation can influence the response and this should be taken into account when interpreting results, because a change from a resistant to a sensitive phase may

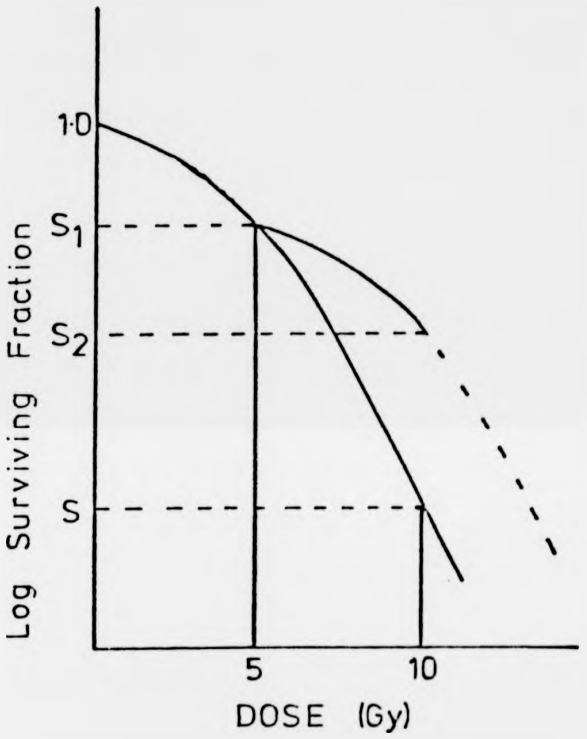


Figure 1.2: The effect of dividing a dose of 10 Gy into two fractions of 5Gy, separated by an interval of time sufficient for repair of SLD.

offset any sparing due to fractionation. In most cases there is a good correlation between the extent of repair of sublethal damage which takes place and the magnitude of the initial shoulder on the survival curve.

The split dose studies of the type pioneered by Elkind and Sutton are possible in only a limited number of systems, mainly those *in vitro*. However, in many situations, it is possible to measure Dq by matching single and split doses which produce equivalent biological damage. If two dose fractions are delivered, spaced by a time interval sufficient for the repair of sublethal damage to take place (assuming no change in the sensitivity of the cells), a total dose D_2 is required to produce the same biological damage that a single dose D_1 would produce. The quantity D_2-D_1 is equal to Dq, with a knowledge of Dq and D_0 the value of n can be estimated using the relationship $Dq = D_0 \log_e n$. Typical values of Dq for normal tissues estimated in this way are; mouse epidermal cells, 3.75 Gy (Withers, 1967) and 5.7 Gy (Emery *et al*, 1970); mouse jejunum crypt stem cells, 3.3 Gy (Withers and Elkind, 1970).

(b). Potentially Lethal Damage

Another form of repair is repair of potentially lethal damage (PLD). This is damage which can be lethal, but is capable of being repaired depending upon post-irradiation incubation conditions. If time is allowed between radiation and cell division, repair of PLD can take place. The first studies were performed with mammalian cells *in vitro* (Phillips and Tolmach, 1966). Cell survival was increased, and PLD repaired, if cells were incubated in balanced salt solution (instead of full growth medium) for several hours post-irradiation to inhibit cell division. Other studies of the repair of PLD have been performed using

density-inhibited plateau-phase cultures. Cell survival was enhanced considerably if irradiated cells were allowed to remain in the density-inhibited state for 6 to 12 hours post-irradiation before being subcultured and assayed for colony-forming ability (Hahn and Little, 1972).

This type of recovery is dose-dependent (the higher the dose the more the recovery), and the time scale is similar to that for SLD repair. Repair of PLD has been shown to occur *in vivo* in tumour systems. In this case, repair is observed by significantly enhanced cell survival if several hours are allowed to elapse between irradiation of the tumour *in situ* and removal of the cells from the host to assess their reproductive integrity. This was first shown by Little *et al* (1973) for the EMT6 mouse tumour, and for other tumours e.g. Lewis lung carcinoma (Shipley *et al*, 1975).

When cells are resting (e.g. in G_0) in normal tissues that proliferate only very slowly, PLD repair can occur. This has been reported in a number of such tissues. For example, Reinhold and Buisman (1975) studied rat capillary endothelium and demonstrated that the D_0 value was increased from 1.68 Gy to 2.73 Gy when the stimulus to proliferate was delayed by 32 days. Gould and Clifton (1979) reported this type of repair *in vivo* in irradiated rat mammary gland cells. The increased survival was characterised by an increase in the extrapolation number and Dq, but there was no change in D_0. A slope change is usually characteristic of repair of PLD *in vitro* and in tumour cells *in vivo* (Hahn and Little, 1972). Gould and Clifton (1979) suggested that the type of repair they had observed was a special form of PLD repair which

they termed in situ repair. PLD repair has also been reported for rat thyroid cells (Mulcahy et al, 1980), rat hepatocytes (Jirtle et al, 1982) and mouse hepatocytes (Fisher, 1985) using similar transplantation techniques.

(c). Slow Repair

Irradiation of the whole thorax of mice leads to death between 40 and 180 days post-irradiation from radiation pneumonitis (Phillips and Margolis, 1972). When two (or more) doses of X-rays were given with intervals between doses of one hour up to five weeks, Elkind repair of sublethal damage occurred in the first few hours, followed by a slower repair with a half time of about 10 days (Field and Hornsey, 1977). The possibility of this later repair phase being due to repopulation has been investigated by Coultas et al (1981). They irradiated mice with 10 Gy X-rays to both lungs and then injected with ^{3}H-thymidine at intervals of eight hours. Injections were started at a variety of times between 1 day and 5 weeks after irradiation. Cell labelling in the alveolar areas was examined by autoradiography. Type II pneumocytes have been suggested as a possible critical target in radiation damage to lung (Coultas et al, 1981). However, labelling of type II pneumocytes (and other alveolar cells including capillary endothelial cells) did not rise above normal levels until about 10 days post-irradiation, by which time half the slow repair had already taken place.

There is no evidence that the slow repair phenomenon is due to repopulation, and it has been suggested that this process could be an intracellular biochemical repair process like Elkind repair (Hornsey and Field, 1980). There is evidence that a "slow repair" phenomenon occurs also after single doses. Stevenson and Curtis (1961) found that there

was a decline in chromosome aberration frequencies in liver cells over a period of many months after acute X-irradiation of mice; but no such decline was observed after exposure to neutrons (Curtis, 1967). If the loss of aberrations was due to cell elimination, at aborted mitosis, then this differential would not have been observed. They therefore proposed that the reduction of damage after X-rays resulted from a slow repair process, and that the neutron damage could not be repaired in this way (Curtis, 1967).

Similar observations to these have been made in rat hepatocytes (Tates et al, 1982), and in rat thyroids (Scott et al, 1983) exposed to X-rays or neutrons. Whether this repair mechanism is the same as that observed in lung is not known.

1.2.2. Fractionation

In the radiotherapy of malignant disease, the doses of radiation used (30-60 Gy) are administered as a series of small fractions (of the order of 2-3 Gy).

One important factor in fractionating radiation to produce a given response is the size of the dose per fraction. If a large single dose (e.g. D_1 in Figure 1.3) gives a response S_1, then if this total dose is administered over a number of smaller equal fractions, it can be seen that the same response is not obtained (now it is S_2 or S_3) due to repair between fractions. Alternatively to obtain a similar response (S_1) , a greater total dose (D_2 or D_3) is required. From both viewpoints however, it can be seen that an important factor is the size of the dose per fraction. In general, the smaller the dose per fraction

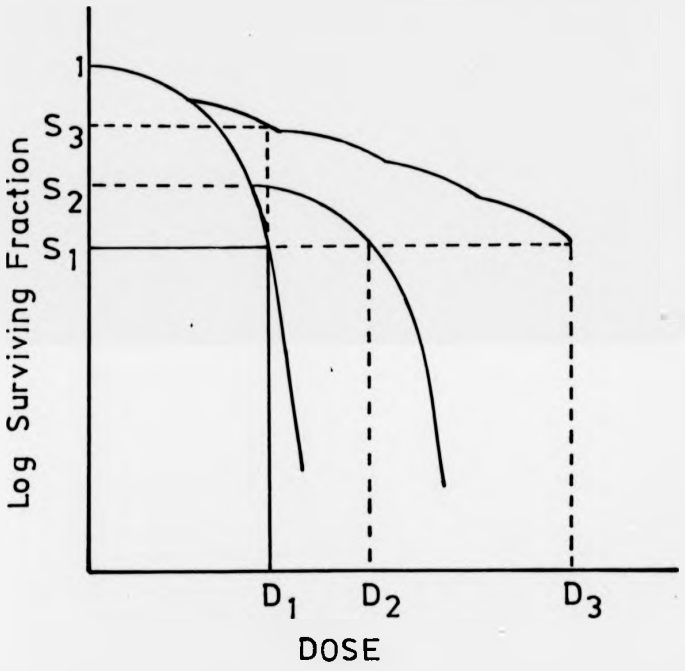


Figure 1.3: Dependence of the size of the dose per fraction on the measured response, or the total dose required to obtain a given response.

then for the same total dose less effect will be seen, or for the same effect, a greater total dose will be required.

Another important factor is the shape of the survival curve particularly at lower doses. If the survival curve has a broader more curved shoulder then this will mean that higher total doses will be required for smaller fractions than if the curve has a more log-linear shape. This has been suggested to apply to late responding in contrast to early responding tissues (Thames et al, 1982).

1.2.3. Influence of the Mitotic Cycle on Sensitivity

To observe the radiation response of cells at specific stages of the cycle it is necessary to use a technique that will yield a great majority of the population in the same mitotic stage. Various methods have been used to achieve this synchrony including a mitotic harvesting technique used in vitro or by treating the cells with agents such as hydroxyurea that kills cells in S phase and creates a block at the G_1/S border.

Using these techniques complete survival curves at a number of discrete points during the cell cycle have been measured. This has been done for cell lines in vitro and it has been shown that in general cells are most sensitive in M phase and most resistant in late S phase (see Figure 1.4). The hydroxyurea block technique has been employed in vivo, to obtain a synchronous population of e.g. mouse jejunal crypt cells. Irradiation of these synchronised cells indicated that their radiosensitivity varied substantially with the phase of the cell cycle, and that the pattern of the response was similar to that observed for cells in vitro, with a maximum radioresistance of cells in late S (Withers

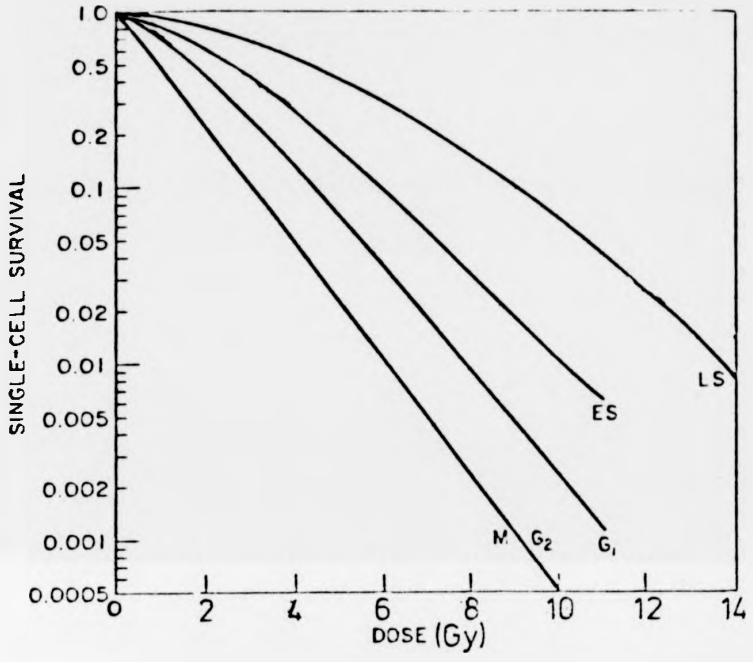


Figure 1.4: Cell survival curves for Chinese hamster cells at various stages of the cell cycle. For cells in mitosis, the survival curve is steep with no shoulder. For cells in late S, the curve is less steep and has a large initial shoulder.
(From Sinclair, 1968).

et al, 1974).

In measurements of hair follicle survival, it has been shown that the growing follicles are more sensitive than resting follicles (Griem et al, 1973).

1.3. DEVELOPMENT OF THE RADIATION RESPONSE: RELATION OF PROLIFERATIVE ORGANISATION TO RADIATION RESPONSES

Most organs and tissues in the adult mammalian organism depend on replenishment of differentiated cells to maintain their integrity and function. These renewal systems are in a steady state equilibrium involving cell production, differentiation and cell death. These processes are usually regulated by complex homeostatic control mechanisms.

The translation of the radiation insult into functional impairment depends on a number of factors. These include the radiosensitivity of proliferative cells, the kinetics of cell renewal, the functional reserve of the tissue, the level of physiological demand to which the tissue is subjected and the way in which the tissue is organised, particularly the distribution of the proliferative and tissue-specific functions among its cells.

1.3.1. Proliferative Organisation

Tissue organisation as an important factor in determining the development of a response of a tissue was considered a number of years ago (Lajtha and Oliver, 1962; Gilbert and Lajtha, 1965). More recently this has been further developed and normal tissues have been classified

into two distinct organisational categories (Michalowski, 1981; Wheldon et al, 1982). The two patterns of proliferative organisation have been termed: (a) hierarchical or "type H" tissue and (b) flexible or "type F" tissue.

(a). Type H Tissue

A diagrammatic representation of a type H tissue is given in Figure 1.5. (a). In type H tissues clonogenic proliferation and tissue-specific function are mutually exclusive cellular abilities. In such tissues, the ability to self-replicate indefinitely (i.e. clonogenic ability) is restricted to a small, sub-population of stem cells, which are not capable of the function usually associated with the mature differentiated cells of that tissue. The functionally competent and irreversibly post-mitotic mature cells are derived from the stem cells. Mature cells develop by a process of differentiation during which intermediate "transit cells" progressively lose their capacity for proliferation, as their degree of maturity increases.

Mature cells are lost at a rate characteristic of the tissue, as a result of cellular senescence, and their numbers are maintained by a continual replacement by stem cells into the differentiation pathway. Stem cell number is maintained by self-replication of stem cells themselves. When they are not proliferating the stem cells rest in a quiescent state (G_0). This hierarchical scheme applies to a number of tissues for example haematopoietic tissue, seminiferous epithelium, alimentary canal and epidermis.

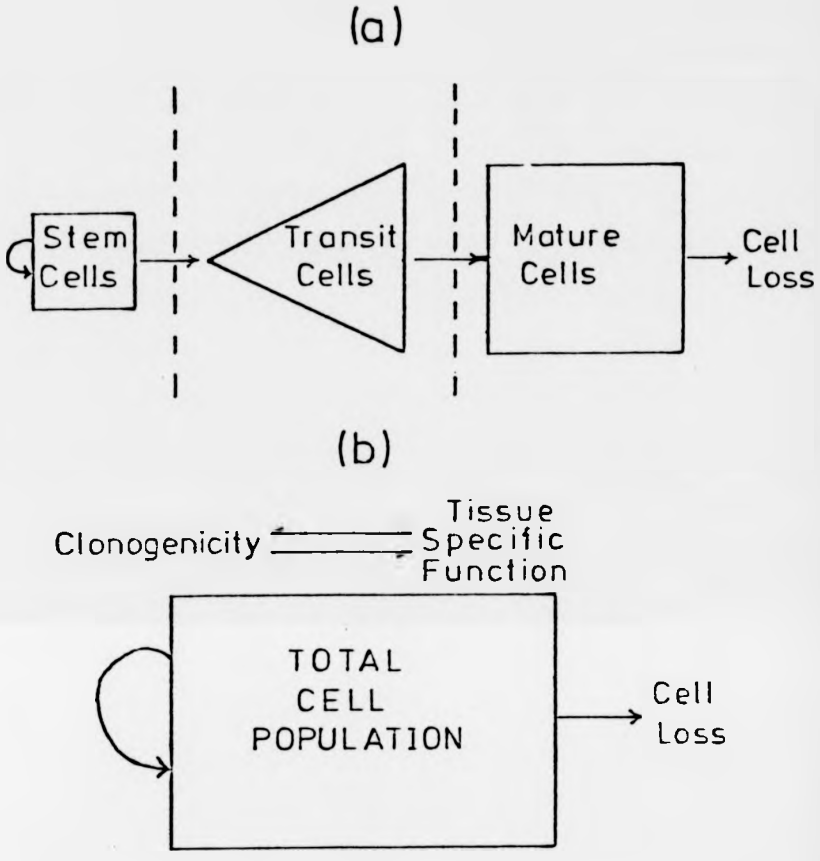


Figure 1.5: Two types of proposed proliferative organisation. (a) Hierarchical, type H, population in which a small number of clonogenic stem-cells provide a transit population which ultimately differentiate into mature functional cells. (b) Flexible, type F, population in which all cells are capable of either function or proliferation. (After Wheldon et al, 1982).

(b). Type F Tissue

Other tissues do not appear to conform to the above type-H model. For example, liver cells are usually engaged in tissue specific functions, but are capable of proliferation if required. Following partial hepatectomy, initiation of near-synchronous DNA synthesis occurs in a high proportion of the hepatocytes, which then proliferate until liver regeneration is complete (Goss, 1978). This burst of proliferative activity has been shown to result from the reactivation of previously quiescent hepatocytes (Lajtha, 1979). Wheldon et al (1982) suggested that this type of proliferative recruitment of G_0 hepatocytes also occurs slowly under normal circumstances to compensate for cell loss due to cellular senescence. Thus they postulated that some tissues have functionally competent cells which are capable of being stimulated from a quiescent G_0 state to undergo proliferation if required (e.g. after cell depletion) and have termed these type F (flexible) tissues (see Figure 1.5 (b)). Such tissues, besides liver, may be endothelial and neuroglia populations, and in the skin, may be endothelial and other connective tissues. It has also been suggested (Wheldon et al, 1982), that it is possible that most of the cells in type F tissues are capable of indefinite proliferation (e.g. would form colonies), or alternatively may be capable of only a finite number of divisions.

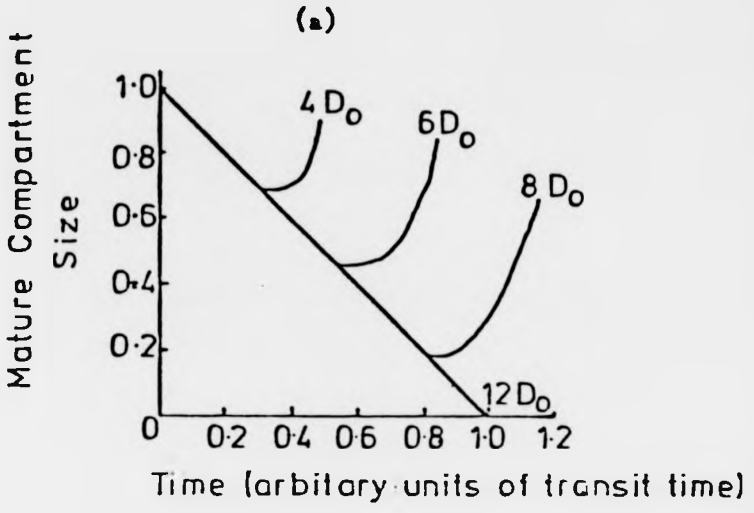
1.3.2. Response to Irradiation of Type H or F Tissues

(a). Type H Tissue

Figure 1.6 (a) show the hypothetical response of type H tissues to irradiation. They usually respond rapidly to radiation and in a very characteristic way. This is related to the resistance of most types of non-dividing mature cells, and the sensitivity of the stem cells when

they are stimulated into proliferation. Following high doses of irradiation, there is a continued loss of mature cells at the normal rate but there is little cell production because the stem-cells will be sterilised and they and the existing transit cells may undergo only one or two divisions. Hence the mature cell population will decline at a rate characteristic of each particular tissue. The decline will stimulate proliferation in the stem-cell population in an attempt to compensate for the reduction in mature cells, and this induced proliferation will express the lethal injury in the stem-cell population. The decline in mature cells will continue until a critical level is reached which results in tissue failure. It has been shown that the time to fully denude the epidermis (15 to 29 days for different sites) and the gastrointestinal mucosa (3 days) in mice, corresponds to the transit time through the amplifying and mature cell populations (Potten, 1981). Also, the times of death of mice from the gastrointestinal syndrome (4 to 7 days) and the bone marrow syndrome (10 to 30 days) are correlated with the transit time through the maturing cell populations. A clear example of the relation of cell kinetics to time of death was observed for gut death in mice, with a prolonged latency period as expected for germ-free mice where the transit time is longer (Tsubouchi and Matsuyawa, 1973). Also, it should be noted that the time to full denudation is independent of dose in the high dose range, where all stem cells are sterilised.

After low doses, where sufficient of the stem-cells survive, there will be regeneration of the mature cell population. The time to full recovry is dose dependent i.e. dependent on the level of survival of the stem cells. The rate of recovery of the mature cells depends on the cycle



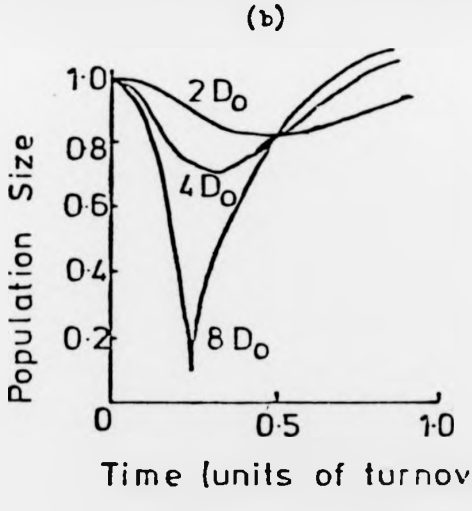


Figure 1.6: Hypothetical response to radiation insult of (a) type H or (b) type F populations. (Taken from Wheldon et al, 1982).

time of the progenitor cells and the rate of differentiation into the amplifying cell populations. The doubling time of the stem-cells during regeneration will be ~~less~~ more than the cycle time because of the differentiation of stem cells at each division.

(b). Type F Tissue

Figure 1.6 (b) shows the pattern of depletion and of regeneration of a type F tissue exposed to different doses of radiation. It has been assumed, in this case, that (a) all cells are capable of proliferation and function; (b) the proportion of proliferating cells is regulated by a homeostatic control mechanism; (c) irradiated cells have a probability of retaining clonogenicity which is an exponential function of dose, and (d) they do not necessarily die at the first mitosis but may complete a number of divisions.

Unlike a type H tissue, the rate of cellular depletion of a heavily irradiated type F tissue could accelerate with time. As the first radiation-sterilised cells attempt division and die an increased number of quiescent cells may be called into proliferation, so precipitating the death of more sterilised cells, calling still more cells into cycle. This increasing rate of depopulation would occur more quickly after higher doses as initially more cells are sterilised. Thus homeostatic controls would be expected to act more swiftly after these doses.

The pattern of regeneration of a type F population also differs from that of the type H. The model suggests that type F tissues may recover more rapidly from large doses of radiation than from lesser doses (Figure 1.6(b)). This is because the avalanche would proceed more

rapidly after higher doses, so that heavily irradiated tissues may fully express damage and be repopulated by clonogenically competent cells before less heavily irradiated tissues have reached the nadir of the depletion curve. However, if only a minority of cells can self-replicate indefinitely, or the cells had only a limited capacity for renewal, then the repopulation would occur more slowly after higher doses.

Another important influence on the development of the response of a tissue to radiation is the kinetics of those cells responsible for replacement of mature cells at the end of their metabolic life. Thus, these would be the stem cells in type H tissues, and potentially all cells in type F tissues. Generally the type H model is one which decribes tissues with a high turnover of cells due to the continual loss of differentiated cells. The type F model, though, describes a population which because cell loss due to senescence is low, has a much lower rate of cell division.

The consequence of this difference in kinetics is manifest in the speed at which the radiation response develops. The rapid renewal systems (i.e. type H) are generally the first to show effects of irradiation, because replacement of differentiated cells within a relatively short time is required.

In type F tissues, though, because of the relatively low mitotic rate, cell death occurs only gradually. Thus a long period of time may elapse before sufficient damage accumulates for functional impairment to be observed. The development of the radiation response may be influenced

therefore, not only by the structure of the irradiated population of cells, but also by the kinetics of the population at risk. The type F responses are exemplified by the occurrence of radiation pneumonitis, following lung irradiation of mice. The time of appearance is dose-dependent, occurring a few months following irradiation (Travis et al, 1980; Collis and Steel, 1982).

1.4. DOSE RESPONSE CURVES FOR TISSUES

1.4.1. Acute Effects

Dose response curves for these acute responding tissues can be obtained in terms of the severity of response versus dose, for assessments made at given times after irradiation. These curves generally show a threshold region of dose where injury is minimal and unimportant regarding the continued function of the tissue, followed by an increasing severity of injury after higher doses. This pattern is described as a non-stochastic effect. This contrasts with a stochastic effect, where the incidence, but not the severity, increases with increasing dose. For example, leukaemia is a stochastic effect arising from a single transformed cell. The endpoint is the same at all doses, and of course the incidence increases with increasing dose (at low doses). On the other hand, skin desquamation is a non-stochastic effect, arising from the combination of the same effect (death) occurring in many cells. The numbers of affected cells increases with dose, and hence so does the severity of the desquamation. The distinction between stochastic and non-stochastic effects is shown diagrammatically in Figure 1.7.

Alternatively, dose-response curves can be presented using the

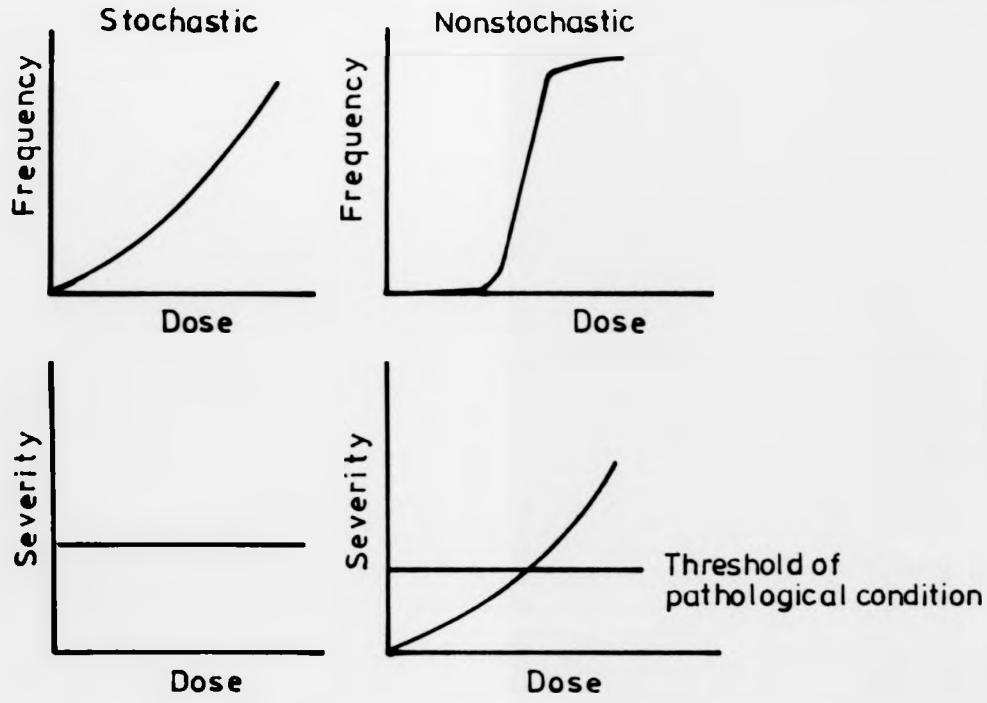


Figure 1.7: Characteristic differences in dose-effect curves between stochastic and non-stochastic effects. Non-stochastic effects vary in severity as well as in frequency with the dose. The upper and lower figures illustrate how the frequency and severity of a non-stochastic effect defined as a pathological condition, increases as a function of dose in a population of individuals of varying susceptibilities.

proportion of tissues where the injury is less than a specified level. This level could be for example death following irradiation of a critical organ e.g. bone marrow, gut, kidney etc. In this case the dose-response curve is S-shaped or sigmoid on a linear plot, showing a threshold region, then a range of dose when the proportion of unaffected samples declines rapidly from 100% and tends to 0% after high doses when all samples are affected (Figure 1.7). In the ideal case, the sigmoid curve could have "sharp corners", because a dose would be reached when all samples would be depleted below the critical level, and the curve would drop down to zero promptly at this dose. However, this never happens in practice. The reason is that there is always some heterogeneity which smoothes out the curve to some degree.

There are various reasons for this heterogeneity (Hendry and Moore, 1984). First, it can be introduced by the cytotoxic agent itself, in other words when all cells do not receive the same dose. This is indeed the situation with radiation where lethal events occur at random (see Section 1.1.1 on cell survival curves). Hence if the specified level of injury is directly related to the number of surviving stem cells in a tissue, and each such cell contributes equally and indepenently to the repopulation of the tissue, then the steepest dose-response curve that will be seen will be determined directly by D_0, the (inverse) sensitivity of the "target" cells. Secondly, if their contribution to repopulation and rescue is not independent, but it increases with an increase in the surviving number, then the chance of rescue will decline faster with increasing dose. This would steepen the dose-response curve. Thirdly, if there is a large variation between the initial samples in either their content of stem cells, or in stem cell sensitivity, the composite dose-response curve will be shallower.

Fourthly, if there are 2 potential target-cell populations e.g. parenchymal and vascular, which are differentially sensitive and which are each depleted to critical level over slightly different ranges of dose, this could increase the steepness of the dose-response curve (Hendry and Moore, 1984).

In experimental systems where variations between samples can be minimised, it has been shown that the D_0 value for the target cells measured using colony techniques, is the major determinant of the steepness of the dose-response curve (Hendry and Moore, 1986). With human material, where there is more variation between samples, the dose-response curve becomes shallower depending on the amount of initial variation.

Dose response curves for tumours and for normal tissues form the basis to optimal treatment in radiotherapy, where patients are treated to tolerance and cure rates are determined. The steepness of the dose response curve for normal tissues determines the accuracy with which doses must be prescribed. The position and steepness of the curve for tumours determine first the cure rate at a tolerance dose, and secondly the degrees of benefit which can be achieved by changing the doses slightly or by using sensitising agents.

1.4.2. Late Responding Tissues and their Influence on Hierarchical Tissues

Acute effects in hierarchical tissues can be modified by stromal responses e.g. Peel et al (1982) (Thulium versus Strontium) for skin, the tumour bed effect (TBE) for tumours. Long term effects in hierarchical tissues may be mediated by stromal responses e.g. long term marrow aplasia. Late responding is that the injury is progressive over a long period of time, and hence the shape of the dose-response curve may depend on the time at which the assessment is made. Also, there are considerations of the interpretation of the steepness of the dose-incidence curve which are not yet resolved. These are as follows:

(1). Structural units of renewing tissues e.g. intestinal crypts, testicular tubules, can be repopulated by single surviving stem cell. However, in such units constituting late-responding tissues e.g. lung alveoli, thyroid follicles, it may be the case that several surviving cells are necessary to reconstitute the unit because of the limited division potential of these cell types. This would have the effect of steepening the dose-response curve.

(2). In the case of the bone marrow, the testis, and the gut, all rapidly renewing tissues and all responding rapidly to irradiation, mature cells are fed into a common pool or onto a common surface to provide the functional component of the tissue. Hence it is likely that the contribution of the respective progenitor cells to tissue rescue is independent of their position in the tissue. In contrast, in a linear tissue like the spinal cord, it is likely that if a middle segment is inactivated, survival of other segments will be ineffective in maintaining cord function and paralysis could occur. This effect would steepen the dose-response curve, particularly for large field sizes

where many such segments are irradiated. This effect may also apply to acute necrosis in large area of skin, and to late strictures in the intestine.

1.5. FRACTIONATION EFFECTS

In terms of normal tissue tolerance in man, the overall treatment time (T) and number of fractions (N) are considered as important parameters in radiotherapy. On the basis of Cohen's (1966) publication suggested separating the factors for overall treatment time T (days) and number of fractions N, Ellis (1969) presented an empirical formula based on experience with acute skin reactions:

$$\text{total dose} = (\text{NSD})\ N^{0.24}\ T^{0.11}$$

Where NSD is known as the nominal standard dose: its "units" are designated as the ret. The NSD is a "constant" referring to the effective maximum single dose which a particular normal tissue can tolerate in radiotherapy. It will depend, therefore, on the field size and to some degree on the judgement of the physician. It is not considered reasonable to extrapolate the Ellis NSD formula to less than N = 4 or T = 5 and it is questionable whether or not the formula holds for very large values of N and T. In addition, the exponent of N and T will vary to some extent from tissue to tissue. The limitation of the concept of NSD is that the formula is applicable only at the level of normal tissue "tolerance". However, various modifications have been made to make it possible to apply the formula to levels of injury lower than tolerance. One of these is the concept of "Partial tolerance" (PT).

$$PT = \text{NSD}.\ (N/N_{TOL})$$

Where N_{TOL} is the number of fractions to give full tolerance and N is

the number actually given. For convenience in radiotherapy, Orton and Ellis (1973) introduced TDF (time dose factor) which is proportional to partial tolerance, but is independent of specific values of NSD.

$$TDF_f = N\ (d\ 100)^{1.538}\ (T/N)^{-0.169}\ 10^{-3}$$

Where d is the dose per fraction in Gy.

An alternative generalization of the NSD formula is that of the Cumulative Radiation Effect (CRE) (Kirk et al, 1971), where

$$CRE_f = (\frac{T}{N})^{-0.11}\ dN^{0.65}$$

Where N is the number of fractions given and T is the overall time for those fractions including the first and last treatment days. CRE is applicable to effects less than tolerance.

The Ellis formula predicts that total doses will always increase with increasing numbers of fractions, and there is evidence that this is not the case. The linear-quadratic formulation has been used to a large extent recently and this predicts that iso-effective doses will tend to a plateau at very low doses per fraction.

$$\text{Iso-effect} = -\ln S = n\ (\alpha d + \beta d^2)$$

When n fractions are given of dose d, and α and β are constants. α usually is several times β so that at low doses the α term is dominant and the total dose (= n x d) for the iso-effect tends to a constant value. Earlier studies with skin demonstrated that no further sparing was achieved when the dose per fraction decreased below 3 Gy (Dutreix et al, 1973), but other data showed further sparing down to 1.9 Gy per fraction (Douglas and Fowler, 1976). Recent studies have shown that the fractionation data for skin fit the α/β formulation down to 0.75 Gy per fraction (Joiner et al, 1986).

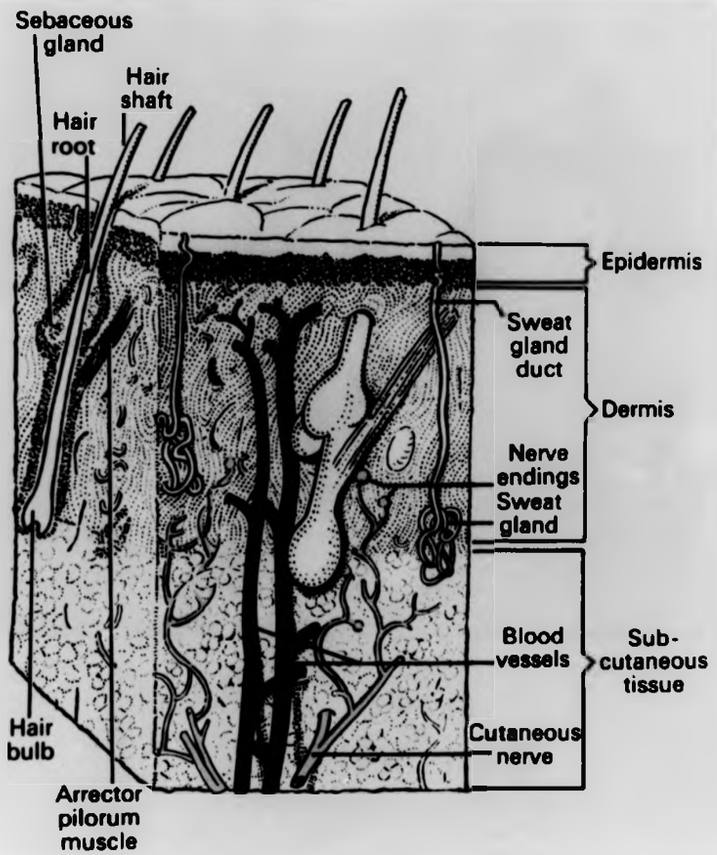
In general, the α/β ratio for the acute effects of skin (i.e. skin desquamation) in most species (e.g. mouse, rat, pig and human) is between 8-14 Gy. For late effects in pig skin (late contraction) the α/β ratio is between 2 and 5 Gy (reviewed by Fowler, 1984). For telangiectasia in pig and human skin the α/β ratio is about 3 Gy (e.g. Turesson and Notter, 1986).

1.6. SKIN STRUCTURE AND FUNCTION

The skin completely covers the body, is continuous with the membranes lining the orifices of the body and is one of the largest and most active organs. Its functions include, protection of the deeper organs from injury and the invasion of micro-organisms, thermal regulation, excretion, and it serves as a sensory organ and as a barrier to water loss. Skin is essentially made up of two components, the epidermis and the dermis, which are separated by a basement membrane (Plate 1.1). The epidermis can be sub-divided into several layers corresponding to the maturity of the functional cells. Most of its cells undergo a progressive sequence from active proliferation to specialisation and finally to dead keratin. The epidermis, therefore, can be regarded as a typical example of self renewing tissue.

The dermis has a less obvious organisation but apart from structural support it contains a capillary bed, which does not cross the basement membrane, upon which the epidermis is dependent for nutrients. Under the dermis is another layer comprising connective tissue. The hypodermis contains blood vessels and it often modified into a fatty tissue which serves both as a supply of energy and as a thermal regulator.

Plate 1.1 : The main structures in the skin.



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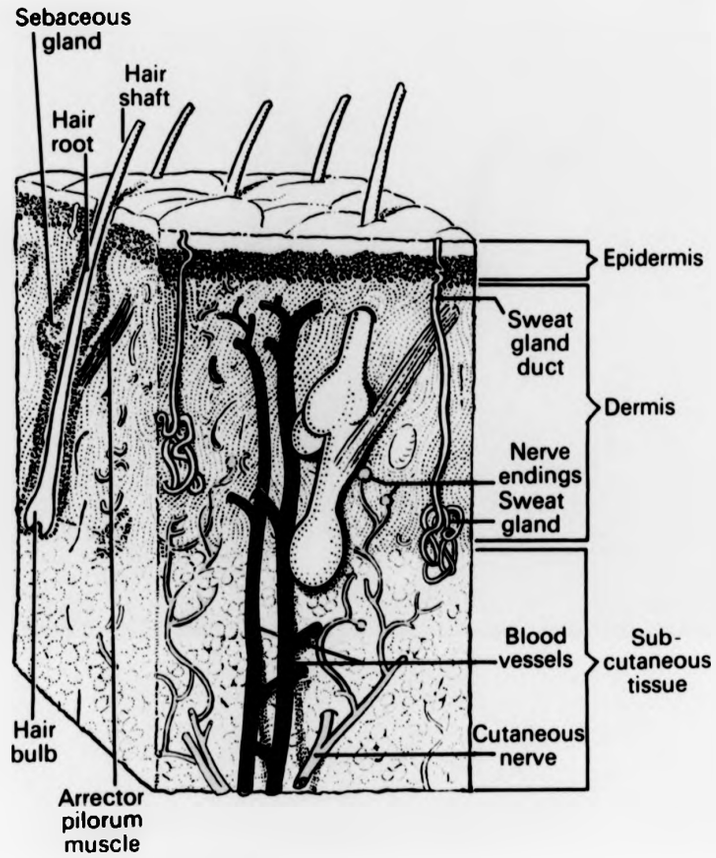


1.6.1. The Structure of Epidermis

The epidermis is the most superficial part of the skin and is composed of the basal layer, stratum spinosum, stratum granulosum, stratum lucidium and the stratum corneum.

Basal Layer

This is a single layer of cuboidal cells and is sometimes called the stratum germinativum because in normal epidermis cell proliferation occurs almost exclusively in this layer. In hyper-proliferative states such as psoriasis, cell proliferation can also occur in the stratum spinosum. The basal cells are attached to the basement membrane by hemidesmosomes, and to suprabasal cells by desmosomes. Cell to cell contact in the basal layer also occurs by extensive interdigitation of microvilli (see Skerrow, 1978 for review). The basal keratinocytes have elongated, basophilic nuclei and prominent nucleoli. Although the basal layer is the least differentiated in the tissue, the cytoplasm of the basal keratinocytes contains numerous tonofilaments. The basal cells appear to be morphologically similar, however, they are not as homogeneous as at first appeared. Christophers (1971) using fluorescein-isothiocyanate (FITC) staining of cryostat-sections of skin demonstrated that the majority of the basal cells did not stain. However, at irregular intervals cells which did stain were found, these had a thin cytoplasmic connection to the basement membrane with the bulk of the cell reaching into the stratum spinosum. As all the suprabasal cells, especially the stratum corneum stain with FITC, this was interpreted that these cells were in transit from one layer to the next and were maturing towards terminal differentiation.

Stratum Spinosum

With the transition from basal layer to the stratum spinosum, keratinocytes become much larger, showing a three-fold increase in volume. The cell also becomes flattened and overlies several basal cells. This is particularly well demonstrated in mouse epidermis (Allen and Potten, 1974) where the stratum spinosum is typically one or two cells thick. Human epidermis has a spinous layer considerably thicker than in mice. The spinous cells usually show considerably more desmosomal attachments than basal cells. The accumulation of tonofilaments increases in the spinous layer and they are assembled into tonofibrils. The membrane is also undergoing changes, binding of concanavalin A is weak in the basal layer but increases suprabasally (Nieland, 1973). In the upper layers of the spinosum, membrane coating granules become apparent (Matoltsy and Parakkal, 1965).

Stratum Granulosum

This layer is one or two cells thick in the mouse and is characterized by the appearance of keratohyalin granules in the cytoplasm. The origin and function of these granules is unclear. They contain sulphur-rich protein (Matoltsy and Matoltsy, 1970) and are associated with ribosomes and tonofilaments. It was suggested that the keratohyalin is intimately involved in keratinization (Brody, 1964) and that it plays an important role in the formation of the inter-filamentous matrix in fully mature squames (Matoltsy and Matoltsy, 1972).

Stratum Lucidum

This is the layer between the lowest stratum corneum and the uppermost granulosum. All cellular organelles disappear and the cell is filled with tonofilaments and keratohyalin (Brody, 1964).

Stratum Corneum

This is a superficial layer of the epidermis and consists of large flattened cells whose cytoplasm has been almost entirely replaced by keratin. Nuclear fragments can occasionally be observed in the lowest levels. In the mouse the stratum corneum is about 5-10 cells thick, in man this is increased to 10-25 layers. Overall the thickness of skin in animals with a thick pelage such as mice, rats and rabbits is thinner than that of less hairy animals such a man, pig and guinea-pig (Montagna, 1971; Winter, 1972; Zotterman, 1976). Desmosomes become less apparent in the stratum corneum and the membrane becomes thickened.

1.6.2. Characteristics of Mouse Tail Epidermis

As discussed by Potten et al (1983), the epidermis on rodent tails is characteristically much thicker (more strata) than in skin from the back. Also, tail skin has a very characteristic pattern of hair follicle distribution. The follicles, which are grouped in threes, are situated in such a way that the indentation of the epidermis leading to the follicles results in the formation of mounds of cornified cells or 'scales' between the follicles. Thus it can be subdivided into the perifollicular region (interscale) and interfollicular (scale) regions. The pattern of keratinisation differs in these two regions such that the interscale region has a granular layer and keratin with a lower cystine content and a lower protein-bound sulphydryl and disulphide levels. The scale region lacks a granular layer and the keratin has a higher cystine and protein-bound sulphydryl and disulphide content. However, the retention of nuclei in the granular and corneal layers that is a frequent feature of parakeratinisation is not a distinctive feature in tail epidermis.

The distribution of Langerhans cells in tail epidermis is non-random and appears to be related to the differences in the pattern of keratinisation (Schweizer and Marks, 1977) since ATPase positive cells (Langerhans cells) are largely absent from the scale regions but common in the interscale regions.

1.6.3. The Basement Membrane

The basement membrane is composed of glycoproteins and a thin layer of collagen-like fibrils that follow the contour of the underside of the epidermis. These fibrils are cemented in an amorphous ground substance.

The basement membrane was originally thought to be produced by the connective tissue (Gersh and Catchpole, 1960). However, basement membrane can form in the absence of connective tissue such as the tooth enamel surface (Schroeder and Listgarten, 1971). Basement membrane type collagen (type IV) can also be synthesised by endothelial cells in vitro (Jaffe et al, 1976; Macarak et al, 1979). There is now evidence that the basement membrane is produced by epithelial cells (Briggaman et al, 1971; Hay and Revel, 1963; Pierce, 1971). The structure of the epidermal-dermal junction has been reviewed by Briggaman and Wheeler (1975), Daroczy and Feldman (1981), Kefalides (1975).

The function of the basement membrane is unclear but it clearly plays some role in sticking the epithelial cells to the dermis and providing an impenetrable barrier to cells and certain large extracellular components. However, it must be permeable to a range of nutrient materials and is in fact not completely impenetrable to cells since certain cell types can cross it, for example some blood cells (especially lymphocytes) and Langerhans cells. Hence, the main functions of the basement membrane is to provide structural support for cells and controls the permeability as was suggested by Fawcett (1966), Kefalides (1975), Heaphy and Winkelmann (1977).

1.6.4. The Dermis

Immediately beneath the basement membrane and the epidermis, and surrounding the various epidermal appendages (hair follicles, sebaceous glands and sweat glands) the tissue architecture is completely different. The epithelial region is composed of densely packed epithelial cells with relatively little inter-epithelial space, whereas

the underlying tissue, the dermis, consists of a thick layer of various fibres, an intercellular matrix, various cell types, a network of both blood vessels and nerve fibres (Zelickson, 1967; Rook et al, 1972; Lever and Schaumburg-Lever, 1975), the whole comprising the connective tissue. This intercellular matrix contains rich networks of minute fibres of elastin, collagen and reticulin as well as mucopolysaccharides, all produced by dermal fibroblasts.

The dermal connective tissue is very dense where it adjoins the epidermis, but it becomes more open in the deeper part where the loose fat is located between its elements. The dermis varies greatly in thickness, and in man is usually thicker on the dorsum than on the abdomen. The surface of the dermis in some regions is covered with minute papillae or pegs. These project upwards and interdigitate with the irregular epidermal downward projections, or rete ridges. This zone, therefore, is known as the papillary layer. These papillae are very vascular and contain a plexus of fine elastic fibres, with their collagen fibres lying perpendicular to the surface. In the papillae there are also many nerve endings. Beneath the papillae, the connective tissue becomes very dense with the collagen bundles running in every direction (Gibson et al, 1965). The presence of this network of thick elastic fibres between the collagen bundles gives the skin its characteristic elasticity. The bundles that lie parallel with the lines of tension of skin are particularly numerous and well-developed.

Oriented smooth muscle fibres are present in the deeper parts of the dermis especially in the skin of the nipple, scrotum and penis, and also in association with the hair follicles (Allectores pilorum) and skin

glands. Striated muscles may reach into the dermis of the face and neck. Sweat glands, sebaceous glands and hair follicles are found at various levels in the dermis. The dermis also contains different cell types such as fibroblasts, fat cells, pigment cells, mast cells, macrophages, lymphoid cells and probably other undifferentiated cells. The most important is probably the true connective tissue cell, the fibroblast which synthesises the various fibrous proteins.

The connective tissue pervades the entire body providing most tissues with a structural support and a matrix through which the vascular and nervous elements spread.

The small cellular processes of the basal cells interdigitate with the basement zone and the papillary dermis (Mihm et al, 1976). The two major moieties of the skin apparently behave and function as a single unit and are clearly interdependent, although the epidermis differs structurally from the dermis. The epidermis protects the dermis and relies on it for its nutrition and respiration, and possibly more complex exchanges occur between the two (e.g. Bullough, 1975; Houck, 1976).

1.6.5. Blood Vessels and Capillaries

The skin circulatory system consists of a complex network of arteries carrying blood to the skin, and veins carrying blood away from the skin. When the major vessels branch and divide, they eventually generate a fine network of capillaries. About 19-30 microlitres of blood exists in every gram of skin in rats and mice.

The vessels form particularly rich networks around structures like the hair follicles. The blood serves as a transport vehicle for nutrients and a wide range of chemical messages, and for the removal of waste material. It also plays an important role in temperature regulation. The skin may also play a role in absorbing atmospheric oxygen, which diffuses into the skin probably down the hair follicle canals and the perifollicular network of capillaries. The importance of this function in terms of total oxygen exchange of the body is unclear. Another important general feature of the vascular supply is its ability to change with time as new capillaries develop or open and old ones disappear or close. This is particularly evident in the network surrounding the hair follicles as they change from resting to growing and then back to the resting stage. This same ability also can be seen in wound healing and skin grafts.

The veins and arteries are the largest vessels with the thickest vessel walls. Both have elastin fibres in the wall, but in the arteries these tend to be well arranged to form an internal elastic lamina. The arteries and arterioles have smooth muscle cells in the vessel wall. A cross-section of an artery would show an internal endothelial layer, a basement membrane, an elastic lamina, a smooth muscle layer, a second elastic lamina and finally an outer coating of collagen and elastic fibres. The capillaries tend to lack muscle and a well organised elastic lamina and hence consist merely of an endothelial cell tube with diameter of about 10 μm.

The cell lining the inner surface of the vascular tubes, the endothelial cells; some of these cells can divide occasionally and can be grown in

culture conditions and respond to injury by a compensatory burst of cell division. The capillary wall ends in a basement membrane manufactured in this case partly by endothelial cells and partly by the neighbouring fibroblasts. A third cell may also contribute in some way and this is the rather poorly defined pericyte. This lines the outer surface of the capillaries and may, in fact, be part of the fibroblast population or possibly a type of endothelial cell. It may be involved in contractile activity and may, in fact, mature into a smooth muscle cell.

It can be estimated that each capillary loop in human skin supplies an area of about 0.04-0.3 mm^2 of surface skin, or between 500 and 5000 epidermal basal cells, and that the loops are separated by only 50-1000 epidermal basal cells (discussed by Potten, 1985).

1.7. RADIATION EFFECTS ON THE SKIN

1.7.1. Acute Effects

The skin response which includes erythema, epilation, desquamation, ulceration and contraction (followed after low doses by regeneration and healing), has been scored and presented in several different ways. The response is generally similar in most species. However, there are some quantitative differences which depend on radiation quality and dose, as well as on the time after exposure, the region of the body studied and the size of the area irradiated.

Regarding the erythema reaction of skin it can be divided into three phases or waves:

1. A reddening that can be observed within a few hours of irradiation. This reaction reaches a peak at 24 hours and then fades to a minimum

over the next 2-3 days. It is known as the initial, or first, erythemal wave and has a threshold of about 3 Gy and an intensity that is largely dose independent over the range 3-6 Gy. This reaction is probably a reflection of capillary changes - dilatation and leakage i.e. increased vascular permeability.

2. The second wave, the main or second erythema wave, begins on about the tenth day, after a latent period of about 8 days from the end of the first wave. The intensity of reaction, which is dose dependent, increases to reach a peak on or after the fourteenth day. This wave declines after about 4 weeks but may be difficult to separate from later changes for example, pigmentation changes.

3. The third wave, and possibly additional subsequent waves, which may occur a month or later after irradiation. The intensity of the third wave is largely independent of dose.

It is clear that the early phases of erythema may reflect (a) changes in capillaries which quickly become dilated and permeable and (b) an inflammatory infiltration. This effect can be seen to extend beyond the boundaries of the irradiated field and this has been interpreted as due to the release of histamine or histamine-like compounds. The later erythema is probably the consequence of other vascular changes and also other processes such as epidermal cell death which consequently reduce the epidermal cellularity and eventually epidermal thickness.

The condition of dry desquamation continues during the period of the second erythema wave. There is a significant reduction in the number of cells in the basal layer of the epidermis by this time. Dry desquamation is usually accompanied by temporary depilation or partial

permanent depilation. There is usually not complete sloughing of the epidermis under these circumstances, and after the second erythema wave, if the response of the epidermis has been limited to dry desquamation, the skin recovers to a fairly normal condition, except for permanent changes in vasculoconnective tissue, local changes in melanin, and perhaps some reduction in the thickness of epidermis, as well as residual changes that may remain in hair follicles and sebaceous glands.

Following erythema, epilation and dry desquamation, there may be breakdown of the epidermis and exudation of plasma. This is moist desquamation which is an important end-point in radiobiological studies. In mouse skin, the peak skin reaction (i.e. moist desquamation) occurs on the 10-28th day after irradiation. If the radiation dose was not very high the irradiated area usually heals within 1-2 months through the coalescence of proliferating epidermal tissues. However, if the dose was very high, the irradiated skin may break down completely and necrosis will occur.

The contraction of skin which takes place in the irradiated area is also a dose-dependent phenomenon, which has a threshold and time course very similar to that of the skin reaction itself (i.e. 12-40 Gy and is greatest at about 3 weeks after irradiation). However, contraction of the skin is assumed to be the consequence of dermal cell population damage (Hayashi and Suit, 1972), such as connective tissue and vasculature.

1.7.2. Late Effects

The late effects occur typically after months or years and are characterized by damage to vascular and connective tissue elements in the dermis. With lesser degrees of dermal damage associated with epidermal necrosis and ulceration, a new epidermis may be formed which is thinner than normal. If there has been sufficient damage to cause a considerable increase in the density and hyalinization of collagen, the basement membrane beneath the basal cells may disappear and rarely reform, so that the new atrophic epidermis is based insecurely on a poor supporting structure and is highly susceptible to trauma, infection, or other stresses (Casarett, 1980).

With greater damage to blood vessels and connective tissues, repair may be partly or even largely a secondary process, which is replacement fibrosis (connective tissue scarring). If fibrosis occur in the deeper tissues, it may compress vessels draining fluid from the skin and thereby contribute to oedema.

In areas where the vasculoconnective tissue becomes inadequate to support the regenerated epithelium, delayed ulcers may develop long after irradiation. This is more likely to occur with superimposed trauma or infection, which the less resilient vasculoconnective tissue of the irradiated dermis is less able to withstand than normal tissue.

The changes in the blood vessels result in vessel destruction and this will alter the levels of essential nutrients, oxygen, chemical messages and waste material, therefore influencing epithelial cell viability and function (Potten, 1985a). There are a few types of blood vessel change

which are related to the late effects of the skin. For example, there may be partial or total occlusion of the vascular lumen, these change frequently referred as "sausage segments" (e.g. Lindop et al, 1969; Hopewell et al, 1978; Hopewell, 1980). The other type of late damage to the vasculature is telangiectasia. This has been reported in human skin many months or years after irradiation (e.g. Turesson and Notter, 1986). It occurs in capillaries and histologically they appear as large dilated vascular channels. Other tissues such as brain have also been discussed (Reinhold and Hopewell, 1980).

1.8. EFFECTS OF FIELD SIZE IN SKIN

It is standard practice in radiotherapy of cancer to adjust the total dose delivered according to the dimensions of the irradiated field. In general, small fields tolerate significantly higher radiation doses than larger fields (Von Essen, 1963; Cohen, 1982; Schulthéiss et al, 1983).

An early demonstration of the dependence of the severity of the radiation reaction on the area or volume of tissue irradiated was obtained by Jolles (1941) in experiments using rat skin. In his studies, fields of 0.075 to 3.0 cm diameter (0.004 cm^2 to 7 cm^2) were irradiated and, for a given dose, the severity of the reaction was found to increase as the field size was increased.

In human skin, the degree of skin desquamation is markedly dependent on the area of skin irradiated. This has been studied in radiotherapy patients (e.g. Paterson, 1963; Von Essen, 1968). However, some of the investigations were confounded by the use of various degrees of reaction (i.e. skin tolerance) acceptable in different field sizes as discussed by Hopewell and Young (1982).

The relationship between dose and irradiated area has been expressed in the form of an equation:

$$D = KA^n$$

Where D is the dose needed to produce a specific reaction, and A is the field area (K being a constant). The value of the exponent n has been quoted as -0.165 (Cohen, 1966) or -0.16 (Von Essen, 1968).
The extrapolation of the above formula to areas greater than 400 cm^2 is uncertain, because evidence for very large areas relates only to lightly penetrating electron beams for the treatment of diseases of the skin, and it is not known if these diseases predispose to increased radiosensitivity. However, it has been concluded that there is little effect of area for areas above 400 cm^2 (Smedal et al, 1962).

Experiments have been carried out with pig skin, comparing irradiation by strontium-90 and thulium-170 (Peel et al, 1982). The percentage of surface dose reaching the basal layer is similar for the two isotopes. However, only about 10% of the surface dose reaches the base of the dermis using thulium-170 compared to about 50% with strontium-90. These studies concluded that there was no field size effect for epidermal reactions with thulium for areas between 5 and 19 mm diameter, in contrast to what was observed with strontium-90. This was considered to be due to the contribution to repopulation from hair follicles, spared more by thulium than by strontium.

Pig skin irradiation with X-rays was studied by Hopewell and Young (1982). They showed that there was no significant effect of field size

when skin areas of 16 cm^2 (4 x 4 cm) and 64 cm^2 (16 x 4 cm) were compared after single doses. The difference between these results and the above human data is possible because of the different shape of the fields and the different level of injury accepted for tolerance in different field sizes in the clinic. In more recent studies in which circular areas of pig skin 22.5 and 40 mm diameter were irradiated with strontium-90 plaques, no field size effect was observed both in terms of the acute epithelial reaction and the healing time (Hopewell et al, 1985).

There are a number of stochastic models which have been presented that relate the probability of reaching a specified level of reaction (e.g. tolerance or necrosis) to the probability of inactivation of an individual independent structure within that tissue (Cohen, 1982; Schultheiss et al, 1983; Wolbarst et al, 1982). With the assumption that the tissue event occurs if at least one of the independent units is inactivated, the field size effect follows directly, since larger fields contain more of those units and hence have a higher probability of injury of at least one unit (Shymko et al, 1985).

Douglas (1982) has analysed several sets of data from the literature relating to field size effects. In his work isoeffect responses to different time-dose schedules were fitted to a linear-quadratic model for cell survival, and the parameters α and β of this model were derived assuming a constant β/α ratio. A similar analysis have also carried out by Shymko (1985) without the constraint of constant β/α, using data from Douglas (1982) and other similar data from the literature. Both of their data showed that large fields are more radiosensitive than small

fields. However, Shymko et al (1985) found that this sensitivity decreases more rapidly with dose per fraction in large fields than in small fields, which is not consistent with the assumption of a constant β/α ratio.

1.9. PLUCKING

Plucking the hair from its follicle or stripping the surface of epidermis with sticky tapes are well established techniques for inducing increased proliferation in both follicular and interfollicular epithelia. Soon after plucking, cells enter S and a new hair growth cycle is started. Interfollicular basal cells are also stimulated in a fairly synchronous way and the LI and MI increase to reach their respective peaks between 12-24 hours post-plucking (e.g. Hamilton and Potten, 1972). The proliferative parameters remain elevated for several days and return to normal by about the 14th day after plucking; about 4 days before the growing follicles re-enter the resting phase (Fry et al, 1970). At the time of maximum stimulation the Tc may drop from 83 hours to only 12 hours (Clausen and Lindmo, 1976).

The skin reaction appears within 7-8 days in unplucked mouse foot skin and arises at the same rate over a wide range of X-ray doses (21-41 Gy), reaching a peak value slightly later the higher the dose (Hegazy and Fowler, 1973). In plucked dorsal skin the reaction appears within 4-5 days and reaches a peak earlier than in unplucked dorsal or foot skin. The decrease in reaction is quicker for low doses than high doses. The differences, by almost a factor of 2 days, between the appearance and peaking of corresponding reactions in plucked and unplucked dorsal skin, were correlated with the differences in the cell kinetics i.e. Tc of the

two tissues when not irradiated of 47 and 100 hours, respectively (Hegazy and Fowler, 1972). The decrease in the time for the maximum involvement of plucked skin was primarily related to a decrease in duration of the G_1 phase. Hegazy and Fowler (1973) suggested that the GF in the basal layer was close to unity for both plucked and unplucked skin. A cellular depletion in the basal layer caused by irradiation would thus be expected to produce an obvious effect on the whole epithelium after a time equal to Tc, plus any radiation-induced division delay. They deduced a 3-6 days division delay, which depended on dose in the unplucked skin. In the plucked skin this division delay was 2-3 days.

1.10. EPIDERMAL CELL SURVIVAL CURVES

Survival curves for epidermal cells have been studied extensively over the last two decades. Both macrocolony and microcolony assay techniques *in vivo* have been developed and used to study the radiosensitivity of the clonogenic cells in the basal layer in mouse, pig and human skin. An *in vitro* assay system has also been established and the radiosensitivity of keratinocytes both in human and mouse epidermis has been studied.

1.10.1. In Vivo

(a). Macroscopically visible colonies - Macrocolonies.

In 1967 Withers developed an ingenious technique which provided means of estimating the radiosensitivity of epidermal cells *in vivo*. After appropriately high doses, only one or two clonogenic cells survive within a test area of skin, and these divide rapidly forming clones that can be counted. The principle of this technique is that a proportion of

epithelial cells in a defined area of mouse dorsal skin is isolated from the surrounding skin by an annulus of lethally irradiated (30 Gy) epithelium, thus preventing the centripetal migration of non-irradiated cells to the experimental area during the post-irradiation period. The test areas, which are shielded from irradiation of the annulus, are irradiated subsequently with a desired test dose. The small numbers of epithelial cells surviving in the test areas proliferate to become visible nodules within 10-20 days after irradiation. It was assumed that each nodule developed from one or more surviving cells. Survival was deduced from the observed frequency (f) of the test areas (of varying sizes between 0.00093 and 1.13 cm^2, depending on the dose), not containing a clone using the Poisson distribution:

$$\text{i.e. } S = \frac{-\log_e f}{NA}$$

Where S is survival, f is observed, A is the area and N is constant.

For a given dose, the test area size was adjusted so that the frequencies of clone formation could be measured. After determining the surviving cells per unit area at several doses (i.e. 9.4 Gy to 23.1 Gy), a dose-survival curve was obtained with a D_0 value of 1.35 Gy and $n = 1.4 \times 10^6$ cells/cm^2. This technique was soon followed and used by various authors using different conditions. For example, using single and fractionated doses of X-rays (Emery *et al*, 1970), single and fractionated doses of fast-neutrons and electrons (Denekamp *et al*, 1971 and 1974), and single doses of heavy-ions (Leith *et al*, 1971). All of these studies provided D_0 values ranging from 0.95 to 1.5 Gy.

Instead of irradiating the test area with varying sizes and measuring

the probability of no survivors as developed by Withers (1967), other macrocolony techniques involved irradiating the test area with exactly the same size and counting the number of colonies in that area. In a segment of mouse tail (e.g. 3 cm) irradiated with doses ranging between 30 and 38 Gy, nodules can be seen by the naked eye within the damaged epithelium between 19 to 27 days after irradiation. The numbers of macrocolonies can be counted and expressed per unit length or per unit area of epidermis, and a survival curve obtained. The curve for tail has a D_0 value of 3.5 Gy and extrapolates to a value of 2×10^4 cells/cm^2 at zero dose (Hendry, 1984).

Using a similar approach, there has been one attempt to obtain epidermal cell survival information on human skin *in situ*. This made use of the fact that two patients after radical mastectomy received a series of small doses to the chest wall and consequently accumulating considerable total doses. Observation of macrocolonies on different strips of skin receiving slightly different total doses were made. The number of macrocolonies per cm^2 was estimated either using the Poisson formula or directly counting the number of these colonies, and a very small portion of the dose-response curve was constructed. This indicated a D_0 of 4.9 ± 1.5 Gy for fractionated doses (Arcangeli *et al*, 1980).

(b) Microscopically Visible Colonies - Microcolonies.

Macroscopically visible colonies developed within 2-3 weeks after irradiation. These were formed from individual cells which divided rapidly and doubled their numbers within less than 24 hours (Withers, 1967c). Hence these growing colonies should be present microscopically at earlier times. One way of detecting these microcolonies was by using an autoradiographic technique to seek foci of rapidly dividing cells.

Clumps of rapidly dividing cells have been identified in autoradiographs of epidermal sheets on the third day after irradiation and plucking (Al-Barwari and Potten, 1976). The colonies were scored in known areas, and a survival curve over a wide range of X-ray doses was obtained. This gave a D_0 value of 2.33 Gy X-rays and extrapolated back to the ordinate at 1.23×10^4 cells/cm^2.

Using the same technique, there was a study on unplucked mouse skin 13-14 days after irradiation. A survival curve with a D_0 of 4.4 Gy was obtained which extrapolated to 4.6×10^3 cells/cm^2 (Keech, 1982).

Attempts have been made to measure the survival of colony-forming cells in pig skin using histological sectioning techniques. Three doses (17, 23 and 27 Gy) were delivered to various skin sites, biopsies were taken and colonies were identified in sections at 17-28 days after irradiation by the presence of runs of healthy-looking basal cells. Different colony sizes were defined by the length of these runs. If small colonies were defined by runs of 20-100 cells (< 500 μm in length), a portion of a survival curve was obtained, having a D_0 of 2.7 Gy. Colonies of 100-300 cells (500-1500 μm) provided a curve with a D_0 of 5.7 Gy and colonies with more than 300 cells (> 1500 μm) gave a curve with a D_0 of 16.2 Gy (Archambeau et al, 1979). The problems associated with the high values of D_0 are discussed in Section 4.1.2.3.4.

1.10.2. In Vitro

It is now possible to grow human and mouse keratocytes in culture. This system has been used to measure the survival of the colony-forming cells, by irradiating the initial cell suspension or intact confluent

cultures. Human keratocytes assayed *in vitro* gave a D_0 of 0.74 Gy (Dover and Potten, 1983) and 0.92 Gy (Parkinson *et al*, 1986) which are lower than values for mouse cells irradiated either *in vivo* (0.95 - 4.4 Gy) or *in vitro* (1.18 - 1.39 Gy) (Parkinson *et al*, 1986). The initial slope ($1/\lambda$) for these human keratocytes was 3.7 Gy (Parkinson *et al*, 1986) which is similar to the D_0 of 4.9 Gy obtained from human cells assayed *in situ* using fractionation protocols (Arcangeli *et al*, 1980). The D_0 value for mouse cells *in vitro* (1.39 Gy) is similar to that *in vivo* (1.35 Gy) using dorsal epidermis.

1.11. RESIDUAL INJURY

Re-treatment of previously irradiated sites in patients must be done with caution as any residual injury in the tissues will reduce tolerance to subsequent treatment. Residual injury can be measured by giving a "priming" treatment (a single dose or a series of doses) below tolerance then followed sometime later by a test treatment and measuring the reduction in the test dose required to produce a given response, compared with treating unirradiated age-related controls. The reduction in the test dose can be expressed as a percentage of the priming dose and this is called the "% remembered dose" (Brown and Probert, 1975; Denekamp, 1975).

Residual injury has been studied in a number of tissues in different ways. One of the earlier demonstrations was by Weinbren *et al* (1960) in irradiated rat liver. They have shown latent chromosome abnormalities at times up to one year after irradiation, when cells were stimulated into division in the anterior lobe by ligating the peduncle of the posterior lobes. Curtis (1967) showed that such abnormalities decreased

in number with time after irradiation, and Fisher (1985) showed that there was a corresponding increase in the clonogenicity of hepatocytes.

In the skin, there is evidence for the persistence of chromosomal lesions in skin fibroblasts for long times after radiotherapy (Savage and Bigger, 1978). Denekamp (1975) and Brown and Probert (1975) showed that by 6 to 8 months after priming treatments of either single or fractionated doses of X-rays, the tissue responsible for early skin damage in mice had almost totally repaired and only about 10% of the 1st dose was remembered. This has been confirmed by others (Field and Law, 1976; Hendry, 1978). The early reaction is the result of killing of the basal epidermal cells, so clearly their repopulation can restore the tissue to near normal. Human skin is also able to repair epithelial damage almost fully, as assessed by early reactions (Hunter and Stewart, 1977).

Brown and Probert (1975) also investigated the end-point of late deformity in the mouse foot. In their experiments, 35-40% of the first course of treatment was "remembered" for late foot deformity when the second treatment was given six months after the first. It was suggested that different components of the limb were responsible for late reactions and that for these there was less repair. However, Denekamp (1977) using mouse feet, and Field and Law (1976) using mouse feet and ears found the relationship between early and late damage remains the same for a wide range of fractionated treatments and re-treatments. This discrepancy has not been resolved. It should be noted that in the experiments of Brown and Probert (1975) the Ellis formula for partial tolerance with time after treatment was also tested and was found to give a fairly close prediction of the results from 1 to 10 months after

the priming treatment.

Using necrosis of the mouse tail as the end-point, Hendry (1978b) showed about 35% residual damage after a second course or after up to 6 courses of irradiation given at 6 weekly intervals.

From these data shown above, it appears that rapidly proliferating tissues such as epidermis can almost fully repair radiation injury, whereas tissues responsible for late injury e.g. vasculature or connective tissues may have a lower recovery capacity and would, therefore, be expected to accumulate more damage during protracted irradiation. The relationship, if any, between the early and late reactions has been the subject of much debate and is still not resolved.

Residual injury has also been investigated after neutrons and argon ions. Hendry et al (1977) using necrosis of the mouse tail observed that the residual injury was greater after a treatment with neutrons (25%) than after X-rays (10%). A similar experiment was also performed on mouse foot skin by Field and Hornsey (1980). Both the early and late deformities were assessed after test doses given 6 months after the priming irradiations. Their results showed that the amount of residual injury was not significantly different for early or late damage after pre-irradiation with X-rays or for early acute damage after neutrons, but was significantly greater for late damage after pretreatment with neutrons. This is in agreement with the findings of Hendry et al (1977). However, Raju et al (1983) investigated residual injury using the end-point of late deformity in the mouse foot and demonstrated that the residual injury for foot deformity for argon ions did not seem to be

higher than for X-rays when the doses of first treatment with argon ions and X-rays were matched to produce similar effects.

1.12. AIMS OF THE STUDY

The aim of this work was to investigate residual radiation injury in the skin at both the cellular and tissue levels under various conditions. The particular aspect of residual injury under study was the injury expressed when the skin was re-irradiated after single, fractionated and repeated irradiations.

Previous data in the literature on this subject have concerned gross skin reaction or necrosis, and there are no data showing how the changes in skin tolerance after priming treatments are related to deficiencies in the target cell populations concerned e.g. changes in cell number or cell sensitivity. Also, there are no accurate data available concerning the threshold dose to induce residual injury, nor concerning the effects of fractionated iso-effective priming doses on residual injury.

It was decided to use the mouse tail, a site where assays for skin reactions, skin healing/necrosis, and macrocolonies are already developed (Hendry *et al*, 1976; Hendry, 1984). Although the dorsum has been used extensively for radiobiological investigations it was not ideal for the present studies because of the contraction of the skin following irradiation which would affect the size of the area and the position to be re-treated. Also, it was decided to develop a microcolony technique for tail epidermis in order to extend the range of dose which could be used, and obtain more accurate estimates of sensitivity.

The main items under investigation are as follows:

1). In the microcolony technique what are the optimal assay conditions? e.g. dose range, sampling time, histological preparation, identification of colonies, colony counting etc.

2). What is the radiosensitivity of the microcolony-forming cells as well as the recovery factor in the normal tail epidermis, and how does this compare with values measured using other techniques?

3). What is the correlation between the different assays namely skin reactions, microcolonies, macrocolonies and skin healing? The reasons for this investigation are: (a) to compare the present data with other data in the literature using particularly the micro or macrocolony techniques. (b) to interpret the differences observed using skin reactions in terms of changes in the target cell populations which are responsible for the reactions; from the dose-response curves for healing a dose-response curve for the target cells can be deduced mathematically and this can be compared with direct measurements of cell survival using colony assays.

4). As the length of irradiated tail could be varied, it was possible to study the effect of field size at the cellular and tissue level. Hence the current view that cell survival should be independent of field size (Hopewell and Young, 1983) could be tested directly using colony techniques. Also, it was possible to test the hypothesis that field-size effects should diminish with low doses per fraction (Shymko et al, 1985).

5). Can this microcolony technique be used to study re-treated skin where epidermal architecture and pigmentation are changed after the first dose, and what modifications are necessary to the technique?

6). What is the threshold dose for residual injury in the skin after single and fractionated doses? The reason for this study is that

although morphologically or histologically the skin may appear normal, it may still contain latent radiation injury.

7). Would the size of dose per fraction in iso-effective first treatments affect the degree of residual injury? This is important regarding the current interest in hyperfractionation because of the sparing of late responding tissues (Fowler, 1984). Also, using the colony techniques it is possible to determine whether dosage changes for iso-effects are due to changes in cell sensitivity.

8). Three repeated tolerance doses were used in order to produce probably the maximum residual injury, so that dosage and any sensitivity changes would be large and easier to detect.

9). Is the degree of residual injury measured using 3 end-points (i.e. microcolony, macrocolony and healing techniques) the same? And, is the change in tolerance after 3 tolerance doses due to a sensitivity change or a change in the target cell number?

SECTION TWO

MATERIALS AND METHODS

2.1. RADIATION SOURCES AND DOSIMETRY

2.1.1. Pantak X-Rays

A beam of X-rays was generated from a Pantak X-ray machine operated at 290-300 kVp, 10 mA and fitted with a beam hardening filter of half value layer = 2.3 mm Cu. It was calibrated using an air ionisation chamber. The variation in absorbed dose did not exceed 5% across the jig when 3 cm of tail was irradiated at f.s.d. of 35 cm (Figure 2.1), and there was less than 3% variation when 4 cm of tail was irradiated at 70 cm f.s.d. (Figure 2.2). The exposure was delivered automatically using monitor units with a conversion factor to give the actual radiation dose absorbed by the tissue. When operating at 300 kVp, 10 mA the dose-rate for the 3 cm of tail at 35 cm f.s.d. was 200 cGy per minute and for 2 cm and 4 cm of tail at 70 cm f.s.d. was 50 cGy per minute.

2.1.2. 10 MeV L-Band Linear Accelerator

The travelling-wave electron linear accelerator used in the field-size studies operated at an energy of 10 MeV and delivered 2 μsec pulses at a rate of 50 pulses per second and 0.5 ampere peak current. The dose per pulse to the mouse tails was of the order of 30 cGy and a mean dose rate of 15 Gy per second (900 Gy per minute) was obtained at a distance of 3.4 metres from the accelerator end window with a 0.3 mm aluminium scatter plate at the end of the drift tube. Under these conditions beam flatness was within ± 1.5% over a field diameter of 14 cm. It was important to determine the dose distribution across the diameter of the field.

Ionisation chamber measurements are not suitable for this purpose as in very high dose-rate electron beams ion recombination in the ionisation

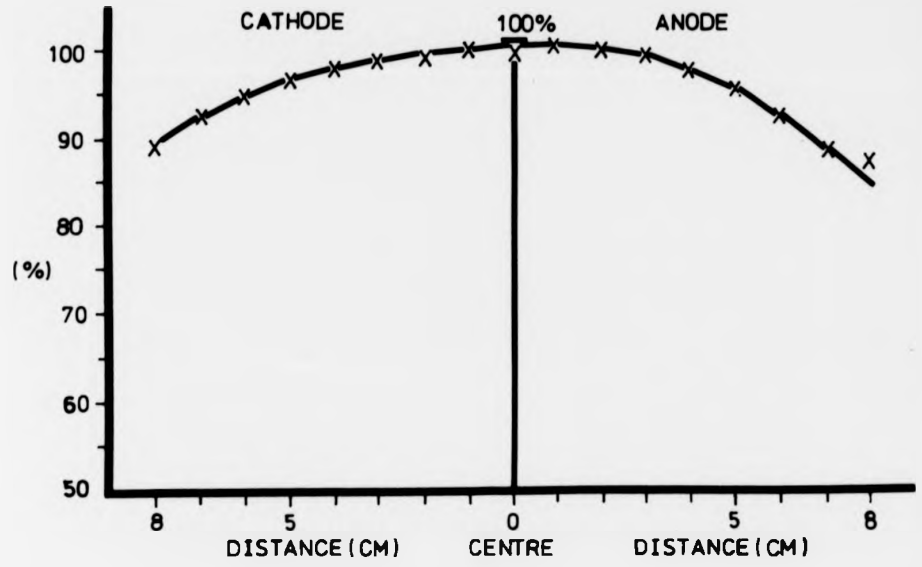


Figure 2.1: Dose distribution profile at f.s.d. 35 cm using PANTAK x-ray unit.

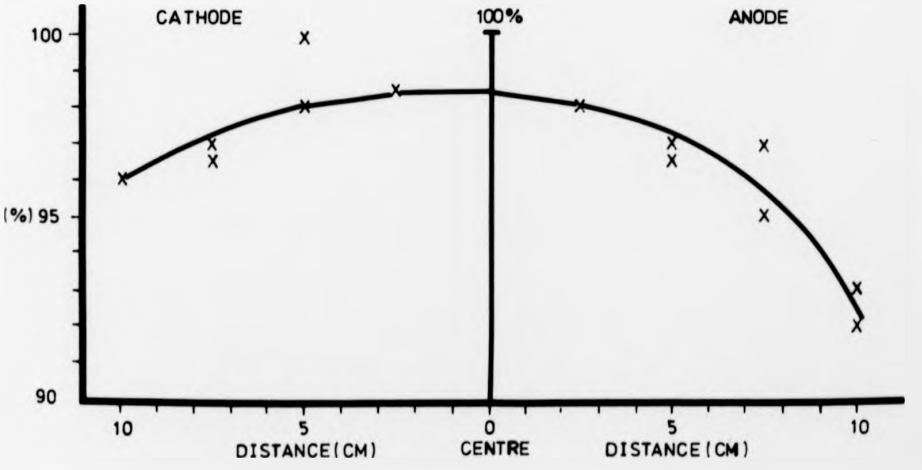


Figure 2.2: Dose distribution profile at f.s.d. 70 cm using PANTAK x-ray unit.

chamber would introduce uncertainties in the interpretation of the readings. Therefore, measurements were made with both thermoluminescent dosimetry and colour changes in PVC. The latter proved to be more convenient. Hence, measurements were made using PVC strips to estimate the variation in the ratio of absorbed dose to nominal dose with the length of the irradiated tail. The results in Figure 2.3 show the optical density measured after two nominal doses and for three tail lengths of 2, 4 and 6 cm. In order to achieve the same density, and therefore the same absorbed dose, it was found necessary to give 3% more nominal dose to the 6 cm length compared with the 2 and 4 cm length.

Ferrous sulphate dosimetry is a most reliable and widely used method. An air-saturated solution of ferrous sulphate in sulphuric acid is used. This dosimetry technique was used throughout the electron beam studies, to calibrate each exposure. The technique is described briefly as follows: A dilute sulpuric acid solution was prepared containing approximately 10^{-3} M ferrous sulphate i.e. 10^{-3} M Fe^{2+} in 0.4 M (0.8 N) sulphuric acid. The solution was contained in polystyrene tubes given a series of radiation doses. After the irradiation, measurement was by spectrophotometric determination at 304 nm of the ferric ions formed, using an unirradiated solution as a blank. The molar extinction coefficient of ferric ion in 0.8 N (or 0.4 M) sulphuric acid i.e. 2205 M^{-1} cm^{-1}. The absorbed dose received by the ferrous sulphate dosimeter in 0.8 N sulphuric acid was calculated from the formula:

$$Dm \text{ (in cGy)} = 4.42 \times 10^{5} [1-0.007 (t - 20)] OD/G$$

where OD is the absorbance (optical density) of the irradiation solution at 304 nm, measured in 1 cm cells with unirradiated solution as a blank, G is the yield of the reaction for the radiation in use, and t is the temperature in °C at which the absorbance is measured.

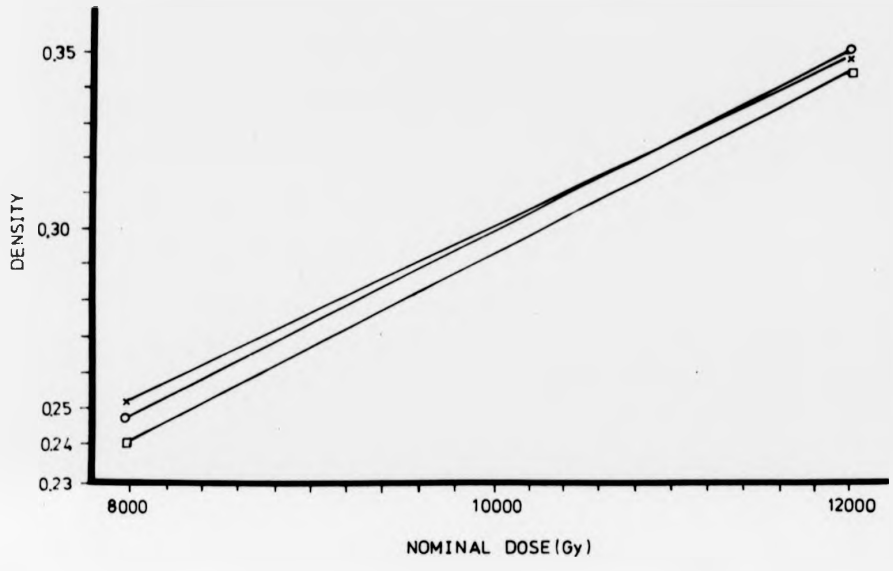


Figure 2.3: Mean dose distribution from 10 MeV L-band linear accelerator measured at 3 meters S.S.D. using PVC dosimetry. X, 2 cm tail; O, 4 cm tail; □ , 6 cm tail. Density in arbitrary units. Note that an average 6 cm tail has about 3% less dose than either 2 or 4 cm tail.

2.2. IRRADIATION SET-UP

2.2.1. X-Rays

The same set-up was used for all X-ray irradiation except the field size experiments which will be discussed briefly at the end of this section. The mice are loaded into adjustable perspex tubes so that the whole body is inside the tube. The mouse is secured with a cap through which the whole tail is exposed. Twelve mice are then radially positioned in the jig, (Plate 2.1) thermostatically controlled at 37°C, with their tails inside radial holes of a disc. The disc is 13 mm thick, radius 90 mm, positioned on top of the jig. It contains 12 radial holes into which the tails are inserted. The jig is fitted with a gas inlet and has an electrical heating coil to maintain a constant air flow of 142 litre/hour and temperature of 37 ± 0.1°C in the tails during irradiation. Shielding of the tail is achieved using a series of lead rings, so that the correct length of tail is irradiated. Most of the irradiated tail sections in the X-ray experiments were 3 cm in length. A 10 cm circle application is fitted, and the X-ray tube is pointed vertically downward. The jig is placed on the irradiation table and centred with the applicator in contact with it. The applicator is removed without moving the jig. A 3 mm lead shield was placed over the mice (Plate 2.1), the X-ray tube was lowered to the predetermined distance which could be checked from the vertical height scale on the X-ray tube stand (Plate 2.2). In the field-size experiment, the same set-up was used, but the f.s.d. was changed to 70 cm. The lead shield was the same as was used for 35 cm f.s.d. When the f.s.d. was increased all of the shield for the mouse bodies is within the main beam and this allows about 2.5% transmission through the 3 mm lead shield into the whole body of the mouse. Hence each mouse received about

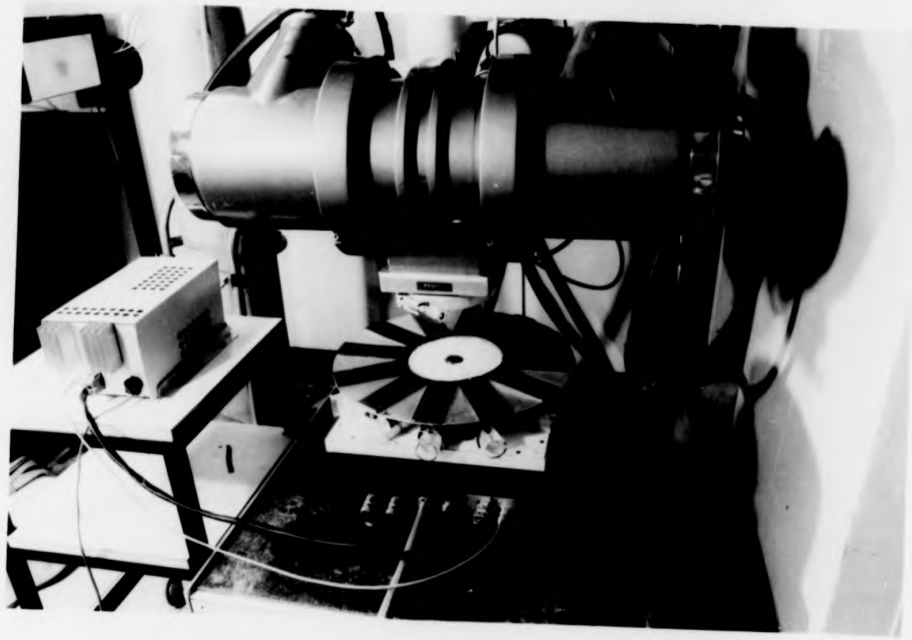


Plate 2.1: PANTAK x-irradiation set-up showing x-ray head, lead shield and mouse tail jig (top view).

Plate 2.2: PANTAK x-irradiation set-up, showing x-ray head, lead shield, mouse tail jig and air cylinder (lateral view).

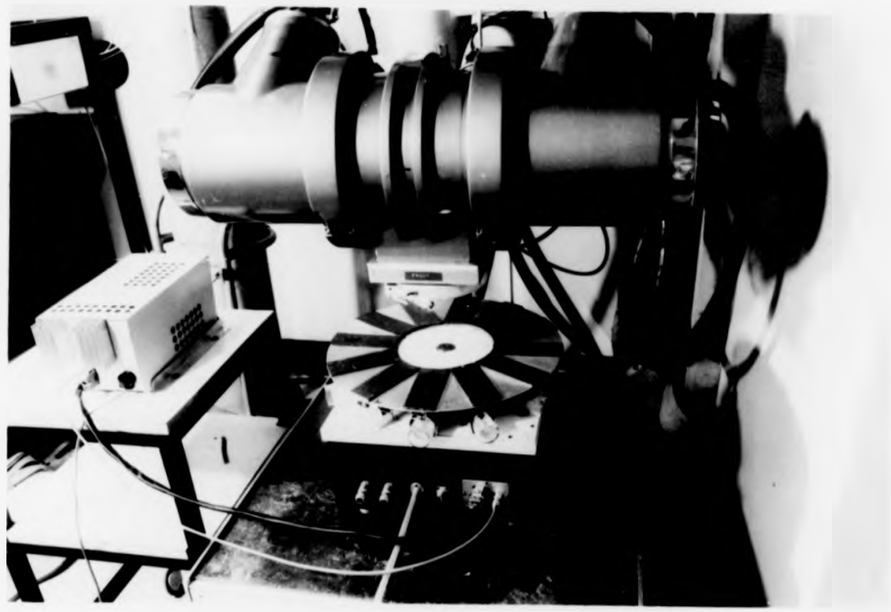


Plate 2.1: PANTAK x-irradiation set-up showing x-ray head, lead shield and mouse tail jig (top view).

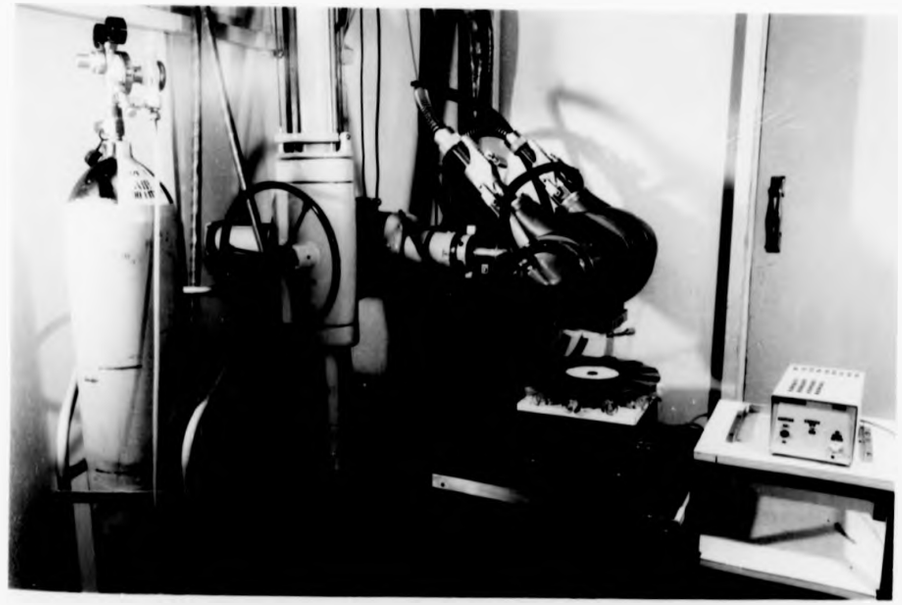


Plate 2.2: PANTAK x-irradiation set-up, showing x-ray head, lead shield, mouse tail jig and air cylinder (lateral view).

75 cGy whole body dose when the total irradiation dose of 3000 cGy was given to the tail.

2.2.2. Electrons

The same jig was used as for the X-ray set-up (Plate 2.3), except for a 3 mm thick circular lead collimator to allow a maximum of 6 cm of tail to be irradiated (Plate 2.4). During the irradiation a constant air flow of 142 litre/hour and a temperature of 37 ± 0.1°C were maintained in the tail holes. The actual setting of the electron beam irradiation is described as follows:

The electron flux incident on the mouse tail jig was measured by means of a Faraday cup monitor mounted at the jig centre, connected to an integrating electrometer. The charge collected per pulse was related to the dose at the mouse tail position by ferrous sulphate dosimetry at the same depth in a phantom designed to simulate the presence of the jig. The typical charge collected per pulse was 0.03 nc giving a calibration for the faraday cup monitor of 915 cGy/nc. The required integrated charge was calculated and set-up on a preset dosimeter attached to the control of the integrator. Therefore, doses given were dependent on the stability of the calibration factor for the cup over the series of experiments, but they were independent of the pulse to pulse variation of the accelerator itself.

2.3. ANIMAL HUSBANDRY

Female B6D2F1 (C57BL/6 x DBA2) mice were used throughout the experiments. All the mice were weaned at 3-4 weeks and were supplied by the Animal Services Unit of the Paterson Laboratories at about 12 weeks

Plate 2.3: Irradiation set-up for 10 MeV L-band linear accelerator. The jig was fitted on the vertical stand and the animals were placed in perspex tubes, onto the jig as shown. The temperature regulator (left bottom) and air cylinder are also shown.

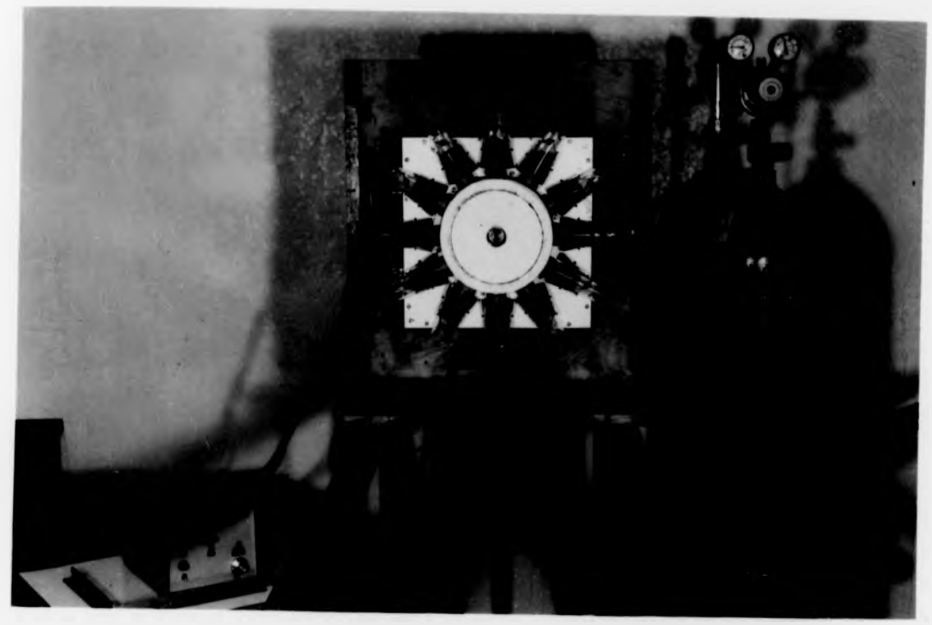
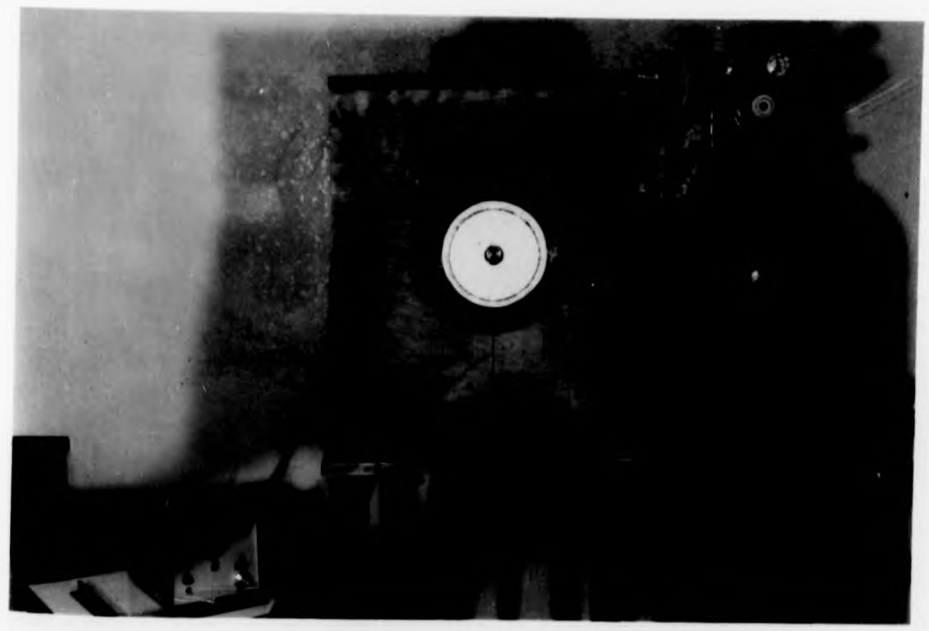


Plate 2.4: Irradiation set-up for 10 MeV L-band linear accelerator, showing the circular lead shield placed in front of the mouse jig to shield the mouse bodies.



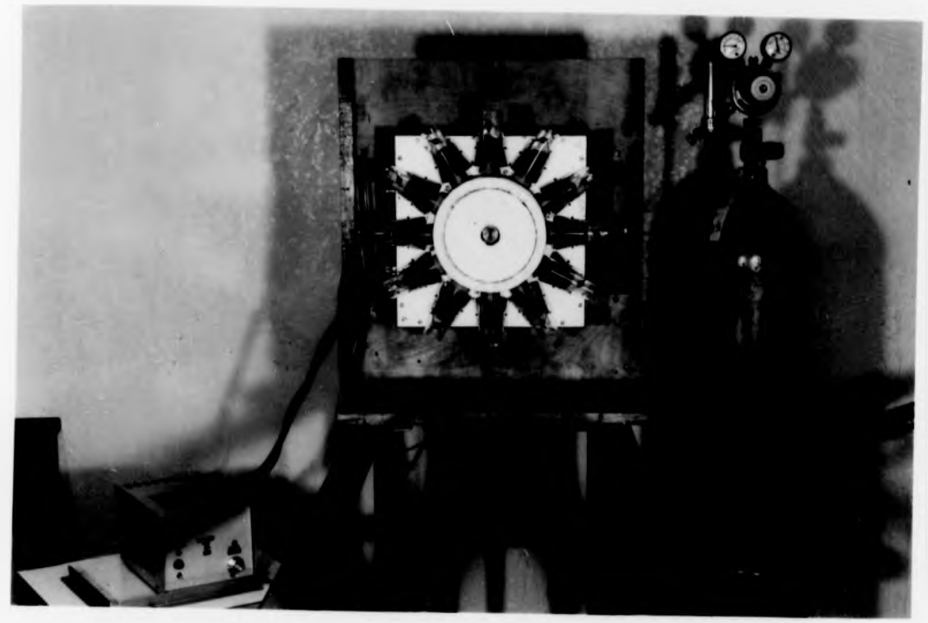


Plate 2.3: Irradiation set-up for 10 MeV L-band linear accelerator. The jig was fitted on the vertical stand and the animals were placed in perspex tubes, onto the jig as shown. The temperature regulator (left bottom) and air cylinder are also shown.

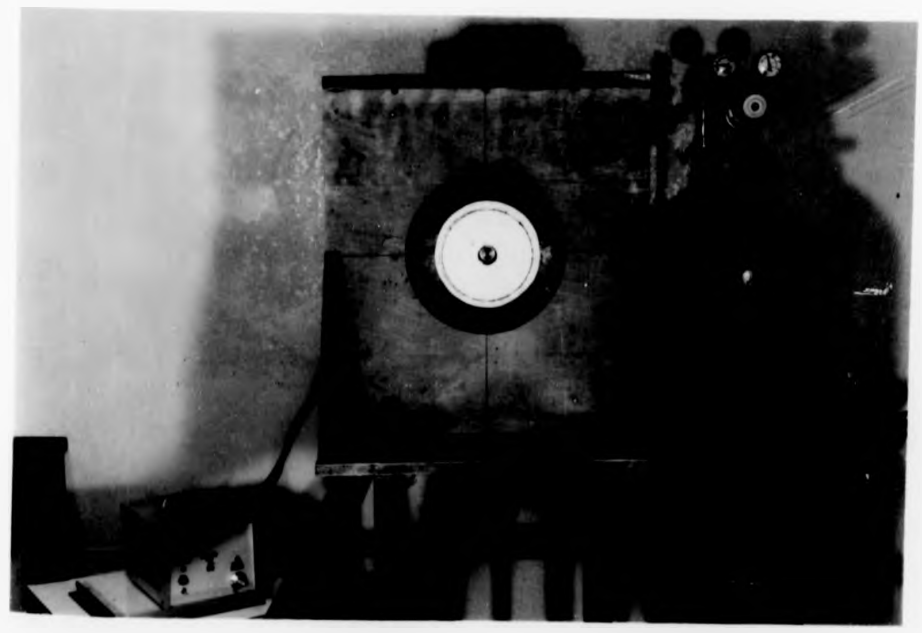


Plate 2.4: Irradiation set-up for 10 MeV L-band linear accelerator, showing the circular lead shield placed in front of the mouse jig to shield the mouse bodies.

of age. They were put on a standard laboratory diet (Labsure economy diet, RHM Agriculture Ltd) with water ad libitum, and housed with a 12 hour light/dark cycle, switching at 7.30 a.m. to light and 7.30 p.m. to dark. The room temperature was kept at 20-22°C, humidity at 50-55%. The cage and soft wood shavings were changed once weekly while the water bottles were sterilised and filled 3 times per week. The mice were housed 6 animals in small and 10 animals in large cages.

2.4. ASSAY TECHNIQUES FOR SKIN DAMAGE AFTER IRRADIATION

2.4.1. Skin Reaction

After irradiation using high doses the skin reaction appeared mostly from about day 12 to about day 32. During this period the skin reaction was scored everyday. Between day 32 and 9 weeks, most of the tails were either undergoing necrosis or healing up, and they were scored twice weekly. They were scored using the arbitrary scale system, as shown in Table 2.1. The relationship between the reaction score and the degree of skin damage is demonstrated in Plate 2.5.

2.4.2. Macrocolonies

Macrocolonies developed from about day 18 to day 32 after irradiation. Typical macrocolonies observed after a first irradiation or after a second irradiation are shown in Plate 2.6. The second irradiation was given at 9 weeks after a first dose of 25 Gy. The mice were observed and colonies counted everyday during the development period, until the colonies either joined together or the tissue necrosed.

(a). Counting Criterion

A surviving colony was chosen to be greater than 2 mm in diameter,

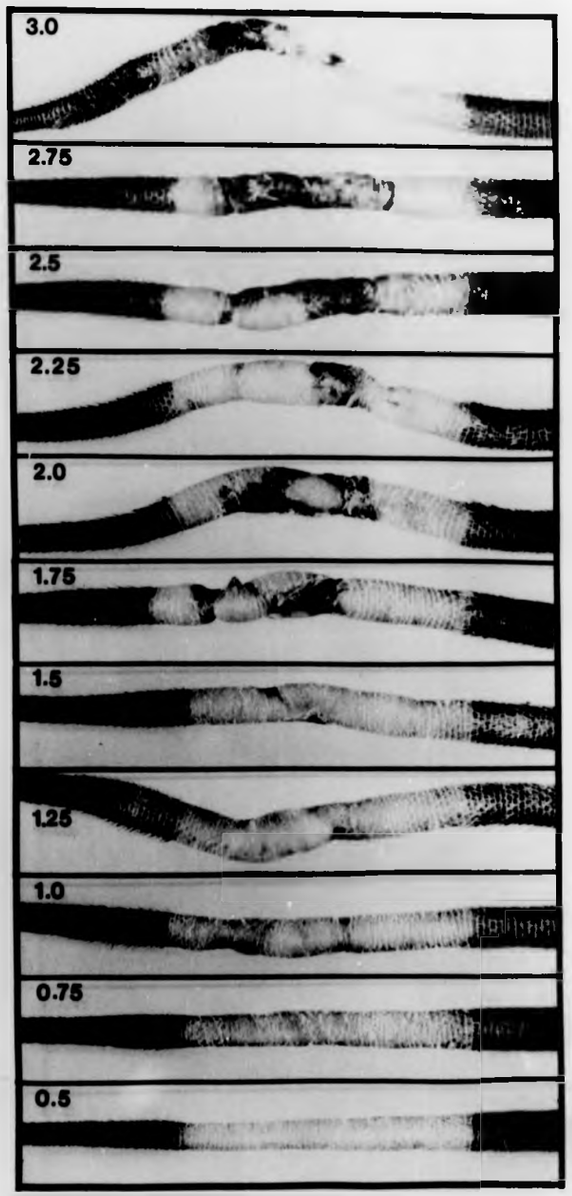
TABLE: 2.1

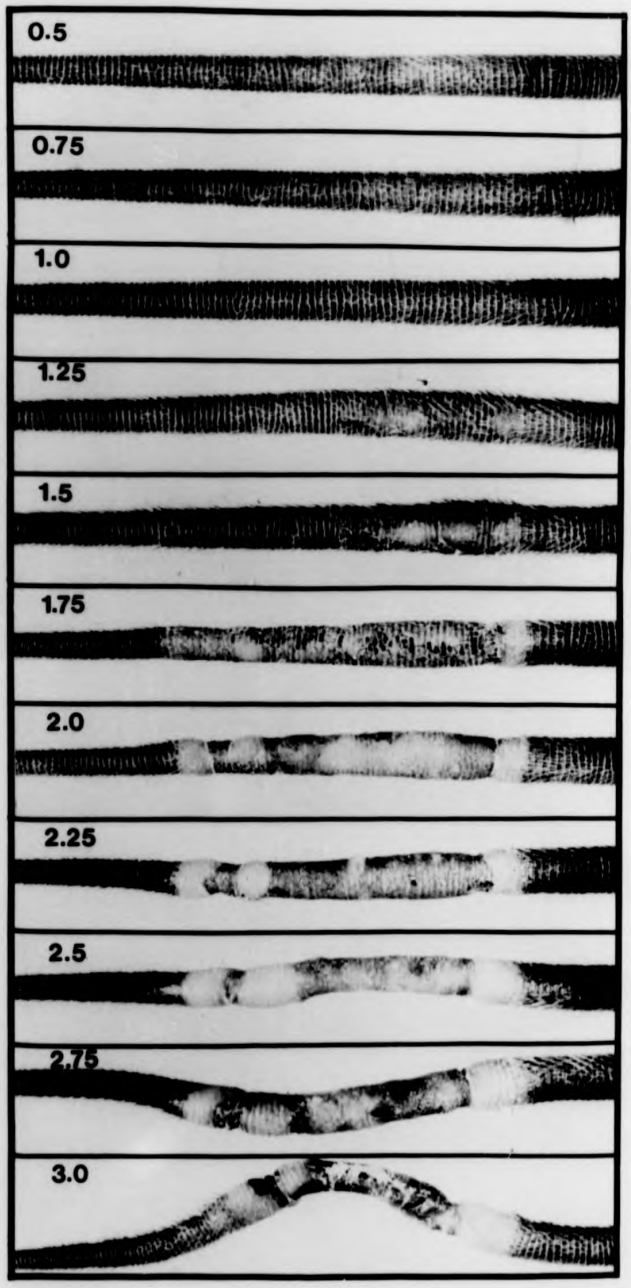
Skin Reaction

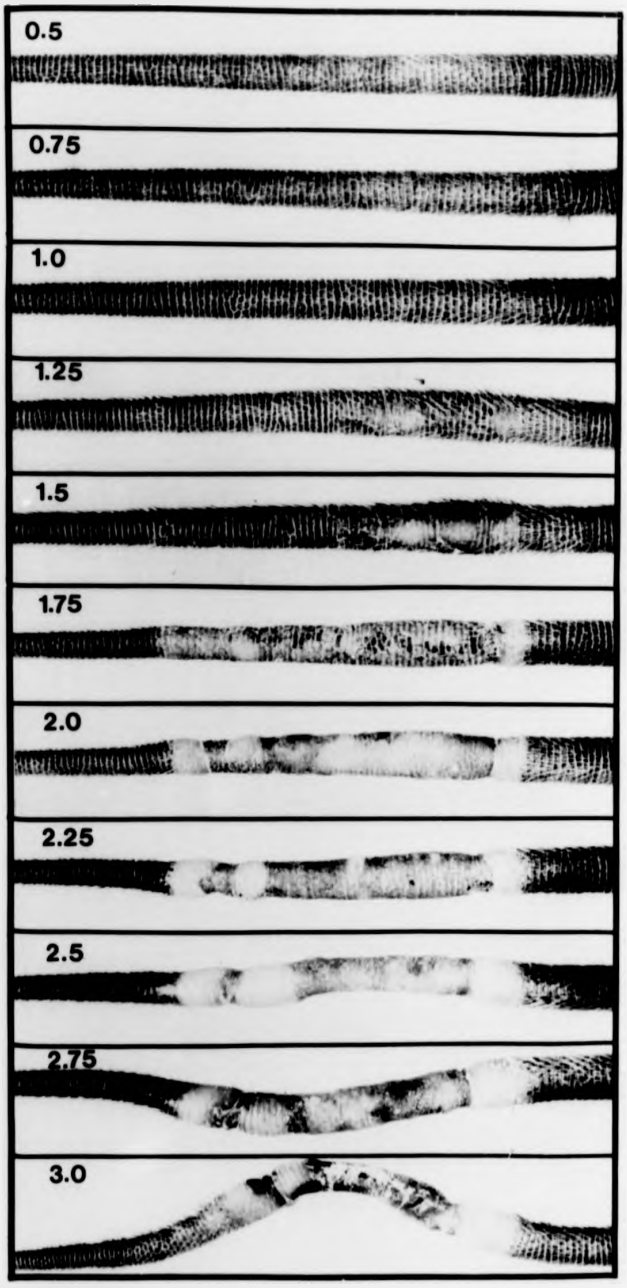
SCORE	REACTION APPEARING	REACTION DISAPPEARING
0.5	Possibly different from normal	Healed
0.75	Slight colour change; some epidermal thickening	Thin epidermis in parts; slight reddening
1.0	Thickened epidermis	Reddening in healed epidermis
1.25	Thickened epidermis with slight desquamation	Slight dry desquamation; final stages of scab sloughing; some reddening
1.5	Moist or dry desquamation over small irradiated region	Small scab persistent; some dry desquamation
1.75	Desquamation over half irradiated area	Smaller scab persistent
2.0	Total moist desquamation	Scab sloughing; still moist in parts
2.25	Scab forming; moist in parts	Scab over part irradiated area
2.5	Hard scab over half irradiated area	Hard scab persistent
2.75	Firm scab; slight bleeding	Scab persistent; slight oedema
3.0	Evidence of bleeding; distal tail oedema	Distal tail oedema nearly severed

Plate 2.5: Skin reactions (2 plates) using the scoring system shown in Table 2.1 .

Plate 2.5: Skin reactions (2 plates) using the scoring system shown in Table 2.1 .




0.5
0.75
1.0
1.25
1.5
1.75
2.0
2.25
2.5
2.75
3.0


0.5
0.75
1.0
1.25
1.5
1.75
2.0
2.25
2.5
2.75
3.0

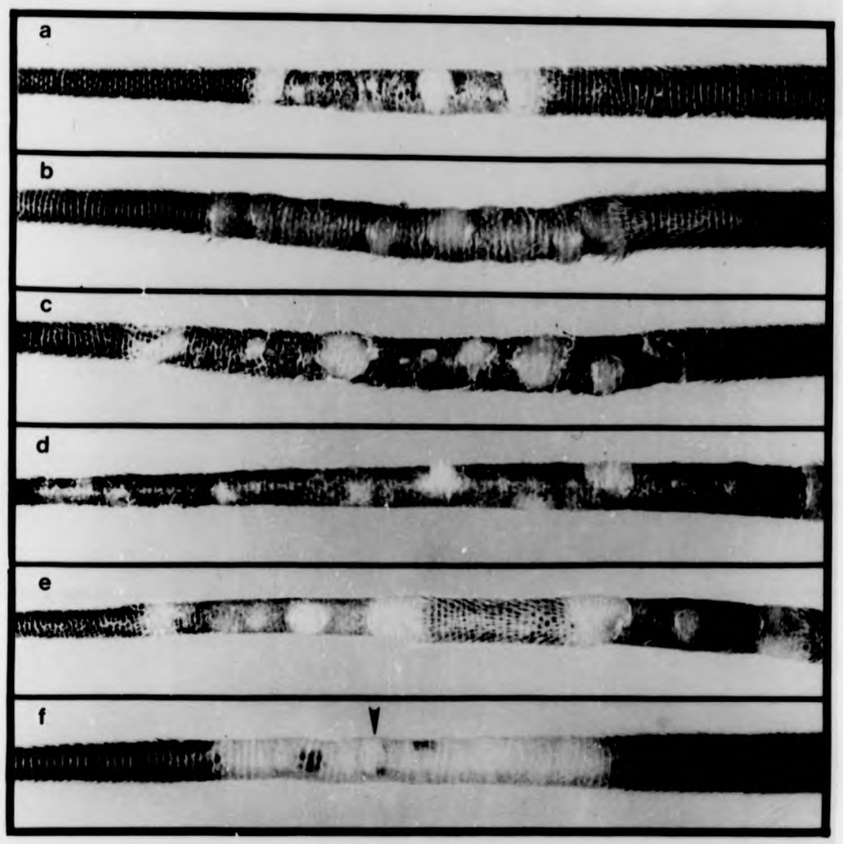
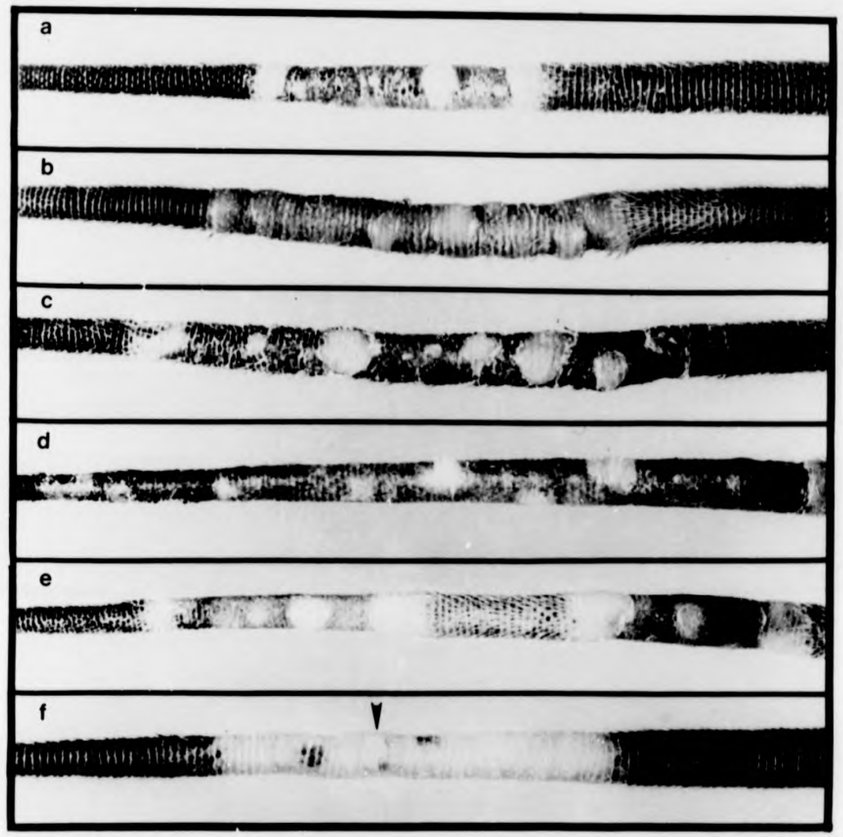


Plate 2.6: Examples of macrocolonies observed in normal tail skin with different irradiated lengths. a,b,c,d and e represent 2 cm, 3 cm, 4 cm, 6 cm and 2 + 2 cm (split area) tails. The arrow points to a typical colony observed in the pre-treated skin (f).

Plate 2.6: Examples of macrocolonies observed in normal tail skin with different irradiated lengths. a,b,c,d and e represent 2 cm, 3 cm, 4 cm, 6 cm and 2 + 2 cm (split area) tails. The arrow points to a typical colony observed in the pre-treated skin (f).



because it was found that many of the tiny colonies i.e. < 2 mm in diameter appeared for only a short time and then disappeared. When counting colonies, 5 mm of tail from the edge of each irradiation field was not scored to avoid problems of cell migration and dose inhomogeneity at the edge of the irradiated field (Hendry, 1984). This was measured by holding the irradiated tail to match with a pre-marked scale. When less than half of the colony was within 5 mm from the irradiated edge as defined by the boundary of the pigment, this colony was included in the total numbers of colonies counted.

(b). Measurement of Irradiated Area

A total of 48 normal mouse tails and 4 sets of 12 irradiated healed tails (i.e. 2, 3, 4 and 6 cm tails) were used for the area measurement. The whole tail was cut from the dead body and fixed in Mirsky's fixative for 3 days. The location of the irradiated portion of tail was determined first on tails which had been irradiated and allowed to heal, so that the irradiated area was defined by a lack of pigment. This location was then used on control tails in order to measure the correct position on the tail. The skin was cut and removed, then mounted between a pair of glass slides for about 10 days until the whole skin was dried and the shape fixed. The irradiation area was measured for each of 12 tail samples and the areas for colony counting were measured in the middle 1, 2, 3 and 5 cm from 2, 3, 4 and 6 cm tails respectively.

(c). Total Number of Colonies Per cm^2

The total number of colonies per cm^2 was the maximum number of colonies counted at a particular day for each mouse during the scoring period, divided by the average skin area measured. The mean of these values

among the mice in a group gave the average total number of colonies per cm^2. The same criteria were used and the same measurements were made for skin receiving irradiation for the first time or receiving test radiation doses after various pretreatments.

2.4.3. Microcolonies

I. Skin Irradiated for the First Time

a). Sampling Criteria

It was noted in the pilot experiment that the level of skin reaction correlated well with the stage of microcolony development (see Results Section). It was decided to use erythema as a criteria for sampling skin irradiated with less than 22.5 Gy and severe erythema for doses more than 22.5 Gy (Plate 2.7). The detailed criteria and these results will be described in the Results Section.

b). Sampling Procedure

From day 10 to about day 21 the tail skin was checked very carefully everyday (in a few cases twice daily) under a lamp. Any mouse whose tail skin met the sampling criterion was selected and injected intraperitoneally with 0.01 mg per mouse of the mitotic inhibitor vincristine sulphate (Eli Lilly and Company Limited) three times with intervals of three hours. They were killed 3 hours after the last injection. The hairs were plucked from the tails using at least 8 applications of sellotape. The skin was dissected through the middle of the dorsal side and fixed in Carnoy's fixative (6 parts absolute methanol, 3 parts chloroform and 1 part glacial acetic acid) overnight. It was then transferred to 70% alcohol.

c). Histological Procedures

Procedure	Duration
Skin in 70% alcohol	
50% alcohol	5 min.
30% alcohol	5 min.
distilled H_2O	5 min.
distilled H_2O	5 min.
5N HCl (approx. 22°C)	90 min.
distilled H_2O	5 min.
distilled H_2O	5 min.
Remove connective tissue and hair follicles	
Display epidermal sheets on subbed slides	
Air dry	
Bring dry slides to distilled H_2O	15 min.
Schiff's staining	90 min.
*sulphurous acid solution (1)	5 min.
sulphurous acid solution (2)	5 min.
Running tap H_2O	30 min.
Air dry	
xylene	4 hours
Mounting with white Xam	

*sulphurous acid solution

concentrated stock solution

50 gm potassium metabisulphite

500 mls H_2O

Working solution

50 mls concentrated stock solution

50 mls 1N Hydrochloric acid

900 mls distilled H_2O

d). Colony Counting

In order to avoid problems of cell migration and dose inhomogeneity at the edges of the irradiated field, the same procedure was used as when counting macrocolonies - a 5 mm length of tail was not scored for colonies at both irradiation edges i.e. only colonies in the middle 2 cm of the irradiated 3 cm length of tails were scored. Before colony counting, each sample under the microscope was marked with a waterproof red pen at the edges of the irradiation field and at 5 mm from the edges. Colonies were identified as foci of high cell density with a mitotic index ranging from about 5% to about 50%, and with a lack of melanin (Plate 2.8a). Less than 1% of colonies were observed between the squame mounds on the tail i.e. associated with hair follicles. A surviving colony was defined as one containing at least 32 cells. This was estimated initially using cell number but later by colony area, since the number of cells per colony was proportional to the area of a colony. This was measured using a Kontron-Mop Videoplan system. The detail of this will be discussed in the Results Section.

II. Pre-Irradiated Skin (Including Aged Control)

a). Sampling Criteria

The same criteria were applied to the pretreated and lightly pigmented skin as for the normal heavily pigmented skin. Doses less than 22.5 Gy to the skin were sampled at the erythema endpoint. For doses more than 22.5 Gy, the skin was sampled when severe erythema had been reached (Plate 2.7).

Plate 2.7: Normal and pre-treated mouse tail skin showing the degree of skin reaction used for sampling. A, erythema (normal skin); B, severe erythema (normal skin); C, erythema (pre-treated skin); D, severe erythema (pre-treated skin).

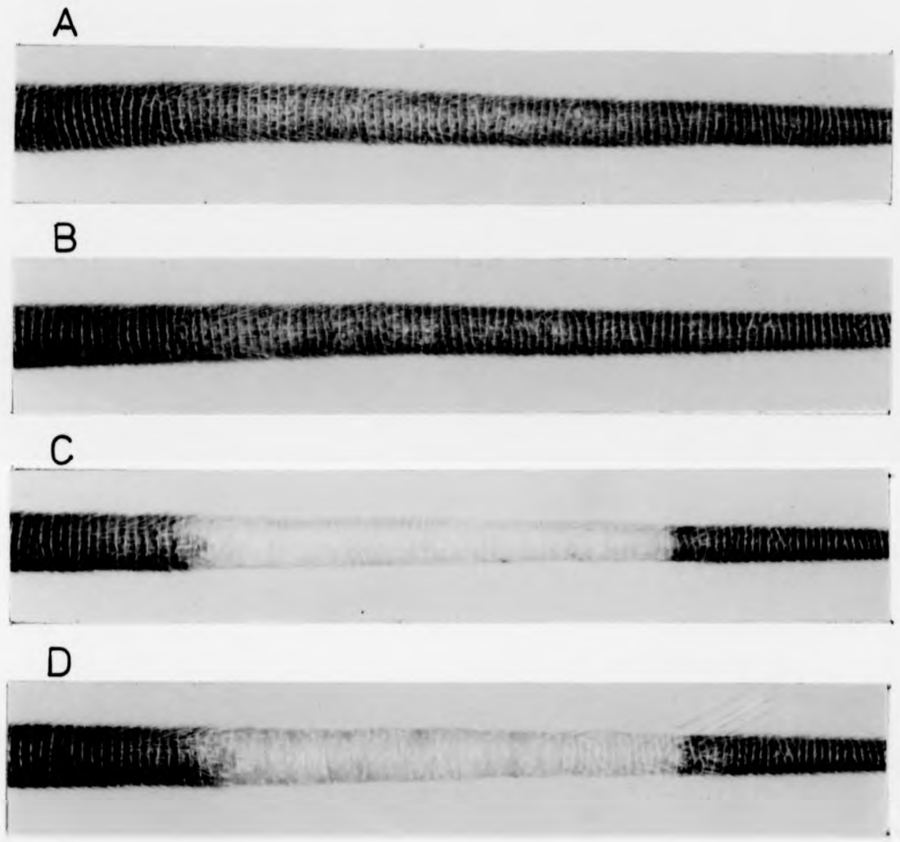
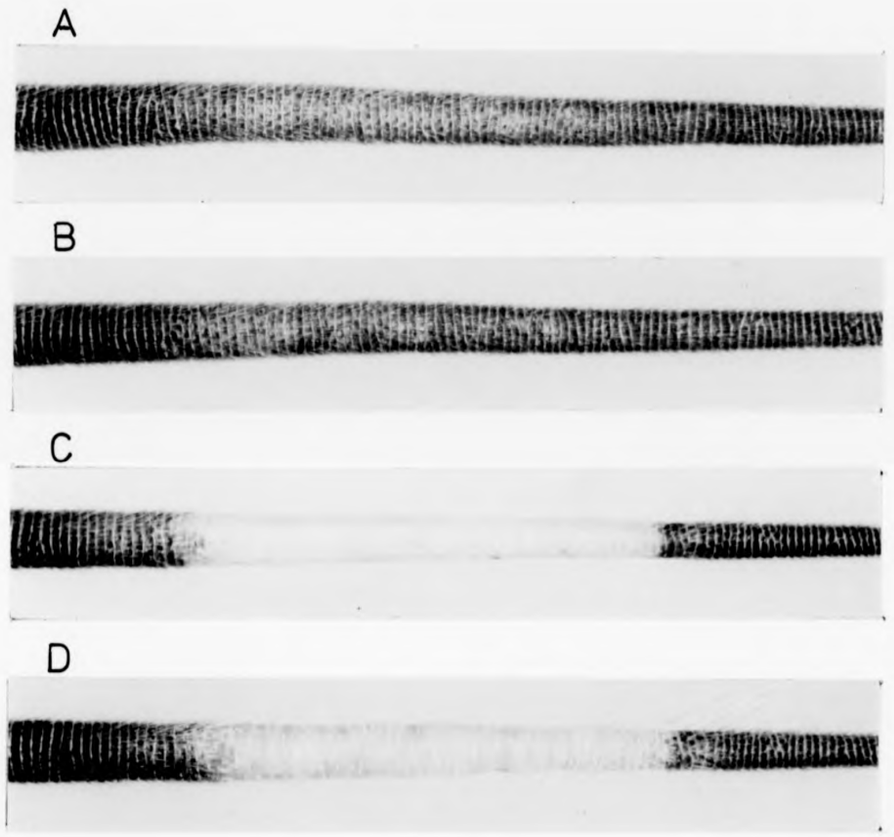


Plate 2.7: Normal and pre-treated mouse tail skin showing the degree of skin reaction used for sampling. A, erythema (normal skin); B, severe erythema (normal skin); C, erythema (pre-treated skin); D, severe erythema (pre-treated skin).



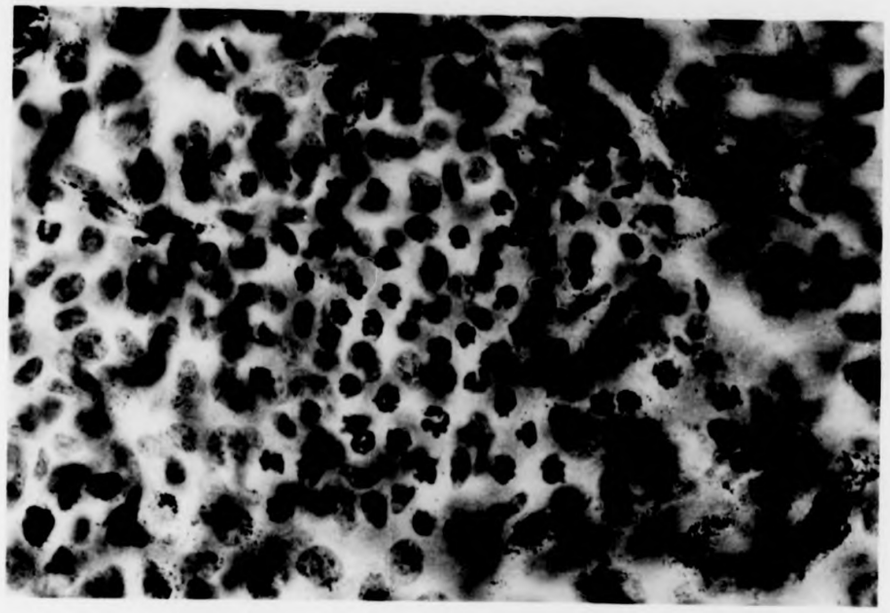


Plate 2.8a: A single microcolony at day 15 after 17.5 Gy, showing a high proportion of mitotic cells. X120.

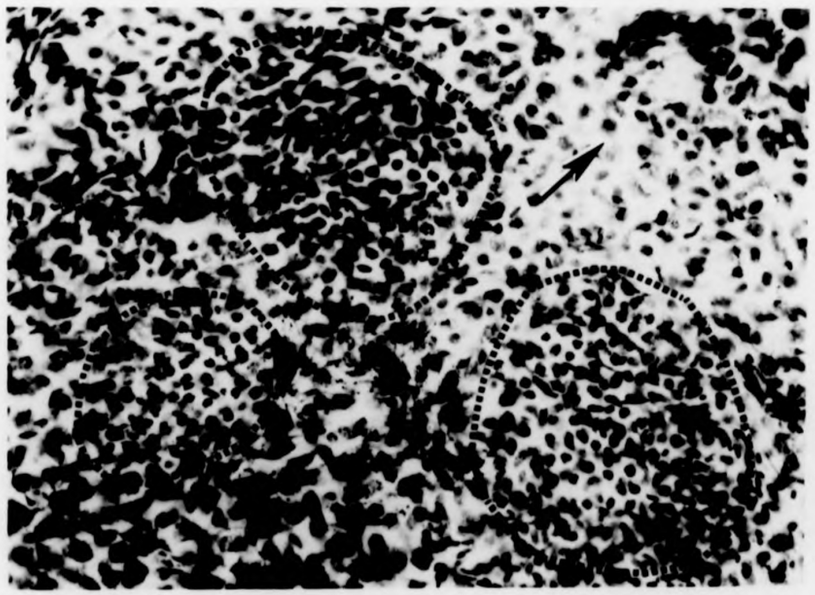


Plate 2.8b: Three neighbouring microcolonies at day 15 after 15 Gy. The arrow points to a hair follicle opening. X60.

Plate 2.8a: A single microcolony at day 15 after 17.5 Gy, showing a high proportion of mitotic cells. X120.

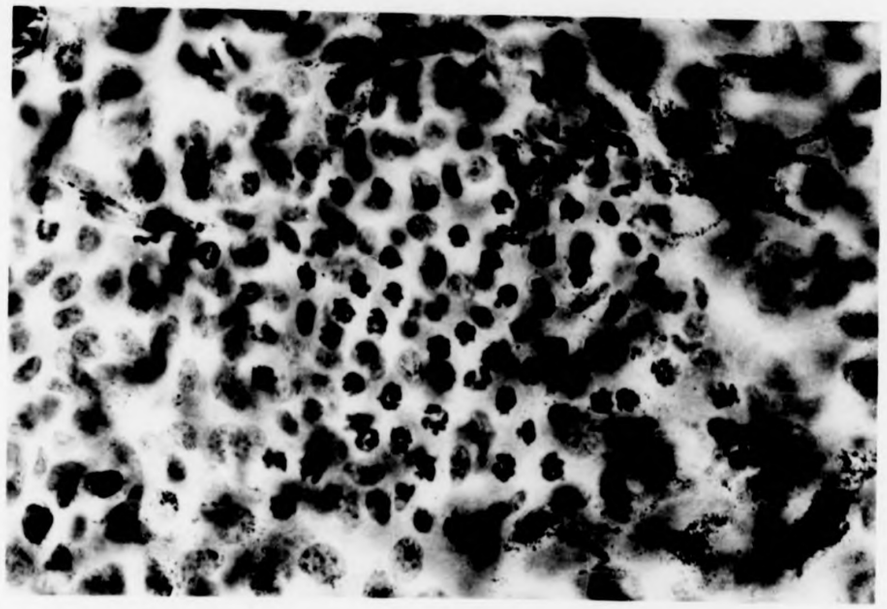
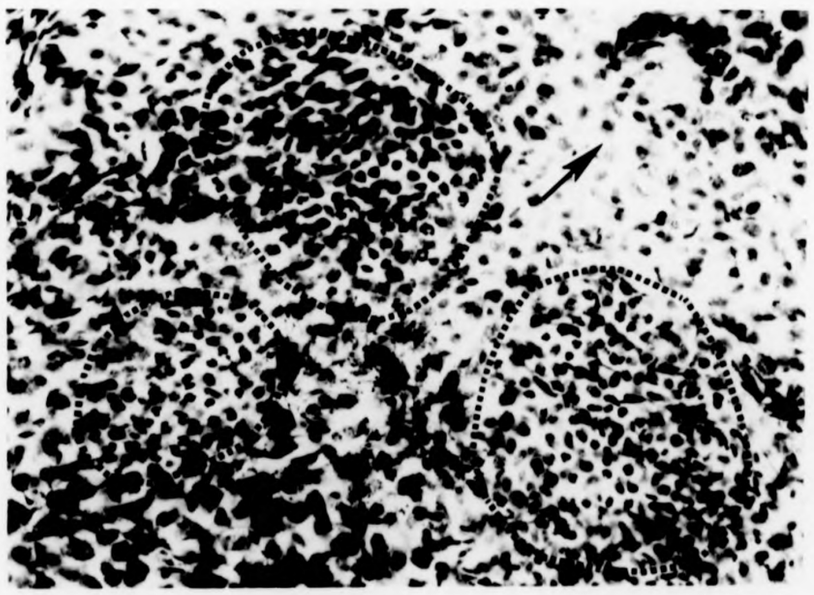


Plate 2.8b: Three neighbouring microcolonies at day 15 after 15 Gy. The arrow points to a hair follicle opening. X60.



b). Sampling Procedure

Three injections of vincristine sulphate were found to be insufficient to identify the colonies against the background of the pretreated skin, as there was no clear demarcation between the degrees of pigmentation in the colony and non-colony areas. This is in contrast to tails receiving the irradiation for the first time where there was a clear boundary between the non pigmented colony area and the heavily pigmented non colony area. Therefore a modification using autoradiography was considered necessary. Between day 10 and day 20 the irradiated mice were checked once each day, although in some special cases it was twice daily. The skin reaction was detected first in the cage under good illumination. The mice were then selected for a further close check to ensure that each mouse receiving the injection met the sampling criterion. After selection the mice were injected intraperitoneally using 2 doses of 925 kBq (25 μCi)/mouse tritiated thymidine with specific activity 925 GBq (25 Ci)/mmol (Amersham International) in 0.25 ml at 1.00 a.m. and 5.00 p.m. The mice were killed half an hour after the second injection, and the hairs removed using at least 8 applications of sellotape with much care. The skin was then fixed in 3% propionic acetic acid (BDH Chemicals) for about 24 hours.

c). Histological Procedures

Procedure	Duration
Separate epidermis under dissecting microscope	2 min.
Fix epidermal sheets in Carnoy's fixative	24 hours
Absolute alcohol	5 min.
70% alcohol	5 min.
50% alcohol	5 min.

Procedure	Duration
30% alcohol	5 min.
distilled H_2O	5 min.
distilled H_2O	5 min.
5 N HCl (approx. 22°C)	90 min.
distilled H_2O	5 min.
distilled H_2O	5 min.
Remove hair follicles under dissecting microscope.	20 min.
distilled H_2O	5 min.
Display epidermal sheets on subbed slides with basal layer upwards.	10 min.
Air dry	24 hrs.
Bring dry epidermal sheets into distilled H_2O.	15 min.
Schiff's staining	90 min.
sulphurous solution (1)	3 min.
sulphurous solution (2)	3 min.
Thoroughly wash with running tap H_2O	30 min.
Air dry	24 hrs.
Ready for autoradiographic procedures.	

d). Autoradiographic Procedures

The stained epidermal sheets on the slides were dipped 10 times in a 1:1 dilution of ILFORD K5 emulsion with distilled water at 37°C. This was made up at 50°C and filtered through a metal mesh to remove bubbles and undissolved emulsion fragments. After they had been allowed to dry they were put in a light-tight box, sealed with black tape and stored in a fridge at 4°C for 4 weeks.

Procedure	Duration
Kodak D19 developer (21°C)	20 min.
2% Glacial acetic acid	1 min.
ILFORD Hypam fixer	15 min.
Running tap water	60 min.
Air dry	24 hrs.

e). Colony Counting

The counting area was marked exactly the same as for the epidermal sheets made from skin irradiated only once. Colonies were identified as foci with high labelling index containing a total of 32 or more labelled plus unlabelled cells (Plate 2.9a or b). The unlabelled cells ranged from about 5 to 30% of the total cells depending on the size of the colony. Unlabelled cells were normally found within the boundary of labelled cells either in between labelled cells (Plate 2.9a or b) or in the centre of labelled foci (Plate 2.10).

2.5. DOSE INCIDENCE CURVES AND SURVIVAL CURVE CONSTRUCTION

a). The healing data for the different doses were analysed using the PROBIT computer program described by Gilbert (1969). This program uses minimum X^2 techniques and it calculates the best estimate of the ED_{50} and the probit width with their associated standard errors. The equation which is used is as follows:

$$\text{probit } (p) = \frac{D - LD_{50}}{W}$$

Where p is the mortality fraction, D is dose, LD_{50} is the dose for 50% response, W is the probit width. There is the facility for pooling different sets of data and fitting one or more common parameters between the different sets of data.

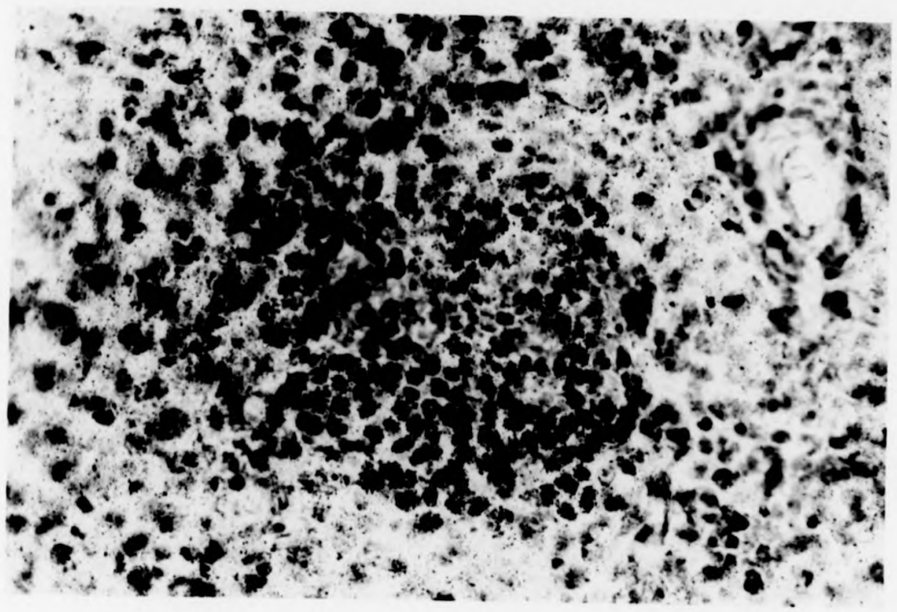


Plate 2.9a: A microcolony in skin at day 14 after 20 Gy showing high proportion of labelled cells with many pigment cells surrounding the colony. Some labelled cells in the middle of the colony are slightly out of the focus. X160.

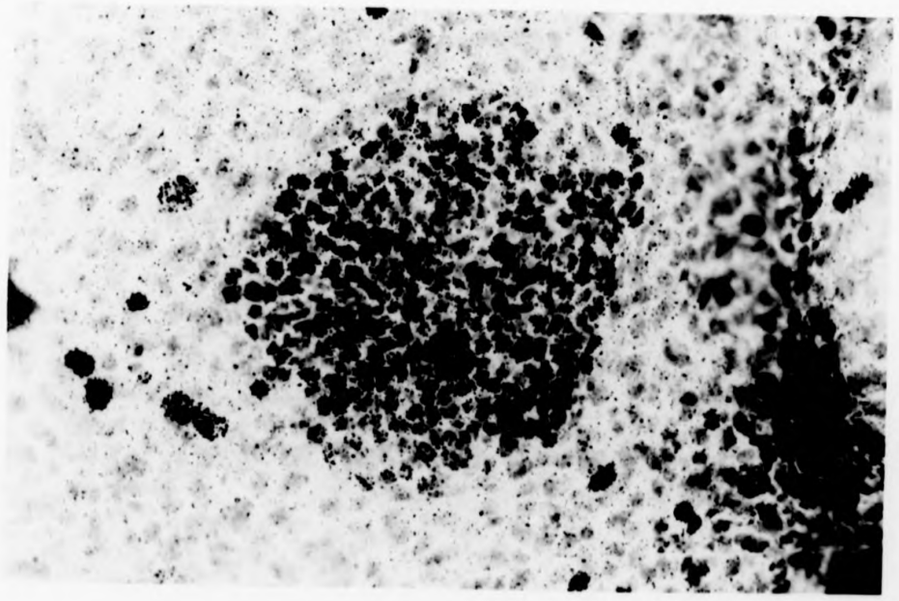


Plate 2.9b: A microcolony in skin receiving 3 previous treatments followed by a test dose of 20 Gy, showing a high proportion of labelled cells with a lack of pigment cells surrounding the colony. X160.

Plate 2.9a: A microcolony in skin at day 14 after 20 Gy showing high proportion of labelled cells with many pigment cells surrounding the colony. Some labelled cells in the middle of the colony are slightly out of the focus. X160.

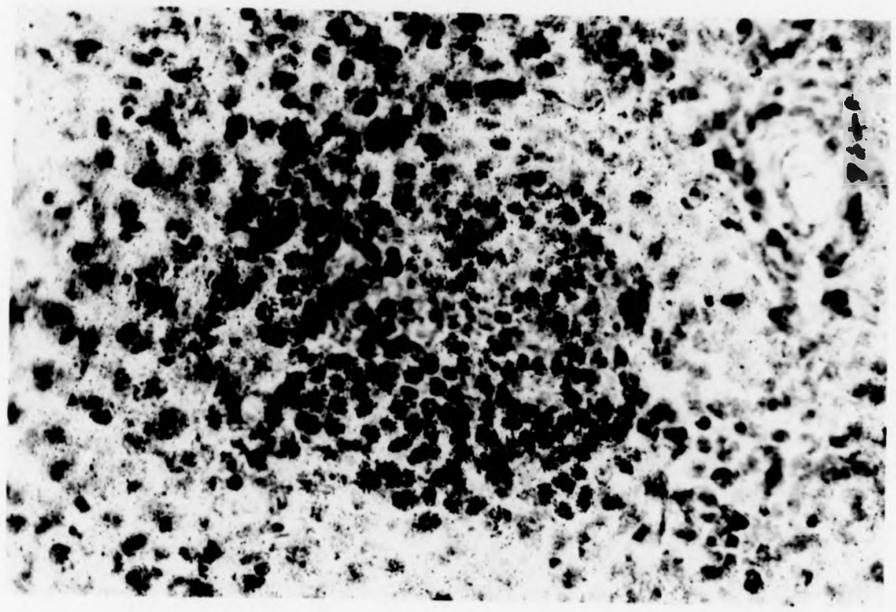


Plate 2.9b: A microcolony in skin receiving 3 previous treatments followed by a test dose of 20 Gy, showing a high proportion of labelled cells with a lack of pigment cells surrounding the colony. X160.

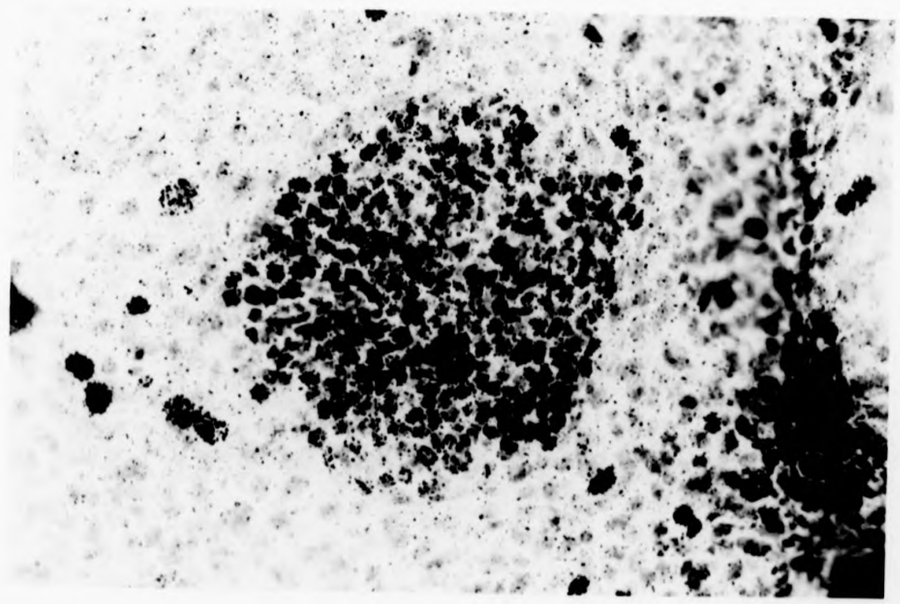


Plate 2.10: A microcolony in skin at 15 days after 20 Gy, showing a significant proportion of unlabelled cells in the middle of the colony. X160.

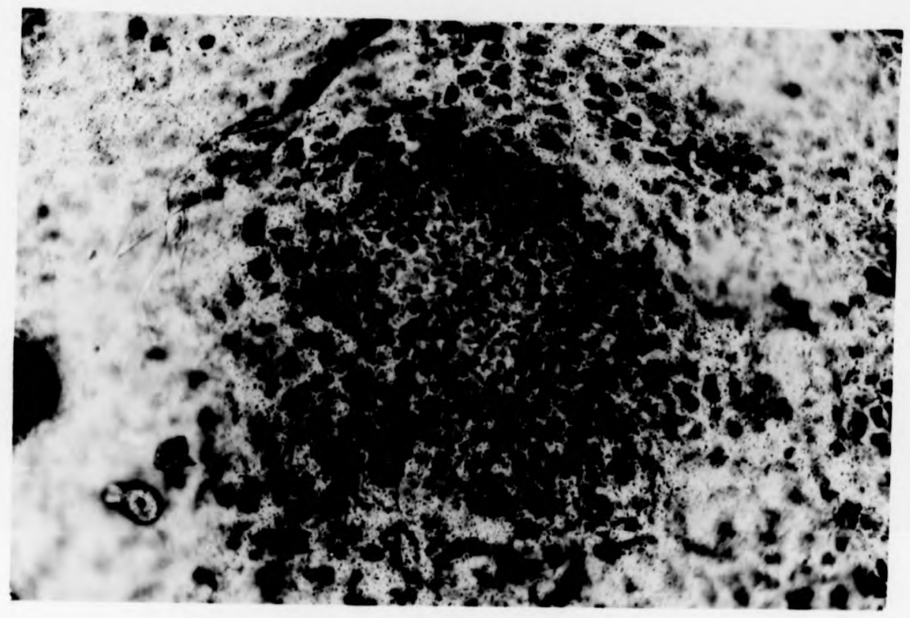
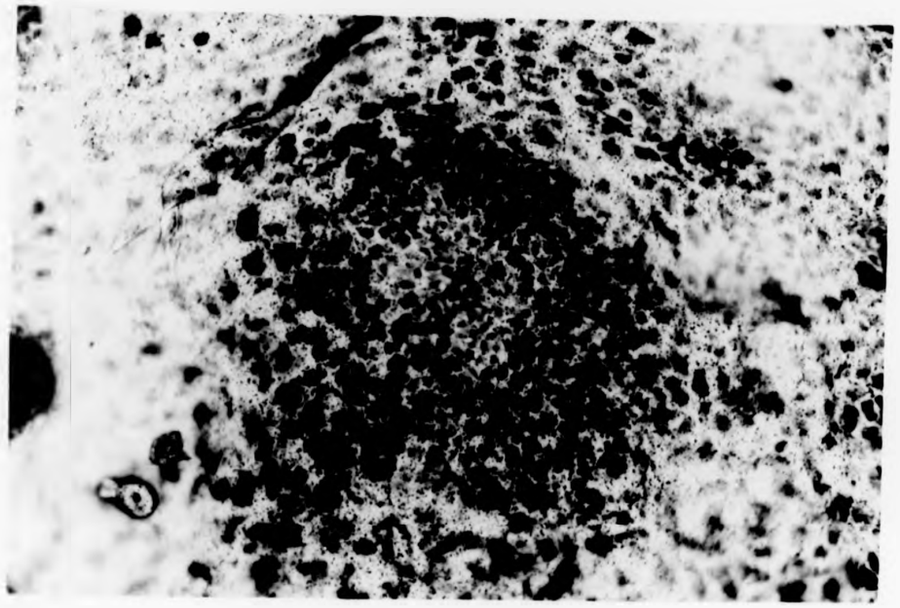


Plate 2.10: A microcolony in skin at 15 days after 20 Gy, showing a significant proportion of unlabelled cells in the middle of the colony. X160.



Another version of this program (DLOGS) was also used (Gilbert, 1974), in which the parameters LD_{37} and D_0 are calculated. D_0 applies for the target cells responsible for the gross tissue effects. The equation which is used is as follows:

$$-\ln(-\ln p) = \frac{D - LD_{37}}{D_0} \qquad \text{equation 2.1}$$

Where p is the mortality fraction, D is the dose and LD_{37} is the dose giving 37% response. It has been shown that D_0 and W are very similar in magnitude and D_0 equals approximately $W \div 1.2$ (Lange and Gilbert, 1968). The same statistical procedure and error calculation are used in both versions of the program. The data which are put into the calculation consist at each dose of the numbers of tails irradiated and the number of tails healed by 9 weeks. Binomial weighting factors are used. An example of the comparison of PROBIT and DLOGS fitting is given in Figure 3.31. Differences between experiments or between for example, the response of control or pre-irradiated skin were assessed using an F test. In this test the value of X^2 calculated for the pooled data is compared with the sum of the values of X^2 for the individual sets of data. If the values of X^2 were not significantly different a common curve could be fitted through all the data. An example of this procedure is as follows: If one set of data gives X_1^2, with N_1 degrees of freedom, then the sum of the values of X^2 for 2 sets of data is $X_1^2 + X_2^2$ with $N_1 + N_2$ degrees of freedom. Hence the average X^2 per degree of freedom:

$$F = \frac{(X^2 + X^2)}{N_1 + N_2}$$

The fitting of the pooled data will give X_3^2 with $(N_1 + N_2 + 1)$ degrees of freedom, because there is one less parameter being fitted. Thus there is $(X_3^2 - (X_1^2 + X_2^2)) = F3$ extra-X^2 for 1 extra degree of freedom. The ratio (F3/F1) is tested against $(N_1 + N_2)$ in a variance

ratio (F) test. e.g. Table V in Fisher and Yates. It is commonly found that when $(N_1 + N_2) >$ about 10, (F3/F1) must be greater than about 4 for a level of significance $P < 0.05$.

b). The colony data were computed using the computer program described by Gilbert (1969). This uses similar statistical procedures and test of significance to those in the above PROBIT analysis, but the equations being fitted are different. We used an equation of the form $S = n.e^{-D/D_0}$ where n and D_0 are the calculated parameters with associated standard errors. The origin on the survival curve at zero dose was unknown: we used an appropriate estimate so that n appeared as a small integer. The value of n was determined from experimental data using split dose experiments where a range of second doses was given after a priming dose.

Much of the colony data was fitted using the DLOGS program. This was made possible by making the level of survival artificially low. In this case $-\ln(-\ln P)$ in equation 2.1 approximates to $-\ln(1-P)$ or $-\ln S$ where S is the surviving fraction. Hence $-\ln S = \frac{D - LD_{37}}{D_0}$

This is identical with the equation $S = n.e^{-D/D_0}$ (see above), where $LD_{37} = Dq = D_0 \ln n$ where Dq is the conventional Quasithreshold dose. Thus, providing the level of survival was made artificially low, so that the above approximation can be made, the DLOGS program could be used also for fitting the colony data and cell survival parameters could be calculated.

c). Correction of macrocolony and microcolony data.

A discrepancy between the microcolony and macrocolony survival curves may be expected if there is coalescence of several microcolonies to form a macrocolony after the lower doses. This possibility was tested at several dose levels by counting the number of pairs (or groups) of microcolonies in a total of between 20 and 80 microcolonies (depending on the dose), where the distance between microcolonies was less than 3.05 mm (the average diameter of a macrocolony). When each pair was counted as 1 colony, predicted values of macrocolony survival could be calculated from microcolony data. To demonstrate this mathematically, a Poisson distribution of surviving cells was assumed. If there are m microcolonies per cm^2 at a given dose, and the average area of a macrocolony is A, there will be (mA) microcolonies in the average area of a macrocolony. The probability of there being no microcolonies in this area is exp(-mA), and of only one microcolony in this area is [mA exp(-mA)]. Also, the probability of there being one or more than one microcolony is [1-exp(-mA)].

Hence the number of macrocolonies will be less than the number of microcolonies by a factor:

$$\frac{1- \exp(-mA)}{mA. \exp(-mA)}$$

A similar exercise to calculate the probability of microcolonies arising from more than one microcolony-forming cell was also calculated.

d). Quantitation of Residual Injury

Residual injury was quantified by two methods. First of all by the ratio of the numbers of colonies in the pre-irradiated skin and the aged control skin receiving a fixed test dose. Secondly, the decrease in the

number of colonies caused by the pre-treatment was converted into a reduction in dose to give the same number of colonies. This was done by assuming that the survival curve for the pre-treated skin had the same slope as the survival curve for the aged control skin (Figure 2.11).

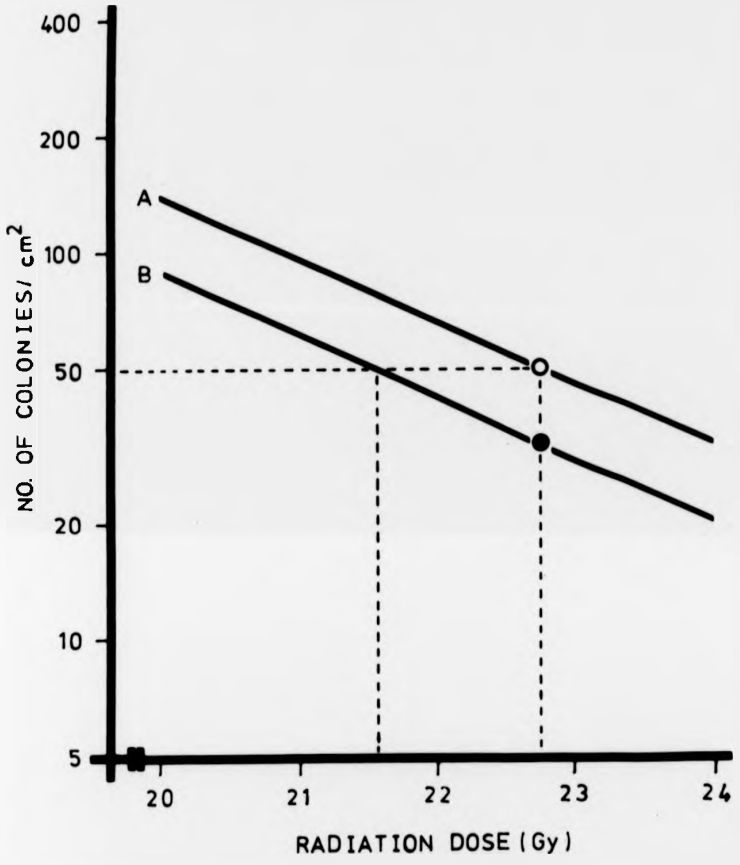


Figure 2.11: The calculation of the percentage residual injury. Curve A (○), survival curve for the aged control skin; curve B (●), assumed curve for the pre-treated skin. When specified level of survival set at 50 colonies/cm^2, the corresponding dose was 22.8 Gy for the control skin and 21.6 Gy for the pre-treated skin. The ratio of these doses was 0.95, so that there was 5% of residual injury.

SECTION THREE

RESULTS

3.1. RESPONSE TO SINGLE DOSES

3.1.1. Effects of Radiation on the Tail Skin: Microcolonies

Because this is a new technique in assaying the effects of radiation on epidermis, a number of experiments was done in order to test the consistency and validity of the new technique. In this section therefore, two types of data will be described. Results obtained from each development stage, and the dose response relationship using this microcolony assay, are presented separately in the following sub-sections.

I. Development of the Microcolony Assay Technique

The development of the microcolony technique was divided into several steps. Each of these steps involved specific problems and it was necessary to solve these before dose-response curves could be measured. In the first step, it was necessary to find out whether any detectable microcolonies were produced by using the dose of radiation or the time of sampling used in similar work published in the literature, then applying a standard histological procedure and looking for microcolonies. The method described by Mitani and Potten (personal communication) was used as a basis in the present work. Many modifications, such as the dose of radiation, the time of sampling, and the histological procedures, were made before the first typical microcolony was found. I then proceeded to the second step namely to find out (1) the best histological techniques for the preparation and staining of the epidermal sheets; (2) the optimum sampling time; (3) the optimum sampling criteria; (4) the range of radiation dose that can be readily assessed using this technique. This resulted in the optimal techniques and procedures attainable, being adopted. They were

described in detail in Section 2.4.3 in the Materials and Methods. The third step involved the problems of colony counting. The relevant questions are listed below: (1) How to determine a surviving colony ? e.g. > 32 cells, high mitotic index, or area of depigmentation. (2) Would any correlation between number of cells versus area be capable of being applied to any dose range, so that colony area could be used as an easier criterion for scoring? (3) Could the samples be assayed using the same criterion but at different time points after irradiation ? (4) Has a day 18 colony the same size as one assayed at day 14 in another sample? Several approaches have been employed to answer these questions and the results will be described separately below.

(a). Sampling Criteria

Regarding the criterion which determines the choice of the sampling time, a test was made using the skin reaction. The choice was based on the belief that the time of a given skin reaction reflected the time of severe cell loss and a certain level of regeneration of colony-forming cells. As it is important to know if the same criterion can be applied to the whole range of doses used, a total of 192 mice was tested using 3 different criteria. At first survival to single doses was measured using "erythema" as a criterion. The data were fitted by eye and are shown in Figure 3.1. As can be seen there was a curvature at high doses in the survival data plotted on a log/linear graph (open circles, Figure 3.1). However, it was noticed that there were many small colonies after high doses, and when the degree of skin reaction used as the criterion was increased to severe erythema, survival after high doses increased by a factor of up to about 5 (crosses, Figure 3.1). No further increase was observed when instead a criterion of slight

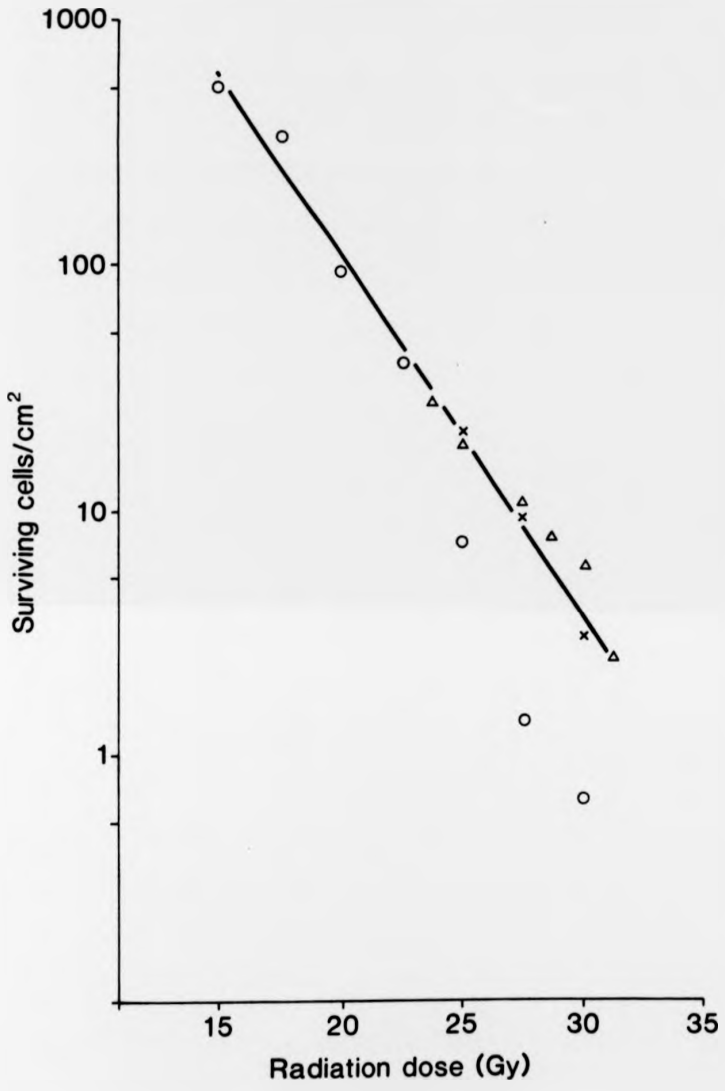


Figure 3.1: Surviving colony-forming cells per cm^2 versus radiation dose. Standard sampling errors are about 3% of the mean after the lower doses, increasing to about 30% after high doses. Tail skin assayed when showing erythema (O), severe erythema (X) or slight desquamation (Δ). Line fitted by eye through the circles up to 22.5 Gy together with the crosses (see text).

desquamation was used (open triangles, Figure 3.1). At 23.5 Gy the level of survival using a criterion of slight desquamation follows the curve generated using erythema as the criterion. This dependence of survival on the criterion used to determine the assay time was therefore a feature only of the higher range of doses. After the lower range of doses virtually all the colonies consisted of more than 32 cells when erythema had appreared. In subsequent experiments it was decided to use the appearance of erythema as the time for sampling for doses up to 22.5 Gy, and severe erythema for higher doses. Severe erythema was not used for doses less than 22.5 Gy because of some confluence of larger colonies at these later sampling times after low doses.

b). Colony Definition

A colony in the present assay system was defined as a focus containing at least 32 cells most of which are actively dividing. An area of depigmentation was normally found (Plate 2.8a) where unpigmented actively dividing cells were growing and spreading outwards. These dividing cells tended to push the existing pigmented cells outwards when the former were increasing their numbers and expanding the colony area, and therefore, in most cases a discrete colony could be identified very easily.

Whether the colony in the depigmented area should contain a high proportion of mitotic cells or not at the time of assessment is an important point. With reference to this, a Kontron videoplan system was used to measure the mitotic index from a total of 240 colonies after 20 and 25 Gy, the number of cells per colony ranged from 10 cells up to

more than 292 cells. The mitotic index ranged from as low as 2% to as high as 97% among the different sizes of colonies including those <32 cells. This range was largely dependent on the size of the colony, but not totally so, since some smaller colonies (e.g. <64 cells) had low mitotic indices, whereas some larger colonies (e.g. >128 cells) had high mitotic indices. In general, smaller colonies tended to have higher mitotic indices than larger colonies. This is shown in the summarised data listed in Table 3.1, and is shown in Figure 3.2. In colonies containing between 32 and 64 cells the mean mitotic index was 31 ± 2.6%, and in a colony containing between 98 and 130 cells, the mean mitotic index dropped to 16 ± 2.1%. When all 240 colonies were analysed and divided into 50 classes, the median mitotic index was about 14%. Based on this analysis, a combined histogram is shown in Figure 3.3. Although mitotic index is a good marker to show an individual colony, it is not good enough to use as the only criterion in determining a colony, because of the very small mitotic index in some colonies. The presence of mitotic cells, irrespective of the mitotic index, together with an area of depigmentation was found to be a more reliable way to identify the surviving colonies. This was used for all colony counting in the vincristine treated samples.

As stated in Section 2.4.3, colonies arising in the re-irradiated skin were identified based on a group of labelled cells instead of mitotic cells. In order to evaluate the relationship between the colony size (number of cells) and the labelling index, a total of 10 tails irradiated with different doses ranging from 17.5 Gy up to 32 Gy were used. The colonies were assessed by counting the number of labelled as well as unlabelled cells, and the results are shown in both Table 3.1 and Figure 3.2. Unlike the mitotic indices the labelling indices were

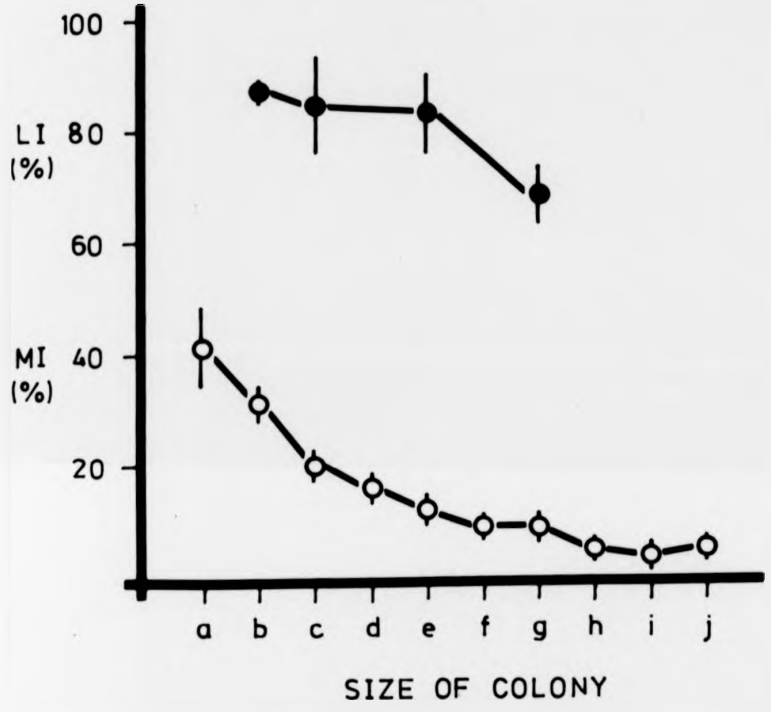


Figure 3.2: The relationship between colony size and mitotic index (lower panel, open circles) or labelling index (upper panel, closed circles). The following letters represent ranges of cells per colony. a: 10-31 cells, b: 32-64; c: 65-97; d: 98-130; e: 131-163; f: 164-195; g: 196-227, h: 228-259; i: 260-291; j: 292 cells. One exception is letter g in the labelling index panel which represents all colony sizes more than 196 cells.

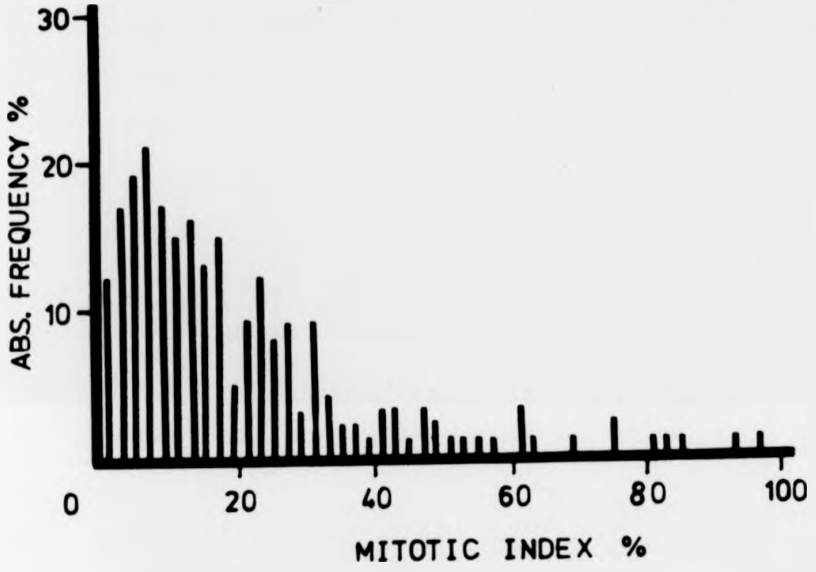


Figure 3.3: Frequency of colonies versus their average mitotic index.

in a much narrower range, from about 70% to 94%. The mean labelling index was about 85% for colonies consisting of 32 cells to 163 cells. When the colony size increased to more than 196 cells the labelling index dropped to about 70%. The identification of a colony from tritiated-thymidine-treated samples depends on the labelled cells which formed a discrete area with a covering of tiny black grains over the colony. The labelling procedure proved to be very efficient when assaying microcolonies in the previously irradiated and unpigmented skin. On the contrary it was very difficult to identify colonies in the non-pigmented skin using mitotic cells as a marker for colony counting. This is because the mitotic index showed a large variation between colonies, and also it is difficult to set a boundary between colonies and background cells without the pigment surrounding the colonies. For this reason, all skin treated previously with tolerance doses was assayed using ^{3}H-TdR and the autoradiographic technique.

TABLE 3.1: The Relationship Between Size of Colony and Mitotic or Labelling Indices

SIZE OF COLONY (NO. OF CELLS)	MEAN MITOTIC INDEX ± S.E. %	MEAN LABELLING INDEX ± S.E. %
10- 31	41±7.0	
32- 64	31±2.6	87±1.5
65- 97	20±1.5	85±8.5
98-130	16±2.1	
131-163	12±2.2	83±7.0
164-195	9±1.1	
196±227	9±1.7	69±4.5*
228-259	5±0.6	
260-291	4±2.0	
>292	5±1.1	

* This value applies for colonies containing more than 196 cells.

c). Relationship Between Number of Cells and Colony Area

The main purpose of measuring the relationship between the number of cells and the colony area was to be able to count quickly the number of colonies using the area of each as a measure of cell number. For this study a total of 268 colonies was used. The number of cells in a colony and its area were measured and analysed using the Kontron videoplan. A linear regression of the number of cells per colony as a function of the area of the colony was calculated and yielded a correlation coefficient of 0.95. In this case the number of 32 cells corresponded to 6290 μm^2 (see Figure 3.4).

d). Comparison of the Relationship Between Number of Cells and Colony Area at Different Doses

The relationship was measured between the number of cells and the colony area in the different dose groups (e.g. 15, 17.5, 20, 22.5 and 25 Gy). A total of 75 colonies with 15 colonies per group was used for this analysis. A wide range of colony size was selected, measured and analysed using the Kontron videoplan system. As can be seen from Figure 3.5 there is no consistent trend towards larger or smaller colony areas when the radiation dose was increased. Good correlation coefficients between numbers of cells and areas for different dose groups were obtained. They were 0.95, 0.95, 0.97, 0.90 and 0.96 for 15, 17.5, 20, 22.5 and 25 Gy respectively.

e). Percentage of Mice Assayed for Microcolonies, and Colony Size, in Relation to the Time of Assay

The assay time for 85% of the mice was between 13 and 17 days after irradiation (Table 3.2). To demonstrate the validity of using a

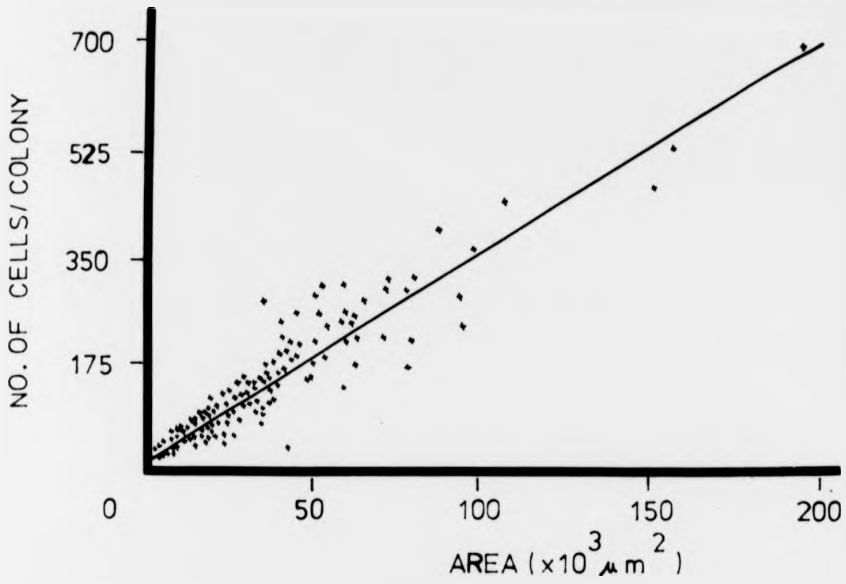


Figure 3.4: The relationship between the number of cells per colony and the area.

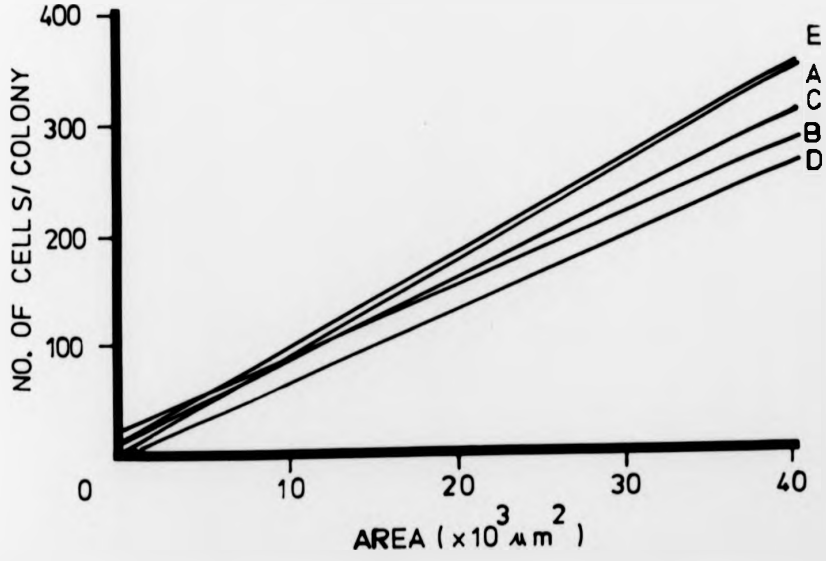


Figure 3.5: Comparison of the number of cells versus colony area after different doses. A,B,C,D and E represent 15, 17.5, 20 22.5 and 25 Gy respectively.

TABLE 3.2: Percentage of Mice Assayed for Microcolonies, and Colony Size

	TIME AFTER IRRADIATION (DAYS)											
	10	11	12	13	14	15	16	17	18	19	20	21
% of mice assayed	1	2	4.5	16.5	19	24.5	12	10	3.5	3.5	1.5	2
Colony No. ÷ mean from all assay times	-	-	-	0.96±0.06	0.96±0.11	1.09±0.1	0.83±0.08	1.05±0.16	1.15±0.13	-	0.88±0.05	-
Mean No. of cells per colony	-	-	-	-	76.5±3.2	68.2±2.7	75.1±2.8	74.9±3.6	78.7±3.5	-	-	-

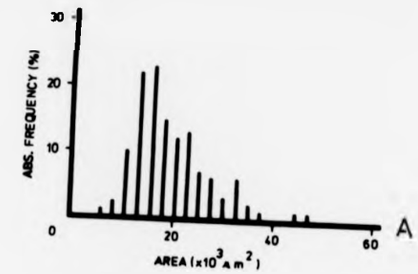
specified level of skin reaction as the criterion for choosing the time for scoring colonies, rather than using a fixed time interval, the mice used over all doses to generate Figure 3.1 were grouped according to the day they were assayed. At each time, the colony count for a particular mouse was divided by the mean count in the group of mice used at a given dose. The average values of these ratios at each day after all doses, are given in Table 3.2. The values do not increase significantly with the time of assay, which would be the case if more colonies are arising at later times. Also, in these same groups, the mean number of cells per colony was similar at all assay times between 14 and 18 days (Table 3.2). The frequency distribution of colony sizes is shown in Figure 3.6 where a sample was assayed on each day between day 14 and 18 after 21.25 Gy.

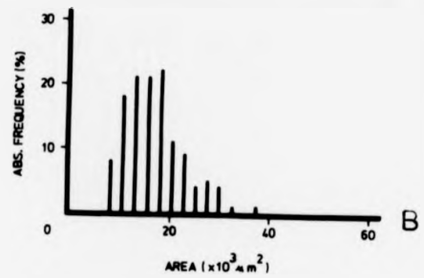
f). Correction of Macrocolony and Microcolony Data

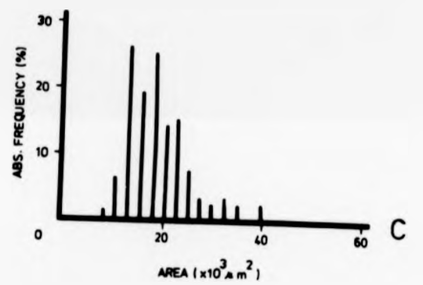
Mathematical ways to correct the macrocolony data for coalescence of colonies were described in Section 2.5(c). To calculate the expected number of macrocolonies arising from the number of microcolonies the following factor (F) was used.

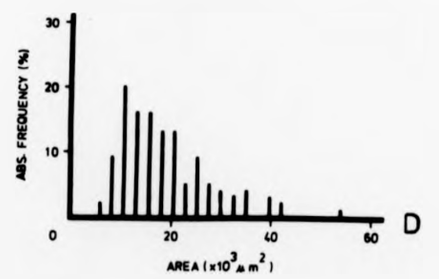
$$\frac{((m.A).e^{-m.A})}{(1-e^{-m.A})}$$

Microcolony counts per unit area were multipled by this factor. Examples of results obtained using this factor are listed in Table 3.3 and Table 3.4. The former table presents predictions of the number of macrocolonies arising from microcolonies, and the latter table shows the effect of microcolony confluence on microcolony scoring. In the case of the macrocolony data, the factor F tends to unity for low values of m (no. of microcolonies per cm^2). The number of macrocolonies at each dose predicted using the microcolony data and the above F factor









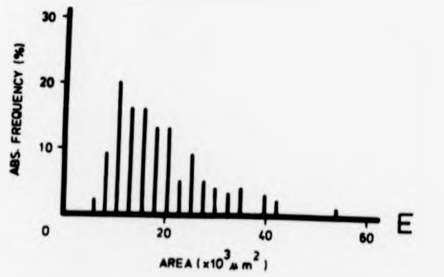


Figure 3.6: Clone-size distributions for five mice each assayed on a different day after 21.25 Gy, from day 14 (labelled A) to day 18 (labelled E). The skin reaction at the time of assay was erythema for all five mice.

Figure 3.6: Clone-size distributions for five mice each assayed on a different day after 21.25 Gy, from day 14 (labelled A) to day 18 (labelled E). The skin reaction at the time of assay was erythema for all five mice.

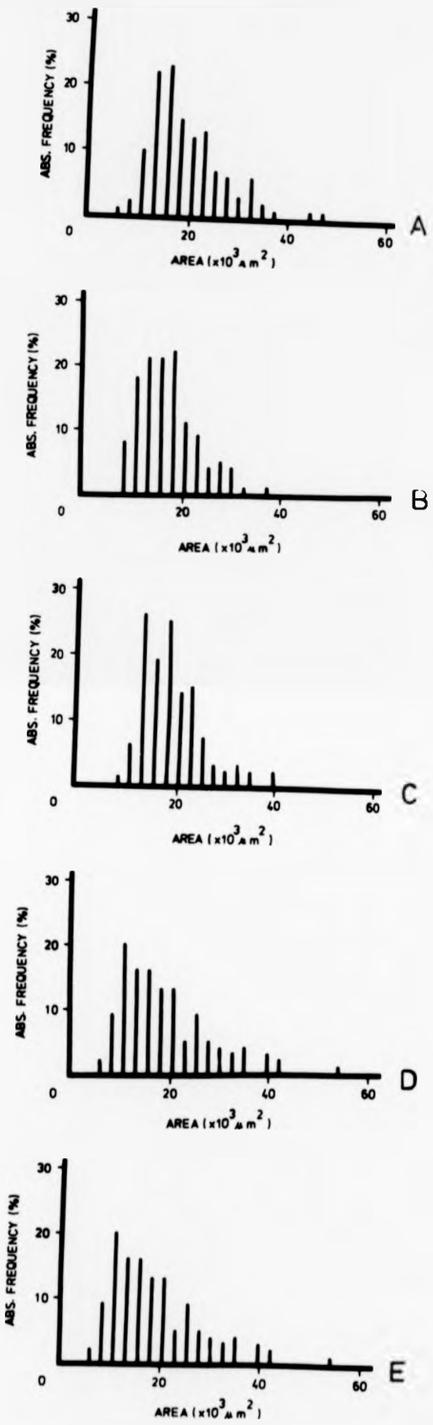


TABLE 3.3: Prediction of Number of Macrocolonies From the Microcolony Data

RADIATION DOSE (cGy)	(a) NO. OF MICRO-COLONIES PER CM^2	(b) CORRECTION FACTOR (F)	(c) PREDICTED NO. OF MACRO-COLONIES PER CM^2	NO. OF MACRO-COLONIES PER CM^2 CALCULATED FROM MEASUREMENTS OF DISTANCE BE-TEEN MICROCOLONIES	OBSERVED NO. OF MACRO-COLONIES PER CM^2
2600	14.76	0.457	6.75	6.18	-
2750	8.47	0.651	5.51	4.04	-
2800	7.06	0.702	4.96	3.97	-
2875	5.35	0.767	4.10	2.94	-
3000	3.35	0.849	2.84	2.24	2.50
3125	2.12	0.903	1.91	1.18	1.90
3200	1.59	0.926	1.47	1.47	1.55
3400	0.76	0.964	0.73	1.18	0.95
3600	0.35	0.983	0.34	0.29	0.58

$c = a \times b$.

TABLE 3.4: Prediction of Microcolonies Arising From More Than One Microcolony

RADIATION DOSE (cGy)	OBSERVED NO. OF MICRO-COLONIES PER CM^2	CORRECTION FACTOR (F)	PREDICTED TOTAL NO. OF MICRO-COLONIES PER CM^2
1500	802	1.067	856
1750	422	1.035	437
1875	224	1.018	228
2000	116	1.009	117
2125	78	1.006	78
2250	40	1.003	40
2500	13	1.001	13
2600	9	1.001	9
2750	6.6	1.001	6.6
2800	6.3	1.001	6.3
2875	4.7	1.001	4.7
3000	3.3	1.001	3.3
3125	1.5	1.001	1.5
3200	1.7	1.001	1.7
3250	1.8	1.001	1.8
3400	1.4	1.001	1.4
3600	0.3	1.001	0.3

(dashed curve in Figure 3.7) was very similar to the number of macrocolonies calculated from the microcolony data by reducing the number of microcolonies to allow for the number of pairs of microcolonies, where the distance between each pair was less than 3.03 mm i.e. the average diameter of a macrocolony (open circles in Figure 3.7). The measured survival curve for macrocolonies (curve B, Figure 3.7) was above the curve for microcolonies after high doses. This was due to the greater weighting of the low dose points in the computer fitting because of the greater number of colonies scored.

In the calculation of the probability of microcolonies arising from more than one microcolony, the parameter A, the average area of a microcolony (in this case a value of 16080 μm^2) and an average number of 80 epidermal cells per colony were used. As shown in Table 3.4, there was only a 6% underestimate of colony number at 15 Gy, decreasing to 1% at 20 Gy. This much smaller correction for microcolonies than for macrocolonies was due to the much smaller size of microcolonies compared with macrocolonies.

g). Split-dose Experiment

An experiment was designed to measure the ratio of survival using equal split doses, and varying the time interval between them. There were six time intervals (i.e. 0, 6, 8, 10, 12, 18 and 24 hours) in this experiment, with 12 mice for each time interval. The highest recovery was found using an interval of 10 hours, giving a ratio of survival levels of 7.1. The ratio was 3.8 using 24 hours. The comparison of the present data with other data in the literature will be described in the Discussion Section.

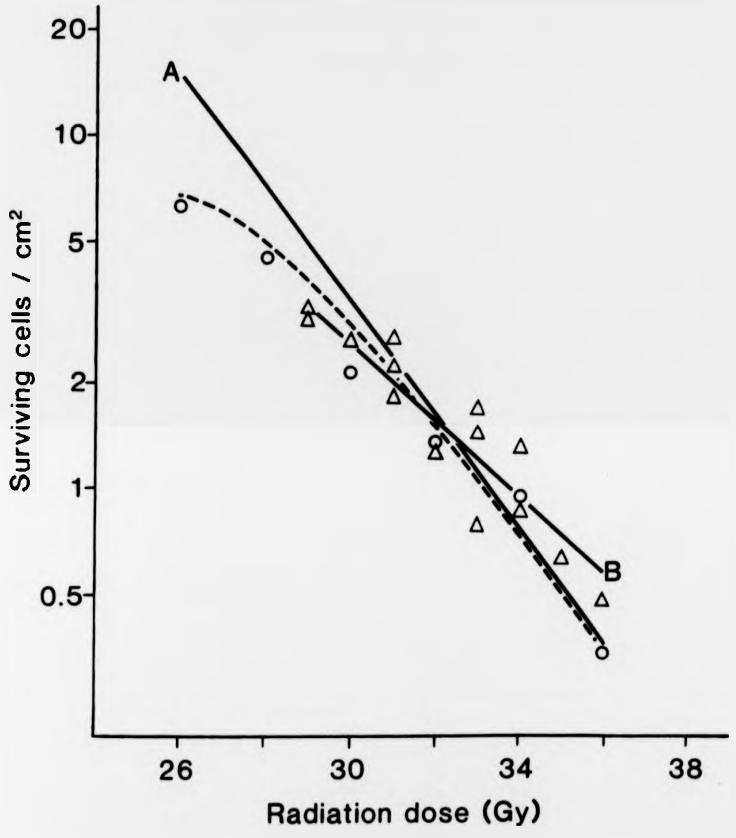


Figure 3.7: Macrocolony and microcolony survival data. (△) and line B. macrocolonies. Dashed curve, macrocolony survival predicted mathematically from the microcolony curve. (○), macrocolony survival predicted from measurements of the distances between microcolonies (see text). Line A taken from Fig.3.8.

II. Survival Curve for Microcolony-Forming Cells

Two experiments were performed using either single or split doses, and a total of 432 mice were used in this investigation. They were divided into 36 dose groups with 12 mice per dose group. 20 dose groups were used for the single dose experiment and 16 dose groups were used for the split dose experiments. Single doses ranging from 15 Gy to 36 Gy were tested. In the split dose experiment, a dose of 17.5 Gy was given 24 hours before the second graded doses were delivered. Second doses ranging from 1.5 Gy to 20 Gy were used. Survival curves were fitted to the data points using a multitarget equation including a single-hit term, in the computer program described by Gilbert (1969). The survival curve measured in the two experiments is shown in Figure 3.8 (left curve). For the pooled single-dose data, the D_0 = 2.70 ± 0.12 Gy and n (right ordinate) = 2.2 x 10^5 per cm^2. In order to produce a complete survival curve for these colony-forming cells, a split-dose experiment was performed where it was assumed that the response to a series of second doses after a priming dose was unaffected by the latter (Hendry, 1979). On the basis of the results obtained using two equal doses of 17.5 Gy, separated by intervals of time from 6 to 24 hours (Figure 3.9), it was decided to obtain a survival curve at 24 hours after 17.5 Gy, which is shown in Figure 3.8 (right curve) using data pooled from 2 experiments. The origin of the second-dose curve is the survival level at 17.5 Gy on the fitted curve for single doses. The D_0 = 3.13 ± 0.29 Gy; n = 2.1 ± 0.4. The data are expressed as surviving colony-forming cells per cm^2 on the right ordinate and as surviving fraction on the left ordinate. The latter procedure was accomplished by assuming that the second-dose curve reflected the initial shape of the single-dose curve (Hendry, 1979), shown as a dashed curve joining the single-dose curve to the

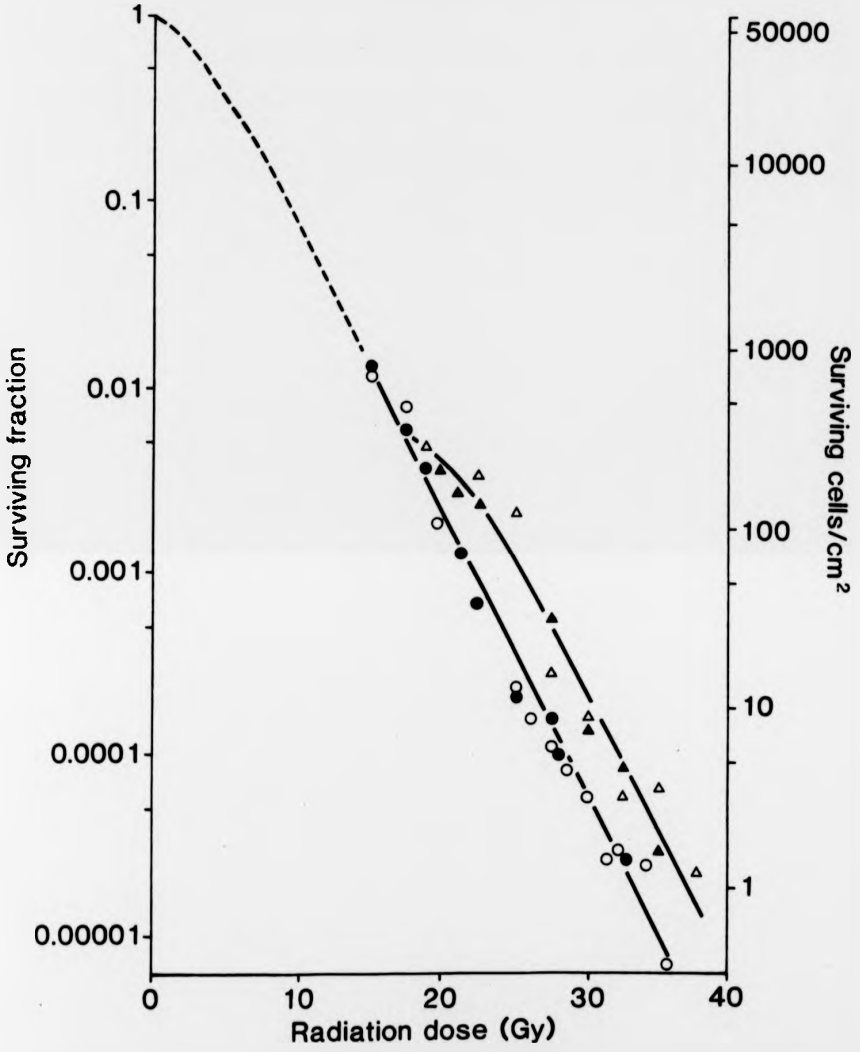


Figure 3.8: Survival after single doses (○,●), or a range of second doses given 24 hours after a first dose of 17.5 Gy (△,▲). The origin of the latter curve is the value of survival at 17.5 Gy on the curve fitted to the single dose data. Open and closed symbols represent two separate experiments. Sampling errors (not shown) as stated for Figure 3.1 . The dashed curve represents the initial part of the single dose curve, assuming it has the same shape as the initial part of the second dose curve.

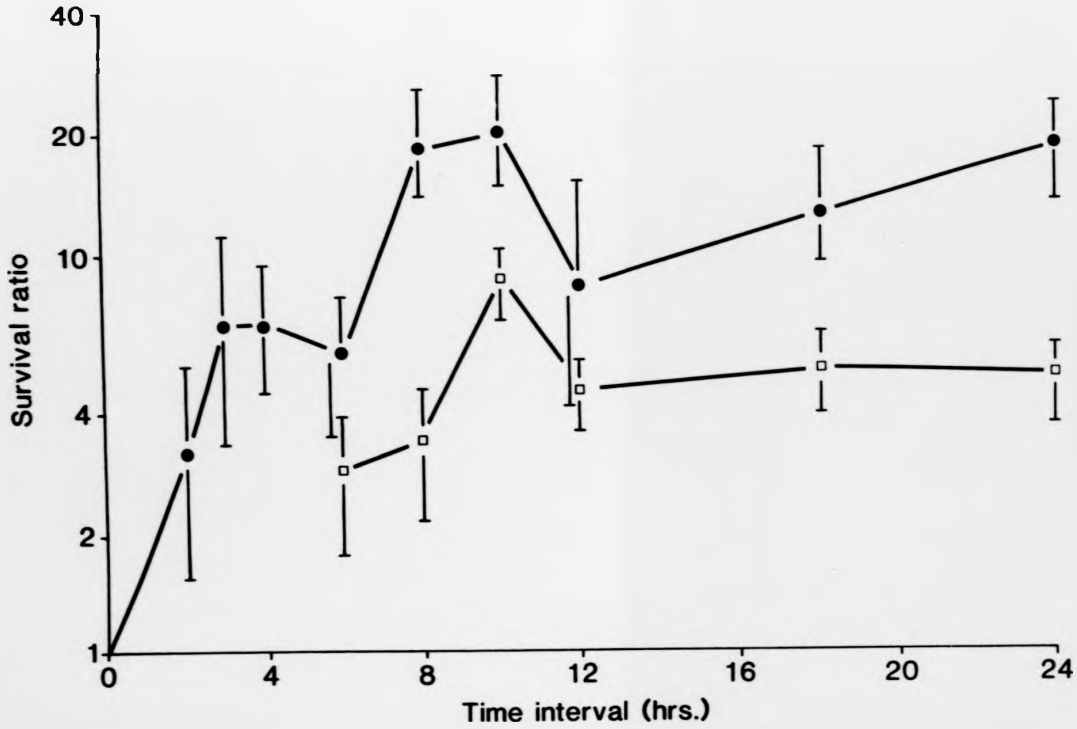


Figure 3.9: Survival ratio versus the time interval, using two equal doses of 17.5 Gy. Present data (□); data taken from Withers (1979a) (●). Bars are 95% confidence limits.

origin in Figure 3.8. In this case the surviving fraction after a first dose of 17.5 Gy was assumed the same as that measured from 17.5 Gy given as a second dose (right curve, Figure 3.8). Hence the total surviving fractions after both first and second doses could be calculated, and these are shown on the left ordinate in Figure 3.8. The origin corresponded to 59000 cells per cm^2 (right ordinate). The "complete" single-dose curve was characterised by D_0 = 2.71 ± 0.16 Gy, a ratio of initial to final slopes of 0.35 ± 0.10, and n = 3.8 ± 1.4 (left ordinate) or 2.2 x 10^5 cells per cm^2 (right ordinate).

3.1.2. Effects on Tail Skin of Single Doses Given at Different Ages

A total of 55 aged control groups with 12 mice per dose group were used in this study. Their age varied from 12 weeks up to 39 weeks at the time when the test dose was given. They were tested using the macrocolony and skin healing techniques, and an example of the gross skin reaction is also given. These data were accumulated from 5 separate experiments which comprised the aged controls for most of the residual injury experiments. They were irradiated under the same conditions (i.e. 37°C, 3 cm tail, 35 cm f.s.d). The data were analysed using the DLOGS computer program described in Section 2.5. Regarding the microcolony data, there were two types of experiments performed using different ages and different sample treatments. They will be described as follows.

a). Gross Skin Reaction

The response of the tail skin at various times after various doses of 300 kVp X-rays is shown in Figure 3.10. A dose of 28 Gy produced a moderate reaction i.e. a total moist desquamation leading to scar

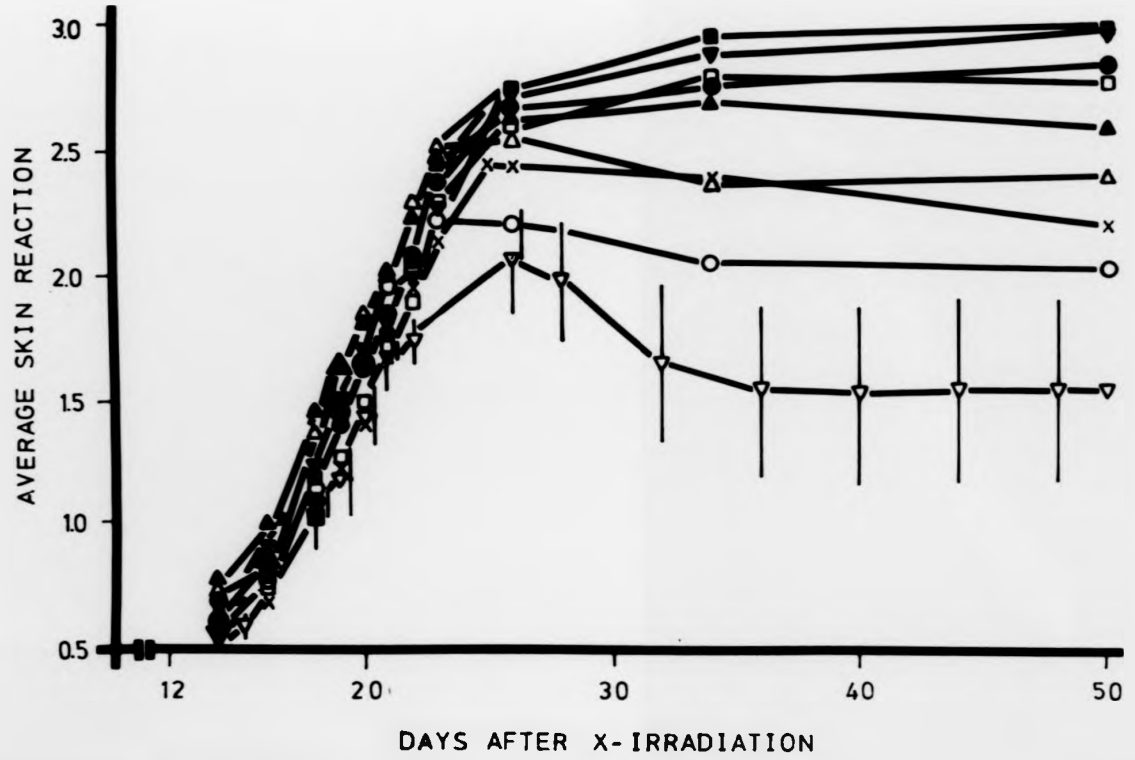


Figure 3.10: Skin reaction scores for mouse tails plotted against time after X-irradiation (28-36 Gy). Symbols ▽, x, ○, △, □, ●, ▲, ■ and ▼ represent doses of 28, 29 30, 31, 32, 33, 34, 35 and 36 Gy respectively. The standard error limits of the individual mouse values are shown on the 28 Gy curve only. Each point is the mean score from 12 animals.

formation. The reaction first appeared on about day 14 after irradiation and was at its peak by about day 26. The skin returned in most cases to a normal appearance by about day 34. No clear-cut changes can be detected by eye for the first 14 days after exposure to doses between 28-36 Gy. After 14 days, however, there is a steady rise with time in the reaction severity and the time of the increase was largely independent of dose. The peak reaction values were reached at about 26 days for most doses. At high doses the peak reaction was delayed to about day 34 after the treatment. Higher doses caused the same level of skin reaction to appear slightly earlier by about 1 - 2 days. The percentage of tails healing was dose-dependent. In the present experiment most doses were high (i.e. 28 Gy-36 Gy) to produce macrocolonies as well as skin necrosis or healing. Over this range of dose the irradiated skin either completely broke down or healed. In this case it took a very long time to heal, usually 6 to 9 weeks. Healing was evidenced by a decrease in the area covered by a scab and by the breaking-up of the large lesion into several small ones which eventually healed-up. This is due mainly to the re-epithelialisation from surviving colonies or from the field edges. The "healed" skin was initially very thick and smooth when looked at from the surface. This is probably due to hyperplastic changes. The skin later became thinner and rougher when healing was completed. Histological studies showed that the hyperplasia was in the nucleated layer, with a thinner keratinised layer. It should also be noted that the "healed" skin usually lacked pigment and hair and these features are dose dependent (Plate 2.7).

b). Microcolonies

An accurate comparison of the effects of age on the radiosensitivity of microcolony forming cells cannot be made, because for the two ages that we used,namely 12 weeks and 39 weeks, different procedures were used. Vincristine was used at 12 weeks, ^{3}H-TdR was used at 39 weeks, and these separate data are given in Section 3.1.1.II for 12 weeks and Section 3.3.3.d for 39 weeks. For comparison purposes two separate curves are taken from Figure 3.8 with D_0 = 270 ± 12 cGy and Figure 3.50 with D_0 = 219±10 cGy. They are shown in Figure 3.11. Although they showed a small difference in sensitivity, this may be due to the different procedures which were involved rather than a true difference in sensitivity.

c). Macrocolonies

The aged groups of mice and the analysis are detailed in Table 3.5. D_0 values ranging from 359±18 cGy to 607±1 cGy were obtained. When they were all pooled, no significant difference was found between the groups, therefore, the best fit line with D_0 of 572±22 cGy is drawn through all the data and it is shown in Figure 3.12.

d). Skin Healing

The aged groups of mice and the analysis are detailed in Table 3.6. D_0 values ranging from 175±53 cGy to 756±424 cGy were obtained. When these data were pooled, there was no significant difference among them, therefore, the best-fit line with D_0 of 260±27 cGy is drawn and shown in Figure 3.13. The data obtained from both the macrocolony and healing techniques suggested that the sensitivity of mouse skin is independent of age when they were more than 12 weeks old at the time of irradiation.

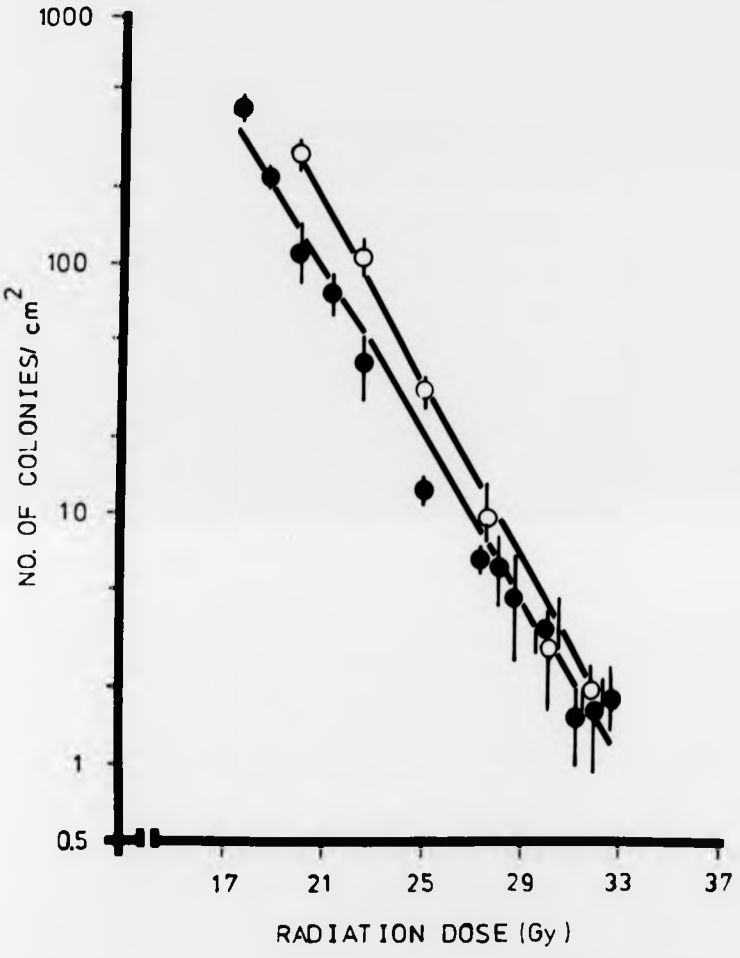


Figure 3.11: Effects of single doses on the microcolony forming cells at different mouse ages. Left curve (closed circles), 12 weeks old; Right curve (open circles), 39 weeks old.

TABLE 3.5: Response of Tail Skin to Single Test Doses with Varying Age of Mice: Macrocolony

DATA SET	NE (NO. OF COL./TAIL)	D_0 (cGy)	X^2	D.F.	X^2/DF	F RATIO	SIGNIFICANCE
12 weeks (1st exp.)	$1.1 \pm 0.6 \times 10^3$	533± 65	7.7	8	0.96		
12 weeks (2nd exp.)	$9.8 \pm 16.0 \times 10^2$	524±209	3.0	3	1.0		
18 weeks	$6.7 \pm 2.6 \times 10^2$	585± 67	32	17	1.88		
36 weeks	$1.9 \pm 0.6 \times 10^4$	359± 18	0.3	5	0.06	0.84	P>0.05
39 weeks	$5.4 \pm 1.5 \times 10^2$	607± 51	42.2	14	2.94		
All pooled	$7.1 \pm 1.0 \times 10^2$	575± 22	96	55	1.75		

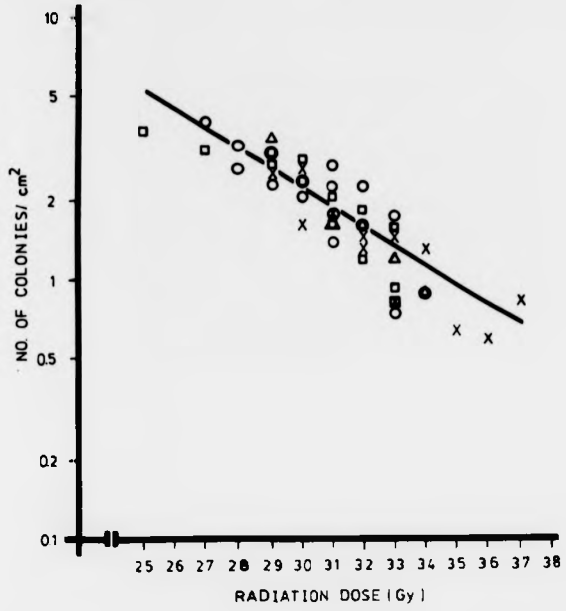


Figure 3.12: Effects of single doses on the macrocolony forming cells at different mouse ages. X, 12 weeks old; ○, 18 weeks old; △, 36 weeks old; □, 39 weeks old.

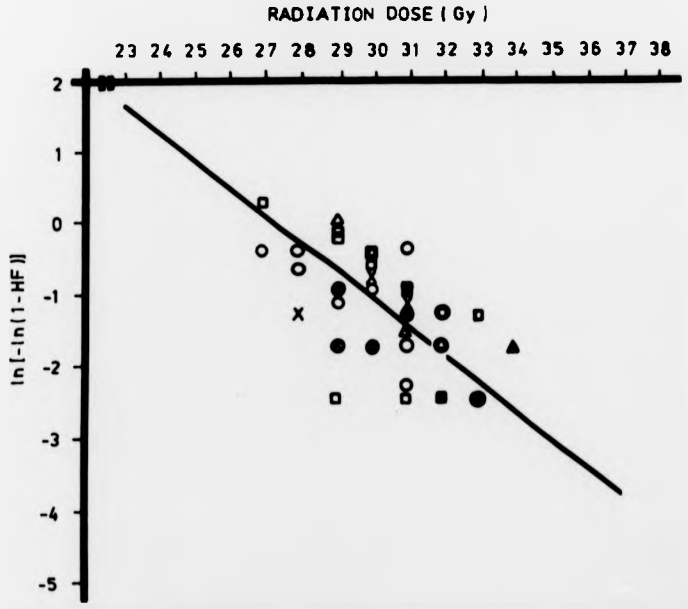


Figure 3.13: Effects of single doses on the skin at different ages: Healing. X, 12 weeks old; ○, 18 weeks old; △, 36 weeks old; □, 39 weeks old.

TABLE 3.6: Response of Skin to Single Test Doses with Varying Age of Mice: Healing

DATA SET	LD_{37} (cGy)	NE	D_0 (cGy)	X^2	D.F.	X^2/D.F.	F RATIO	SIGNIFICANCE
12 weeks (1st exp.)	2777 ± 89	$2.2\pm3.1\times10^{5}$	226± 56	5.1	7	0.73		
12 weeks (2nd exp.)	1815 ± 673	$1.1\pm1.7\times10^{1}$	756±424	1.1	4	0.28		
18 weeks	2610 ± 93	$3.5\pm3.6\times10^{3}$	320± 78	14.3	16	0.89	3.75	P > 0.05
28 weeks	2910 ± 56	$1.7\pm2.5\times10^{7}$	175± 53	2.2	4	0.55		
30 weeks	2867 ± 33	$5.2\pm2.9\times10^{5}$	218± 34	13.2	14	0.94		
All pooled	2736 ± 31	$3.8\pm1.6\times10^{4}$	260± 27	59.9	53	1.13		

3.1.3. Effects of Plucking the Tail Skin

This experiment was designed to study the response of skin to plucking. A total of 9 dose groups with 12 mice per group were used. The tails were tested in three different ways. The hairs and the surface squames were removed by sellotape stripping 18 hours before or immediately after irradiation, and 3 dose groups remained unplucked. The gross skin reaction, macrocolony and healing techniques were used in this study. The two sets of data (i.e. macrocolony and healing) were analysed using the DLOGS computer program and they are described as follows:

a). Gross Skin Reaction

Plucking the tail skin with sellotape (10 applications) resulted in an earlier appearance and earlier peak of the X-ray reactions (Figure 3.14). When the hair was plucked 18 hours before irradiation, the peak reaction appeared at day 18. Plucking immediately after irradiation delayed the reaction by about 1 day. The unplucked skin showed a peak reaction at day 26. The plucking of the skin either 18 hours before or immediately after 28 Gy resulted also in an increased severity of the reaction when compared with unplucked skin, and the percentage of tails healed was decreased to 17% for the skin plucked 18 hours before or immediately after irradiation when compared with 58% for the unplucked skin.

b). Macrocolonies

The treatment regimens and the data analysis are detailed in Table 3.7. The D_0 value varied from 265±18 cGy for 3 groups of mice plucked 18 hours before irradiation to 572 ± 53 cGy for another 3 groups of mice with the skin unplucked. With tails plucked immediately after irradiation, the D_0 was 427±121 cGy. By pooling and re-fitting the

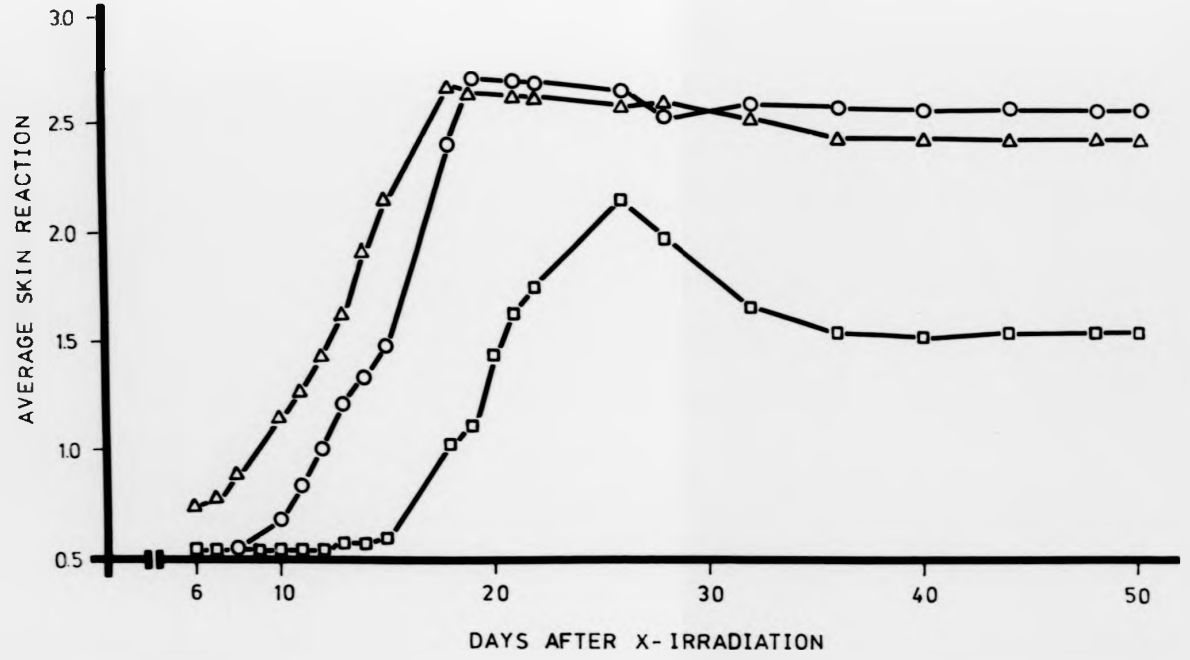


Figure 3.14: Effects of plucking: Skin reaction scores against time after 28 Gy. △, plucked 18 hours before irradiation; ○, plucked immediately after irradiation; □, unplucked.

TABLE 3.7: Effect of Plucking on the Tail Skin : Macrocolony

	DATA SET	NE (NO. OF COL./TAIL)	D_0 (cGy)	X^2	D.F.	X^2/DF	F RATIO	SIGNIFICANCE
A	Plucking 18 hours before irradiation	$1.7 \pm 0.8 \times 10^5$	265 ± 18	0.2	2	0.1		
	Plucking immediately after irradiation	$3.3 \pm 4.5 \times 10^3$	427 ± 121	2.6	2	1.3		
	Unplucked	$7.9 \pm 2.6 \times 10^2$	572 ± 53	1.1	2	0.55	8.5	$P < 0.05$
B	Pooled	$3.8 \pm 4.4 \times 10^3$	422 ± 101	26.3	10	2.63		
	Pooled with common D_0							
C	Plucking 18 hours before irradiation	$2.8 \pm 2.2 \times 10^3$						
	Plucking immediately after irradiation	$3.5 \pm 2.7 \times 10^3$	423 ± 67	9.2	8	1.15	4.02^a	$P > 0.05$
	Unplucked	$4.8 \pm 3.7 \times 10^3$						

[a] = Comparisons of group C (common D_0) with the sum of group A (separate D_0's).

data, a significant difference was shown between the groups ($P < 0.05$). However, the D_0 values were not significantly different, and a common D_0 of 423±67 cGy could be fitted to all 3 sets of data (data C, Table 3.7). These curves are shown in Figure 3.15. Also shown in Figure 3.15 are the three curves fitted separately with different D_0 values.

c). Skin Healing

The treatment regimens and the data analysis are given in Table 3.8. D_0 values ranging from 156±71 cGy to 301±157 cGy were deduced. None of the individual groups was significantly different from a common curve with D_0 of 207±72 cGy fitted through all the data, shown in Figure 3.16.

3.1.4. Effects of Adriamycin Combined with Radiation on the Skin

This experiment was designed to study any additional effects of Adriamycin on the tail skin after irradiation. A total of 8 dose groups with 12 mice per dose group was used. 4 groups of mice were tested using graded radiation doses alone. The other 4 groups were irradiated with the same radiation doses, and 24 hours later, to allow for Elkind repair, each mouse was injected with 10 mg/kg Adriamycin intraperitoneally. Two assay techniques were used in this experiment, namely macrocolonies and healing. The data were analysed using the DLOGS computer program.

a). Macrocolonies

The data analysis is detailed in Table 3.9. D_0 values were 525±219 cGy for radiation alone and 302±38 cGy for radiation + 24 hours + 10 mg/kg Adriamycin groups. The data from both groups were not significantly different, and the data were pooled (data C, Table 3.9). A best fit

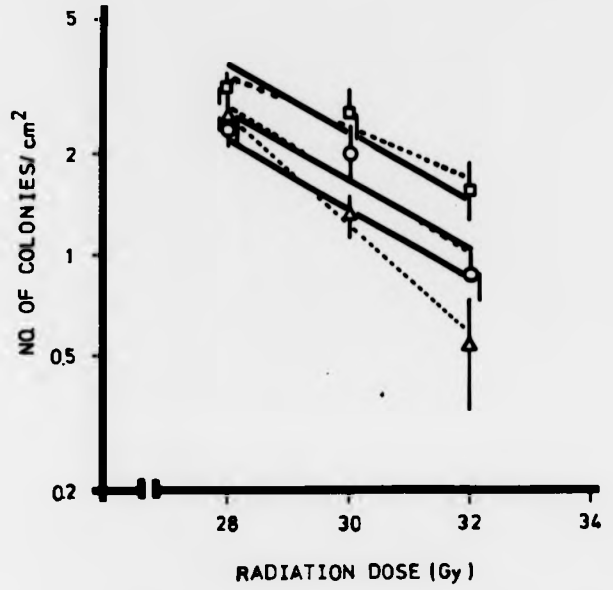


Figure 3.15: Effects of plucking on epidermal clonogenic cells. △, plucked 18 hours before irradiation (bottom curves); ○, plucked immediately after irradiation (middle curves); □, unplucked (top curves). Solid lines fitted with common Do. Dash lines fitted with separate Do values.

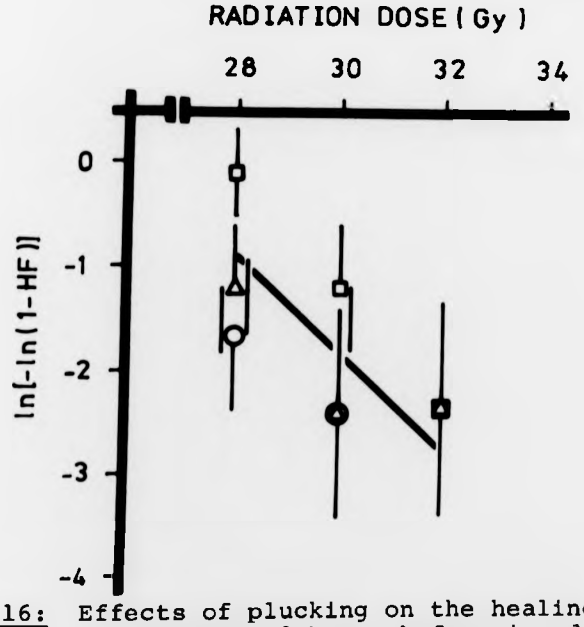


Figure 3.16: Effects of plucking on the healing of skin.
△, plucked 18 hours before irradiation;
○, plucked immediately after irradiation;
□, unplucked.

TABLE 3.8: Effect of Plucking on the Skin: Healing

DATA SET	LD_{37} (cGy)	NE	D_0 (cGy)	X^2	D.F.	X^2/D.F.	F RATIO	SIGNIFICANCE
Plucking 18 hours before irradiation	2396 ± 84	2.8±7.8 $x10^3$	301±157	0.3	1	0.3		
Plucking immediately after irradiation	2549 ± 152	1.3±4.9 $x10^7$	156± 71	0.4	1	0.4		
Unplucked	2776 ± 39	4.9±0.005$x10^6$	180± 39	2.7	1	2.7	1.08	P > 0.05
Pooled	2614 ± 104	3.1±6.1 $x10^5$	207± 72	8.3	7	1.2		

TABLE 3.9: The Effects of Radiation and Radiation Combined with Adriamycin on the Skin : Macrocolony

DATA SET	NE (NO. OF COL./TAIL)	D_o (cGy)	X^2	D.F.	X^2/DF	F RATIO	SIGNIFICANCE
A Radiation alone	$9.6 \pm 16.3 \times 10^4$	525 ± 219	3.0	3	1.0		
B Radiation + 24 hours + 10 mg/kg ADR	$8.5 \pm 7.4 \times 10^4$	302 ± 38	0.7	3	0.23	1.33[a]	P > 0.05
C Radiation alone and radiation + 24 hours + 10 mg/kg ADR pooled	$8.9 \pm 9.5 \times 10^4$	384 ± 72	5.4	8	0.68		

[a] = Comparison of group C with the sum of groups A and B (separate D_0's).

line with D_0 of 384±72 cGy was drawn through all the data and it is shown in Figure 3.17.

b). Skin Healing

The data analysis is detailed in Table 3.10. D_0 values were 760±490 cGy for radiation alone and 260±151 cGy for radiation + 24 hours + 10 mg/kg Adriamycin groups. The data for both groups were not significantly different (data C, Table 3.10), and the data were pooled. The best fit line with D_0 of 368±150 cGy is shown in Figure 3.18.

In summary, although there was a large variation in terms of D_0 and standard error between radiation alone, and radiation combined with Adriamycin, this is basically due to the shorter range of dose used. Hence there was no significant additional effect when 10 mg/kg Adriamycin was given intraperitoneally at 24 hours after the priming dose of irradiation.

Two experiments using 24 mice were also performed using the microcolony technique. Animals were injected with 15 mg/kg Adriamycin 30 minutes before a fixed radiation dose of 21.25 Gy. The results showed that there was an increased effect over the radiation alone, see Table 3.20.

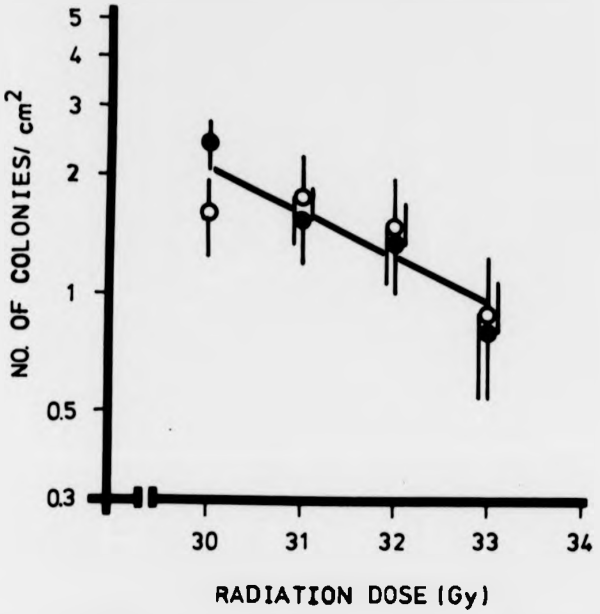


Figure 3.17: Effects of radiation alone, and adriamycin. Combined with radiation, on the macrocolony-forming cells in the skin. ○, radiation alone; ●, radiation + 24 hours + 10mg/kg adriamycin.

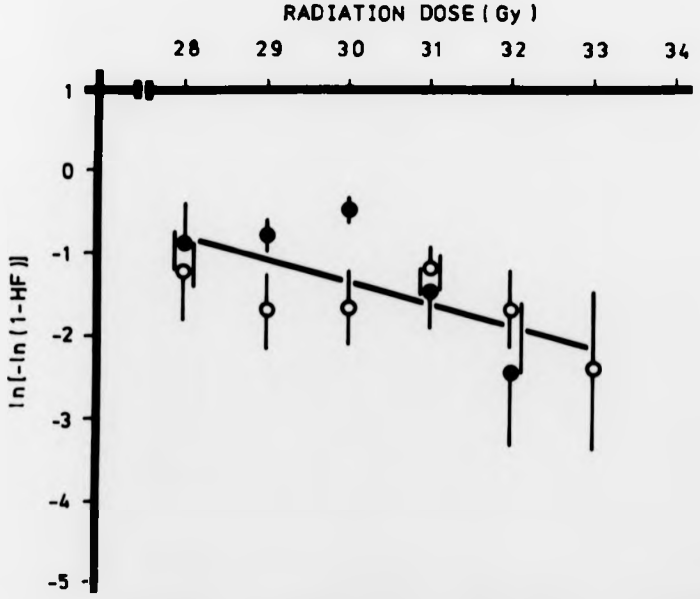


Figure 3.18: Effects of radiation alone, and adriamycin combined with radiation, on skin healing. ○, radiation alone; ●, radiation + 24 hours + 10mg/kg adriamycin.

TABLE 3.10: The Effect of Radiation and Radiation in Combination with Adriamycin on the Skin: Healing

	DATA SET	LD_{37} (cGy)	NE	D_0 (cGy)	X^2	D.F.	X^2/D.F.	F RATIO	SIGNIFICANCE
A	Radiation alone	1809 ± 786	$1.1\pm 1.9\times10^{1}$	760±490	1.1	4	0.28		
B	Radiation + 24 hrs + 10 mg/kg ADR	2681 ± 161	$3.1\pm 7.5\times10^{4}$	260±131	6.1	4	1.53	1.33^{a}	P > 0.05
C	Radiation alone and Radiation + 24 hrs + 10mg/kg ADR pooled	2494 ± 210	$8.8\pm15.3\times10^{2}$	368±150	9.6	10	0.96		

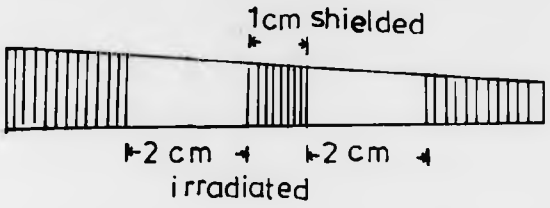
[a] Comparison of group C with the sum of groups A and B (separate D_0's).

3.2. EFFECTS OF FIELD SIZE

Five experiments were performed using single doses with various lengths of tails (i.e. 2, 4 and 6 cm). The areas of skin were 1.33, 3.23 and 4.24 cm^2. Measurements of macrocolonies and healing were made in these experiments. An experiment using single doses with 2 and 4 cm tails was performed using the microcolony technique. All of the single-dose experiments used the 10 MeV electron beam generated by the L-Band linear accelerator as described in Section 2.1.2. The flatness of the field for the different field sizes was checked using both thermoluminescent dosimetry and colour changes in PVC as described in Section 2.1.2. One experiment was designed to irradiate 2 and 4 cm tails using 12 fractions with an interval of 8 hours between fractions. They were irradiated using X-rays. The detailed experimental design will be given for each experiment described in the following sub-sections.

3.2.1. Measurements of the Mean Area Irradiated and Used for Colony Counting

In order to measure the effect of field size using either microcolony or macrocolony techniques, it was necessary to measure the irradiated area as well as the area used for colony counting. Each of these values was obtained from measurements made on at least 6 tails, and they are presented in Table 3.11. Also in this table are shown data for a group of tails irradiated with 2 + 2 cm sections proximal and distal representing the irradiation of a 2 cm length at the proximal part, separated by 1 cm of unirradiated tail then another 2 cm length of tail irradiated at the distal part. A sketch is shown overleaf.



Two measurements were also made to determine the size of the macrocolonies and the length of the re-epithelialised field edge. A total of 70 colonies from 70 tails irradiated with 2, 4 and 6 cm length were chosen and measured, the average size of the colonies being 9.5 ± 0.58 mm^2. The average widths of the re-epithelialised edges were 3.3 ± 0.12 mm for the proximal part and 3.3 ± 0.13 mm for the distal part of tail. These lengths were measured on the day when the macrocolonies were counted, and they represented the unpigmented regenerated margins.

TABLE 3.11: The Mean Area Irradiated and Used for Colony Counting

LENGTH OF TAIL IRRADIATED	IRRADIATED AREA (CM^2)±SE	COLONY COUNTING AREA (CM^2)±SE
2 cm	1.33±0.02	0.72±0.02
3 cm	2.48±0.02	1.70±0.02
4 cm	3.23±0.03	2.43±0.02
6 cm	4.24±0.08	3.56±0.08
2 + 2 cm Proximal	1.59±0.01	0.78±0.01
Distal	1.26±0.01	0.75±0.01

3.2.2. Single Doses and Field Size - Microcolonies

One single-dose experiment was performed using the microcolony technique. There were 11 dose groups with 12 mice per group. 2 cm and 4 cm tails were irradiated with electron doses ranging from 20.29 Gy up to 43.31 Gy for 2 cm tails and 20.84 Gy up to 38.41 Gy for 4 cm tails.

The data were analysed using the PUCK computer program. The analysis is detailed in Table 3.12. D_0 values were 509±21 cGy for 2 cm and 402 ± 22 cGy for 4 cm tails when they were fitted separately. The curves and their D_0 values for the two lengths of tail were significantly different (data A, Table 3.12). This was shown by fitting both sets of data to one curve, or to two curves with different D_0 values, and finding that these fittings were significantly worse than the individual fittings (data C & D, Table 3.12). The separate curves are shown in Figure 3.19. The increase in dose for equivalent effect ranged between factors of about 1.01 to 1.10, with a mean of 1.06. However, survival levels were equal after doses of about 20 Gy.

3.2.3. Single-doses Field-size Experiment - Gross Skin Reaction, Macrocolonies and Healing

Five experiments were performed using single doses. The lengths of tail irradiated were 2, 4, 6 and 2 + 2 cm. Their total irradiated area and the actual area for colony counting are listed in Table 3.11. There were a total of 78 dose groups with 12 mice per group used in 5 experiments. Due to the lack of experience in dose selection in the earlier experiments, the radiation doses in some groups were too low to produce identifiable macrocolonies (i.e. there was coalescence of colonies). These groups were excluded from the macrocolony result, but they were used for the estimation of the fraction of tails healed. For this reason, there were 44 dose groups for macrocolony data, and 78 dose groups for the healing data. Both macrocolony and healing data were analysed using the DLOGS computer program. The data will be described separately. A comparison will be made for the survival parameters fitted using either the PUCK or the DLOGS program for the macrocolony

TABLE 3.12: The Effect of Field Size on Skin Response Using 2 and 4 cm Lengths of Tail: Microcolonies

DATA SET	NE (NO. OF COL./TAIL)	D_0 (cGy)	X^2	D.F.	X^2/DF	F RATIO	SIGNIFICANCE
A 2 cm	$8.6 \pm 1.6 \times 10^4$	509 ± 21	9.7	5	1.94		
4 cm	$2.5 \pm 0.8 \times 10^5$	402 ± 22	31.6	4	7.9	8.3	P < 0.05
B 2 cm and 4 cm pooled	$1.7 \pm 0.5 \times 10^5$	435 ± 23	117.4	11	10.7		
C Pooled with common D_0							
2 cm	$1.9 \pm 0.5 \times 10^5$	435 ± 22	94.8	10	9.48	11.7^a	P < 0.05
4 cm	$1.6 \pm 0.4 \times 10^5$						
D Pooled with common N: different D_0							
2 cm	$1.7 \pm 0.4 \times 10^5$	445 ± 21	81.2	10	8.12	8.7^b	P < 0.05
4 cm	$1.7 \pm 0.4 \times 10^5$	431 ± 20					

[a] = Comparison of group C (common D_0) with the sum of group A (separate D_0's).
[b] = Comparison of group D (common NE, different D_0) with the sum of group A (separate D_0's).

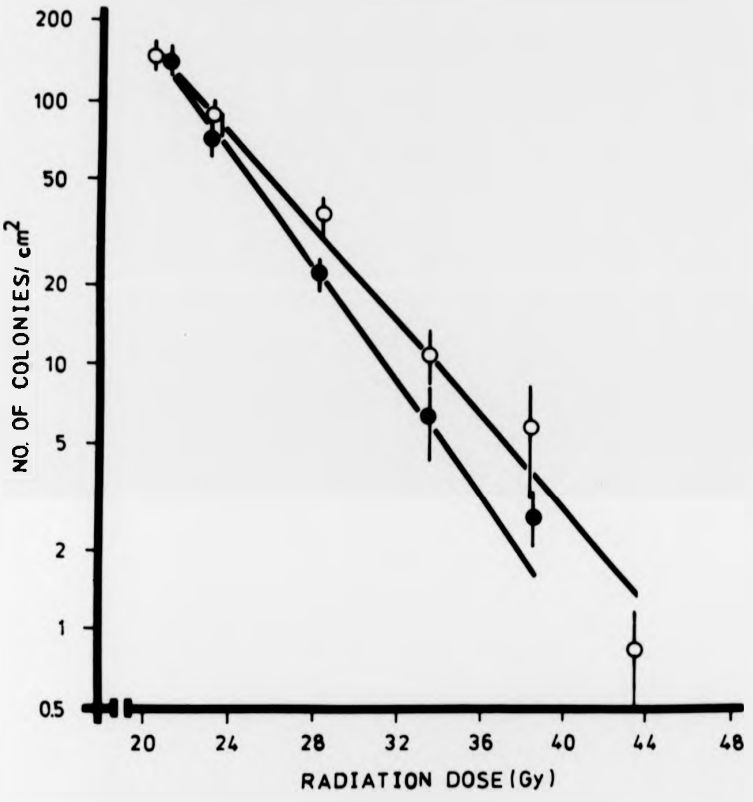


Figure 3.19: Effects of field-size on the sensitivity of microcolony-forming cells. ○ , 2 cm tail; ● , 4 cm tail.

data. Also, the PROBIT and DLOGS programs are compared using the healing data.

a). Gross Skin Reaction

Increasing the size of the irradiated area from 1.33 cm^2 (2 cm tail) to 3.23 cm^2 (4 cm tail) or 4.24 cm^2 (6 cm tail) resulted in an earlier appearance and earlier peak of the skin reactions after similar single doses. Also, it increased the severity of the reaction and prolonged the time for healing (Figure 3.20). When total doses of 52, 56, 60, 64 and 68 Gy were delivered in 12 fractions in 3 days to either 2 cm (1.33 cm^2) or 4 cm (3.23 cm^2) of tail, the time of appearance, the peak of skin reaction, the duration of healing and the percentage of tails healed were not significantly different for the two lengths for dose from 56 to 68 Gy (see Figure 3.21).

b). Macrocolonies

The detailed analysis for the macrocolony data is shown in Table 3.13. D_0 values ranging from 591 ± 56 cGy to 659 ± 76 cGy were obtained for 2, 4 and 6 cm tails. The curves were significantly different, but the slopes were not (data A, Table 3.13). When the data were pooled the common D_0 was 620 ± 24 cGy. The data for 2 + 2 cm (Proximal part) and 2 + 2 cm (Distal part) were not significantly different (data D, Table 3.13). The 2 cm data or the 4 and 6 cm data were not significantly different from the 2 + 2 cm data proximal, and distal (data F & H, Table 3.13). All the groups of 2, 4, 6 and 2 + 2 cm (Proximal) and 2 + 2 cm (Distal) could be fitted with a common D_0 of 620 ± 21 cGy (data J, Table 3.13). Therefore, separate graphs were drawn from data fitted with separate D_0's and they are shown in Figure 3.22, 3.23 and 3.24.

data. Also, the PROBIT and DLOGS programs are compared using the healing data.

a). Gross Skin Reaction

Increasing the size of the irradiated area from 1.33 cm^2 (2 cm tail) to 3.23 cm^2 (4 cm tail) or 4.24 cm^2 (6 cm tail) resulted in an earlier appearance and earlier peak of the skin reactions after similar single doses. Also, it increased the severity of the reaction and prolonged the time for healing (Figure 3.20). When total doses of 52, 56, 60, 64 and 68 Gy were delivered in 12 fractions in 3 days to either 2 cm (1.33 cm^2) or 4 cm (3.23 cm^2) of tail, the time of appearance, the peak of skin reaction, the duration of healing and the percentage of tails healed were not significantly different for the two lengths for dose from 56 to 68 Gy (see Figure 3.21).

b). Macrocolonies

The detailed analysis for the macrocolony data is shown in Table 3.13. D_0 values ranging from 591 ± 56 cGy to 659 ± 76 cGy were obtained for 2, 4 and 6 cm tails. The curves were significantly different, but the slopes were not (data A, Table 3.13). When the data were pooled the common D_0 was 620 ± 24 cGy. The data for 2 + 2 cm (Proximal part) and 2 + 2 cm (Distal part) were not significantly different (data D, Table 3.13). The 2 cm data or the 4 and 6 cm data were not significantly different from the 2 + 2 cm data proximal, and distal (data F & H, Table 3.13). All the groups of 2, 4, 6 and 2 + 2 cm (Proximal) and 2 + 2 cm (Distal) could be fitted with a common D_0 of 620 ± 21 cGy (data J, Table 3.13). Therefore, separate graphs were drawn from data fitted with separate D_0's and they are shown in Figure 3.22, 3.23 and 3.24.

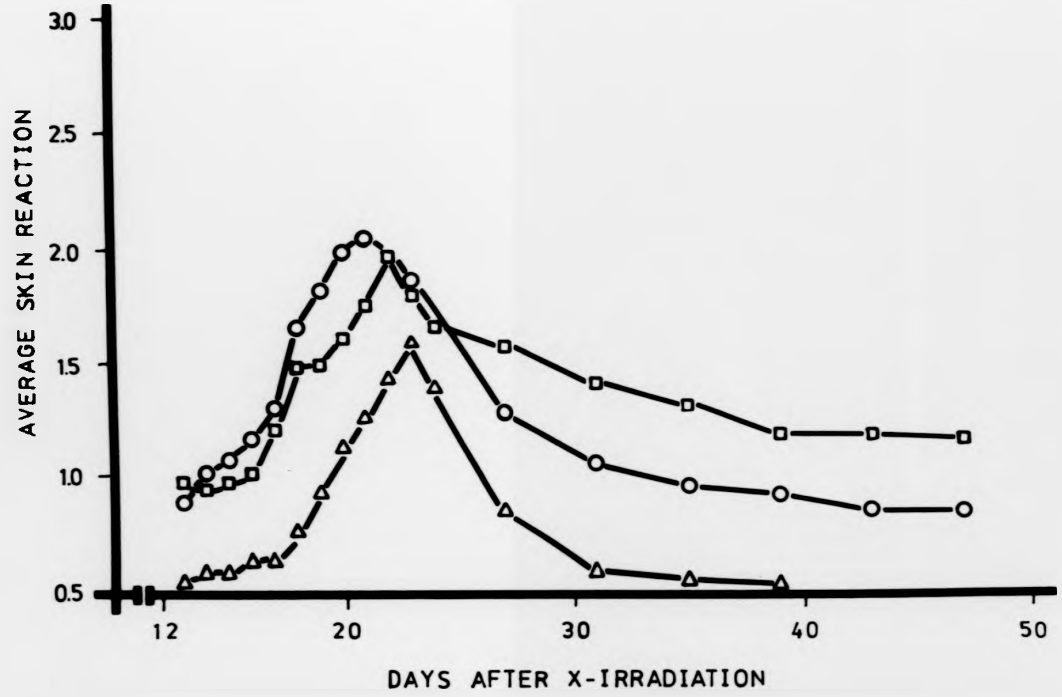


Figure 3.20: The effect of field size (single dose): skin reaction scores plotted against time. △, 2 cm tail 31.83 Gy; ○, 4 cm tail 31.33 Gy; □, 6 cm tail 30.45 Gy. Each point is the mean score from 12 animals.

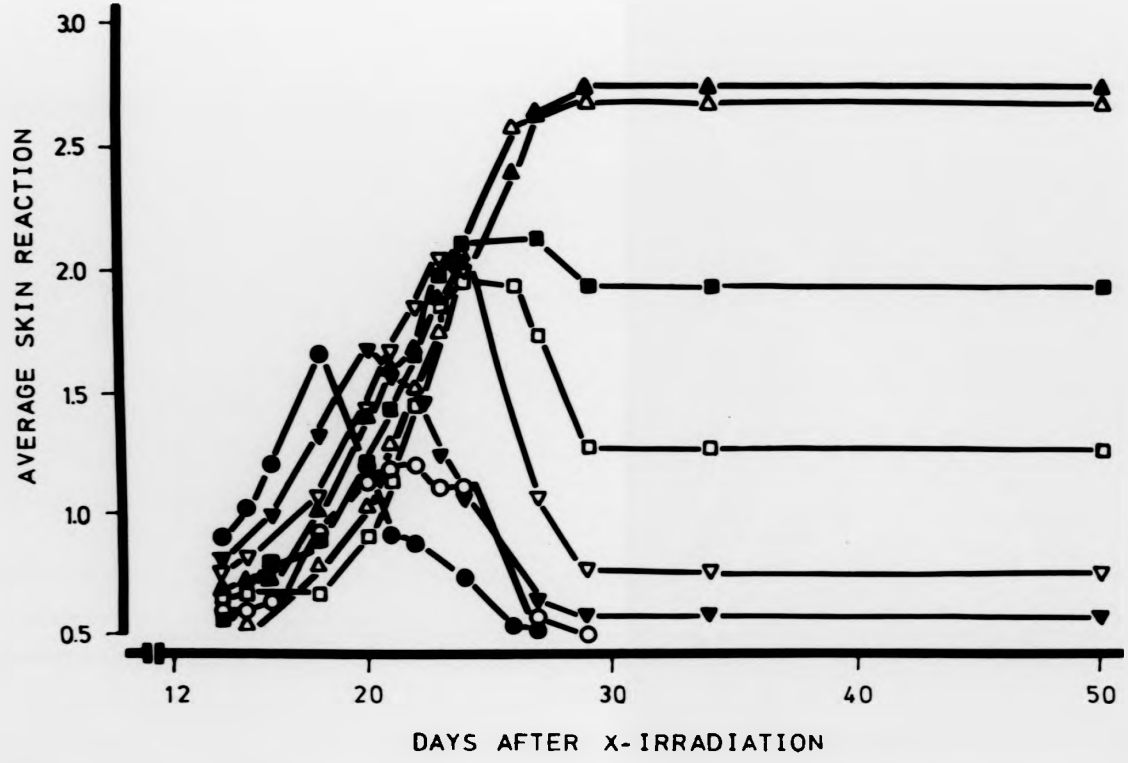


Figure 3.21: Effects of field size (12 fractions): Skin reaction scores against time. Open symbols ▽, □, △ represents 56, 60 and 68 Gy respectively using 2 cm tail. Close symbols ▼, ■, ▲ represents 56, 60 and 68 Gy respectively using 4 cm tail.

TABLE 3.13: The Effect of Field Size on the Skin Response Using Different Lengths of Tail : Macrocolony

	DATA SET	NE (NO. OF COL./CM^2)	D_0 (cGy)	X^2	D.F.	X^2/DF	F RATIO	SIGNIFICANCE
A	2 cm	$1.1 \pm 0.5 \times 10^4$	659 ± 76	15.4	12	1.28		
	4 cm	$6.6 \pm 0.8 \times 10^3$	643 ± 24	50.4	12	4.2		
	6 cm	$9.5 \pm 3.8 \times 10^3$	591 ± 56	25.3	11	2.3	12.9	P < 0.05
B	2, 4 & 6 cm pooled	$7.2 \pm 8 \times 10^3$	634 ± 20	225.2	39	5.77		
C	Pooled with common D_0							
	2 cm	$1.6 \pm 0.3 \times 10^4$						
	4 cm	$8.0 \pm 1.2 \times 10^3$	620 ± 24	93.8	37	2.54	0.52[a]	P > 0.05
	6 cm	$7.1 \pm 1.0 \times 10^3$						
D	2 + 2 cm (Proximal)	$8.9 \pm 1.8 \times 10^3$	667 ± 34	0.4	2	0.2		
	2 + 2 cm (Distal)	$1.4 \pm 0.7 \times 10^3$	944 ± 277	0.2	1	0.2	0.75	P > 0.05
E	Proximal & Distal pooled	$8.6 \pm 1.4 \times 10^3$	670 ± 27	0.9	5	0.18		
F	2 cm	$1.1 \pm 0.5 \times 10^4$	659 ± 76	15.4	12	1.28		
	2 + 2 cm (Proximal)	$8.9 \pm 1.8 \times 10^3$	667 ± 34	0.4	2	0.2		
	2 + 2 cm (Distal)	$1.4 \pm 0.7 \times 10^3$	944 ± 277	0.2	1	0.2	0.59	P > 0.05
G	2 cm, 2 + 2 cm (Proximal) and 2 + 2 cm (Distal) pooled	$9.6 \pm 3.7 \times 10^3$	666 ± 63	18.5	19	0.97		

TABLE 3.13: (CONTD.)

	DATA SET	NE (NO. OF COL./CM^2)	D_0 (cGy)	X^2	D.F.	X^2/DF	F RATIO	SIGNIFICANCE
H	4 cm	$6.6 \pm 0.8 \times 10^3$	643 ± 24	50.4	12	4.2		
	6 cm	$9.5 \pm 3.8 \times 10^3$	591 ± 56	25.3	11	2.3		
	2 + 2 cm (P)	$8.9 \pm 1.8 \times 10^3$	667 ± 34	0.4	2	0.2		
	2 + 2 cm (D)	$1.4 \pm 0.7 \times 10^3$	944 ± 277	0.2	1	0.2	2.62	P > 0.05
I	4 cm, 6 cm, 2 + 2 cm (P) and 2 + 2 cm (D) pooled	$7.6 \pm 0.9 \times 10^3$	623 ± 19	123.4	32	3.83		
J	Pooled with common D_0							
	2 cm	$1.6 \pm 0.2 \times 10^4$						
	4 cm	$8.0 \pm 1.1 \times 10^3$						
	6 cm	$7.1 \pm 0.9 \times 10^3$	620 ± 21	95	42	2.26	0.36[b]	P > 0.05
	2 + 2 cm (P)	$1.4 \pm 0.2 \times 10^4$						
	2 + 2 cm (D)	$1.3 \pm 0.2 \times 10^4$						

[a] = Comparison of group C (common D_0) with the sum of group A (separate D_0's)
[b] = Comparison of group J (common D_0) with the sum of groups A and D (separate D_0's).

Combined curves with common D_0's plus 6 observations for 2 + 2 cm (Proximal) and 2 + 2 cm (Distal) data are shown in Figure 3.25.

A comparison of survival parameters fitted using PUCK and DLOGS programs is shown in Table 3.14. The D_0 obtained from "PUCK" analysis was 655 ± 45 cGy whereas from "DLOGS" it was 620 ± 24 cGy. The chi-square per degree of freedom was 2.46 for the PUCK analysis and 2.54 for the DLOGS analysis. This indicates that either program fitted the data equally well.

An analysis was made of the distribution of macrocolonies along the irradiated tail. Four groups of mice with 12 mice per group received irradiation to different lengths of tail (i.e. 2, 4, 6 and 2 + 2 cm), but similar radiation doses. The radiation doses differed slightly because the electron dose is delivered in pulses (with about 30 cGy per pulse) which vary slightly in number, so that the given dose varies slightly from the nominal dose. For colony counting, the tail which was irradiated to 4 cm in length was divided into 3 equal sections with a 1 cm length in each section (leaving out 0.5 cm at both irradiated edges). Similarly, for the 6 cm length tail, the middle 5 cm was divided into 5 equal sections with 1 cm length in each section. In the case of 2 + 2 cm, only 2 sections in either proximal or distal part of the irradiated tail could be separately assessed. The total number of colonies from each section was counted. The mean colony counting area was measured using at least 6 mice. Therefore, the number of colonies per cm^2 for each irradiated section could be calculated. A ratio was obtained by comparing the individual number of colonies per cm^2 in the

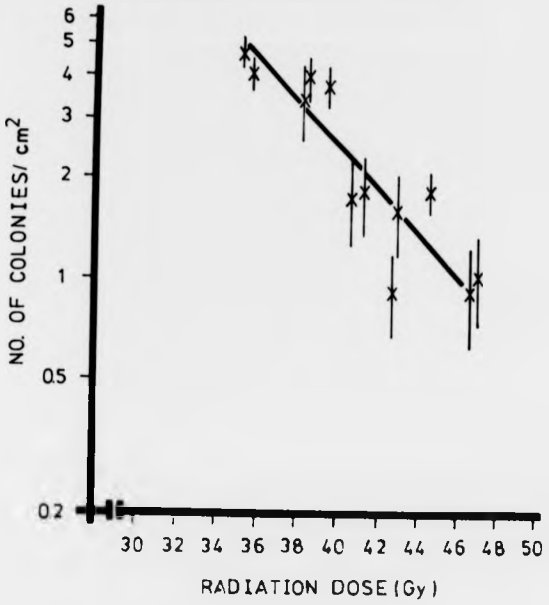


Figure 3.22: Effects of field-size on the response of macrocolony-forming cells. Data for 2 cm tail.

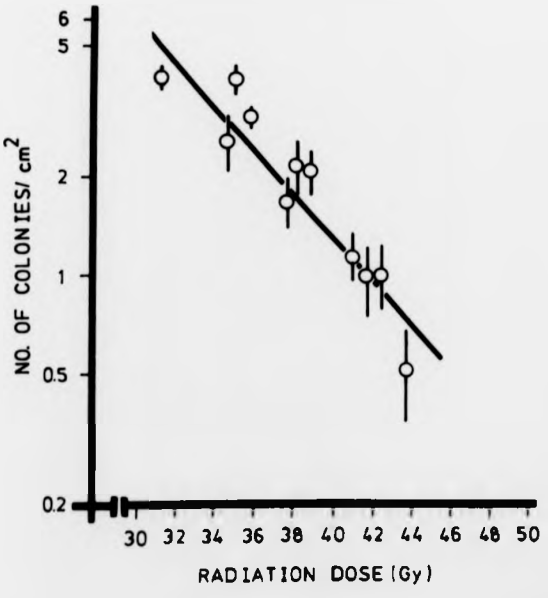


Figure 3.23: Effects of field-size on the response of macrocolony-forming cells. Data for 4 cm tail.

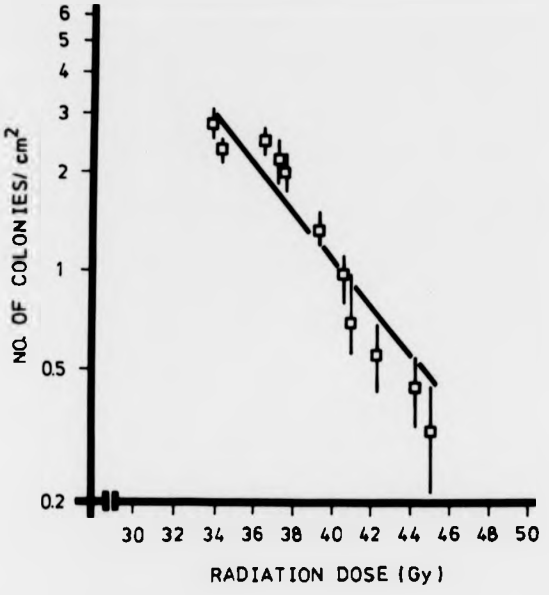


Figure 3.24: Effects of field-size on the response of macrocolony-forming cells. Data for 6 cm tail.

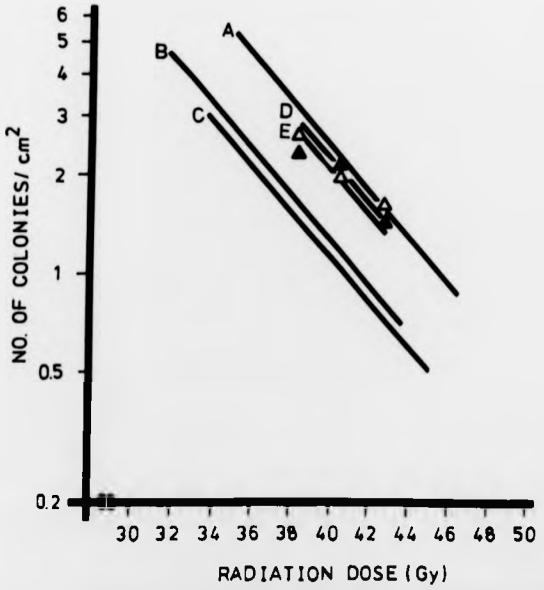


Figure 3.25: Pooled curves for the effects of field-size on the response of macrocolony-forming cells. Curve A, 2 cm tial; Curve B, 4 cm tail; Curve C, 6 cm tail; Curve D (open triangles), 2 + 2 cm (proximal); Curve E (closed triangles), 2 + 2 cm (distal).

TABLE 3.14: Comparison of Survival Parameters Fitted Using PUCK and DLOGS Programs. Field Size Effect Experiment : Macrocolony

DATA SET	NE (NO. OF COL./TAIL)	D_0 (cGy)	X^2	D.F.	X^2/DF
PUCK					
2 cm	$1.1 \pm 0.5 \times 10^4$				
4 cm	$5.9 \pm 2.2 \times 10^3$	655 ± 45	91	37	2.46
6 cm	$5.1 \pm 2.0 \times 10^3$				
DLOGS					
2 cm	$1.6 \pm 0.3 \times 10^4$				
4 cm	$8.0 \pm 1.2 \times 10^3$	620 ± 24	93.8	37	2.54
6 cm	$7.1 \pm 1.0 \times 10^3$				

different sections to the number of colonies in Section 1. This analysis showed that there was no significant difference between the counts in the various separate sections of the 4 cm and 2 + 2 cm length, but for the 6 cm length there was a tendency for the number of colonies per cm^2 to decrease towards the distal part of the irradiated tail. (see Table 3.15).

c). Skin Healing

The data analysis is detailed in Table 3.16. D_0 values were 328 ± 45 cGy, 376 ± 74 cGy and 379 ± 100 cGy for 2 cm, 4 cm and 6 cm tails respectively. The 4 and 6 cm data were not significantly different from each other (data C, Table 3.16), but they were different from the 2 cm data (data A, Table 3.16). All the data could be fitted with common D_0 of 360 ± 41 cGy (data F, Table 3.16). In the case of the 2 + 2 cm (Distal) tail, it was not possible to obtain a separate fitted value because there were too few data points. However, to test any significant difference between 2 + 2 cm (Proximal) and 2 + 2 cm (Distal) the X^2 value and the number of degrees of freedom were obtained by pooling the data using a common D_0 (data H, Table 3.16) and then comparing these data with the pooled data using separate D_0's. The analysis showed a very significant difference between the response of the proximal and distal parts of the tails (data I, Table 3.16). Whether 2 + 2 cm (Proximal) or 2 + 2 cm (Distal) tails respond significantly different from or similar to 2 cm or 4 cm tails is an important point. The analysis showed that 2 + 2 cm (Proximal) was significantly different from 2 cm (data J, Table 3.16) and 2 + 2 cm (Distal) tails responded similarly to 2 cm tails (data L, Table 3.16). However, 2 + 2 cm (Proximal) tails responded differently from 4 cm tails

(data N, Table 3.16) whereas 2 + 2 cm (Distal) tails did not differ significantly in response compared with 4 cm tails (data Q, Table 3.16). The data for 4 cm, 2 + 2 cm (Proximal) and 2 + 2 cm (Distal) tails could be fitted with a common D_0 (data S, Table 3.16). The same also applied when 2 cm, 4 cm, 6 cm, 2 + 2 cm (Proximal) and 2 + 2 cm (Distal) tails were pooled with a common D_0 of 359 ± 40 cGy (data T, Table 3.16), therefore, curves were drawn through the data fitted with common D_0 of 359 ± 40 cGy for all dose groups, this is presented in Figure 3.29. Curves with separate D_0 values for 2 cm, 4 cm and 6 cm tails are presented in Figure 3.26, 3.27, and 3.28. When comparing the individual LD_{37} values from 4 cm, 6 cm and 2 + 2 cm (Distal) tails to 2 cm tails, the dose reductions were 8%, 9% and 8% respectively. An exception is 2 + 2 cm (Proximal) tails which gave an 17% increase in the dose.

A comparison of survival parameters fitted with DLOGS or PROBIT program is detailed in Table 3.17. The common D_0 obtained from DLOGS analysis was 360±41 cGy whereas from the probit width it was 387±42 cGy. The two values are very close to each other if one takes the errors into account. Although DLOGS uses LD_{37} and PROBIT uses LD_{50}, they were not significantly different in terms of the dose ratios between 2 cm and 4 or 6 cm tails. The similar fitting obtained from both DLOGS and PROBIT analysis is shown for the 2 cm tail data using the same scale in Figure 3.31.

3.2.4. 12 Fractions Field-size Experiment - Microcolonies, Macrocolonies and Healing

A total of 12 dose groups with 12 mice per group were used in this

TABLE 3.15: Comparison of the Colony Distribution Along the Sections of Tails Irradiated with Electrons

LENGTH OF TAIL IRRADIATED	SECTION OF THE TAIL (S)	TOTAL NO. OF COLONIES	MEAN AREA FOR COLONY COUNTING (CM^2) ± SE	NO. OF COLONIES PER CM^2 ± SE	RATIO OF COLONY COUNT IN S_2, S_3, S_4 & S_5 COMPARED WITH S_1
2 cm (3933 cGy)		33	0.72 ± 0.02	3.82 ± 0.33	
4 cm (3846 cGy)	S_1	22	0.86 ± 0.02	2.13 ± 0.42	1.0
	S_2	21	0.77 ± 0.01	2.27 ± 0.49	1.07
	S_3	17	0.71 ± 0.01	2.0 ± 0.36	0.94
6 cm (3726 cGy)	S_1	27	0.86 ± 0.03	2.62 ± 0.33	1.0
	S_2	17	0.80 ± 0.01	1.77 ± 0.36	0.68
	S_3	23	0.73 ± 0.02	2.62 ± 0.26	1.0
	S_4	18	0.63 ± 0.02	2.38 ± 0.44	0.91
	S_5	9	0.54 ± 0.02	1.39 ± 0.22	0.53
2 + 2 cm (3843 cGy)	S_1[a]	24	0.78 ± 0.01	2.56 ± 0.21	1.0
	S_2[b]	21	0.75 ± 0.01	2.33 ± 0.30	0.91

S_1[a] = Proximal section of the irradiated tail.

S_2[b] = Distal section of the irradiated tail.

TABLE 3.16: The Effect of Field Size on the Skin Response to Electrons Using Different Lengths of Tail: Healing Endpoint

	DATA SET	LD_{37} (cGy)	NE	D_0 (cGy)	X^2	D.F.	X^2/D.F.	F RATIO	SIGNIFICANCE
A	2 cm	3497 ± 52	$4.3\pm 0.8\times10^4$	328 ± 45	43.8	25	1.75		
	4 cm	3234 ± 80	$5.4\pm 2.6\times10^3$	376 ± 74	40.9	17	2.4		
	6 cm	3207 ± 109	$4.7\pm 2.9\times10^3$	379 ± 100	118.8	22	5.4	8.2	P < 0.05
B	2, 4 & 6 cm pooled	3308 ± 50	$6.2\pm 1.6\times10^3$	379 ± 48	247	68	3.63		
C	4 cm	3234 ± 80	$5.4\pm 2.6\times10^3$	376 ± 74	40.9	17	2.4		
	6 cm	3207 ± 109	$4.7\pm 2.9\times10^3$	379 ± 100	118.8	22	5.4	0.27	P > 0.05
D	4 & 6 cm pooled	3218 ± 67	$4.8\pm 1.9\times10^3$	380 ± 62	158	41	3.85		
E	2 + 2 cm (P) pooled with common D_0, different LD_{37}	4126 ± 91	1.0×10^4	448 ± 177	2.1	2	1.05		
	2 cm	3496 ± 72	$1.7\pm 0.2\times10^4$						
	4 cm	3236 ± 84	$8.1\pm 2.4\times10^3$	360 ± 41	206.7	66	3.13	0.47[a]	P > 0.05
	6 cm	3211 ± 74	$7.5\pm 2.1\times10^3$						
G	2 cm	3496 ± 72	$1.7\pm 0.2\times10^4$						
	4 & 6 cm	3223 ± 58	$7.8\pm 2.2\times10^3$	360 ± 41	205.8	67	3.07	1.31[b]	P > 0.05

TABLE 3.16: (CONTD.)

	DATA SET	LD_{37} (cGy)	NE	D_0 (cGy)	X^2	D.F.	X^2/D.F.	F RATIO	SIGNIFICANCE
H	2 + 2 cm (P)	4120 ± 89	1.0×10^5	356 ± 132	7.4	5	1.48		
	2 + 2 cm (D)	3232 ± 363	$8.7 \pm 20.3 \times 10^3$						
I	2 + 2 cm (P) and 2 + 2 cm (D) pooled	3766 ± 298	$8.9 \pm 25.7 \times 10^3$	414 ± 332	36	6	6	19.3[c]	P < 0.05
	2 cm	3497 ± 52	$4.3 \pm 0.8 \times 10^4$	328 ± 45	43.8	25	1.75		
	2 + 2 cm (P)	4126 ± 91	1.0×10^4	448 ± 177	2.1	2	1.05	8.5[d]	P < 0.05
J	2 cm and 2 + 2 cm (P) pooled	3585 ± 69	$3.9 \pm 0.9 \times 10^3$	434 ± 67	74.8	29	2.58		
K	Pooled with common D_0, different LD_{37}								
	2 cm	3497 ± 48	$3.2 \pm 0.5 \times 10^4$	337 ± 41	42	28	1.5	2.29[e]	P > 0.05
	2 + 2 cm (P)	4118 ± 85	$2.0 \pm 0.3 \times 10^5$						
L	2 cm	3497 ± 53	$5.3 \pm 1.0 \times 10^4$	321 ± 45	52.7	28	1.88		
	2 + 2 cm (D)	3314 ± 223	$3.0 \pm 2.7 \times 10^4$						
M	2 cm and 2 + 2 cm (D) pooled	3486 ± 52	$7.9 \pm 2.4 \times 10^4$	309 ± 42	58.2	29	2.01	2.92[f]	P > 0.05
	4 cm	3234 ± 80	$5.4 \pm 2.6 \times 10^4$	376 ± 74	40.9	17	2.4		
	2 + 2 cm (P)	4126 ± 91	1.0×10^4	448 ± 177	2.1	2	1.05	9.4[g]	P < 0.05

TABLE 3.16: (CONTD.)

	DATA SET	LD_{37} (cGy)	NE	D_0 (cGy)	X^2	D.F.	X^2/D.F.	F RATIO	SIGNIFICANCE
N	4 cm and 2 + 2 cm (P) pooled	3307 ± 139	$2.5\pm 1.3\times10^2$	601 ± 160	85.6	21	4.08		
	Pooled with common D_0, different LD_{37}								
O	4 cm	3231 ± 74	$4.5\pm 1.9\times10^3$	384 ± 65	41.5	20	2.08	-0.66^h	P > 0.05
	2 + 2 cm (P)	4122 ± 112	$4.6\pm 1.9\times10^4$						
P	4 cm	3235 ± 78	$6.7\pm 3.3\times10^3$	367 ± 71	48.3	20	2.42	0.08	P > 0.05
	2 + 2 cm (D)	3208 ± 324	$6.2\pm 7.5\times10^3$						
Q	4 cm	3235 ± 78	$6.7\pm 3.3\times10^3$	367 ± 71	48.3	20	2.42	0.08^i	P > 0.05
	2 + 2 cm (D)	3208 ± 324	$6.2\pm 7.5\times10^3$						
	4 cm and 2 + 2 cm (pooled)	3234 ± 76	$7.0\pm 3.5\times10^3$	365 ± 63	48.5	21	2.31		
	4 cm	3234 ± 80	$5.4\pm 2.6\times10^3$	376 ± 74	40.9	17	2.4	3.94^j	P 0.05
	2 + 2 cm (P) and 2 + 2cm (D) pooled	3766 ± 307	$8.9\pm 26.5\times10^3$	414 ± 338	36.0	6	6.0		
R	4 cm, 2 + 2 cm (P) & 2 + 2 cm (D) pooled	3295 ± 127	$4.7\pm 2.7\times10^2$	536 ± 125	103.2	25	4.13		
	Pooled with common D_0, different LD_{37}								

TABLE 3.16: (CONTD.)

	DATA SET	LD_{37} (cGy)	NE	D_0 (cGy)	X^2	D.F.	X^2/D.F.	F RATIO	SIGNIFICANCE
S	4 cm	3233 ± 93	$5.0 \pm 2.7 \times 10^3$	380 ± 81	77.0	24	3.21	0.03[k]	P > 0.05
	2 + 2 cm (P) and 2 + 2 cm (D) pooled	3790 ± 136	$2.2 \pm 1.7 \times 10^4$						
T	2 cm	3496 ± 71	$1.7 \pm 0.2 \times 10^4$	359 ± 40	214.4	72	2.98	0.327[l]	P > 0.05
	4 cm	3237 ± 82	$8.1 \pm 2.3 \times 10^3$						
	6 cm	3211 ± 76	$7.6 \pm 2.0 \times 10^3$						
	2 + 2 cm (P)	4120 ± 127	$9.5 \pm 2.5 \times 10^4$						
	2 + 2 cm (D)	3226 ± 311	$7.9 \pm 5.4 \times 10^3$						

[a] Comparison of group F (common D_0) with the sum of A (separate D_0's).
[b] Comparison of group G (common D_0) with the sum of 2 cm in group A and group D (separate D_0's).
[c] Comparison of group I (pooled data) with the sum of group H (common D_0, different LD_{37}).
[d] Comparison of group J (pooled data) with the sum of 2 cm in group A and group E (separate D_0's).
[e] Comparison of group K (common D_0) with the sum of 2 cm in group A and group E (separate D_0's).
[f] Comparison of group M (pooled data) with the sum of group L (common D_0).
[g] Comparison of group N (pooled data) with the sum of 4 cm in group A and group E (separate D_0's).
[h] Comparison of group O (common D_0) with the sum of 4 cm in group A and group E (separate D_0's).
[i] Comparison of group Q (pooled data) with the sum of group P (common D_0).
[j] Comparison of group R (pooled data) with the sum of 4 cm in group A and group I (separate D_0's).
[k] Comparison of group S (common D_0) with the sum of 4 cm in group A and group I (separate D_0's).
[l] Comparison of group T (common D_0) with the sum of 2, 4 & 6 cm in group A and group H (separate D_0's).

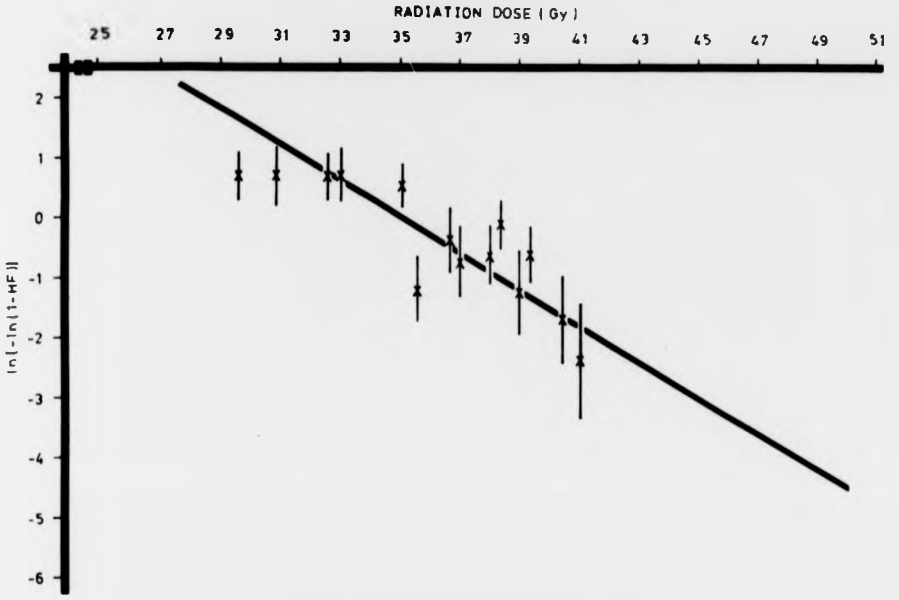


Figure 3.26: Effects of field-size on the healing response of skin. Data for 2 cm tail.

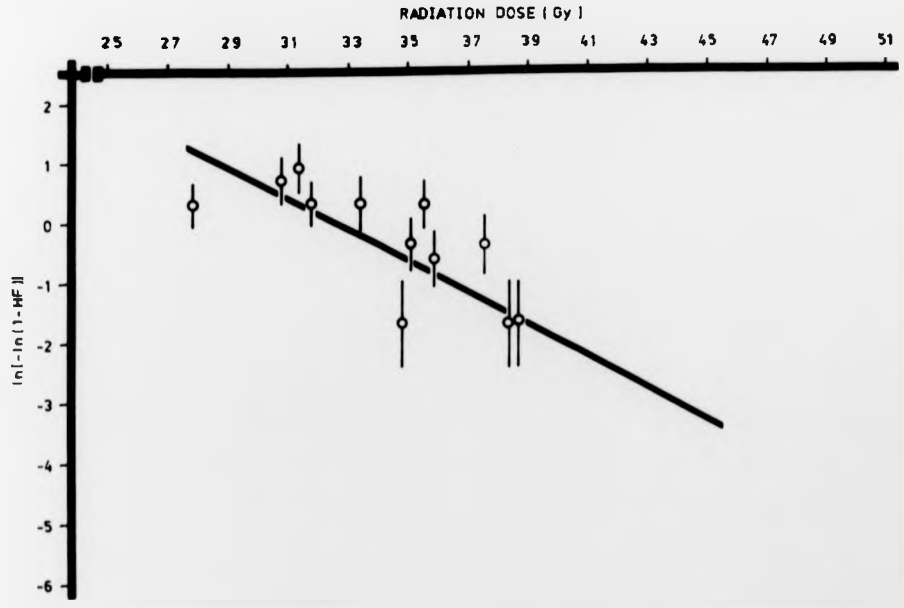


Figure 3.27: Effects of field-size on the healing response of skin. Data for 4 cm tail.

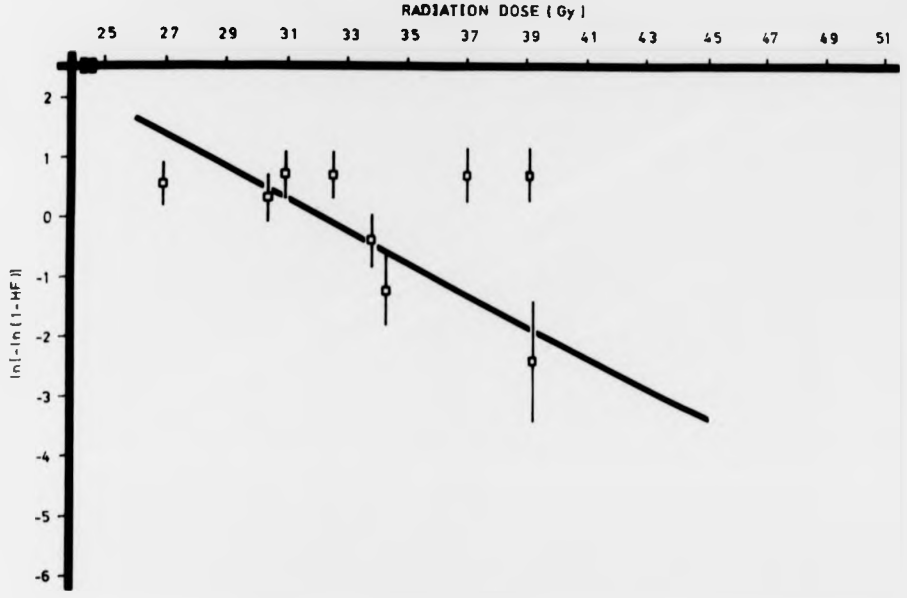


Figure 3.28: Effects of field-size on the healing response of skin. Data for 6 cm tail.

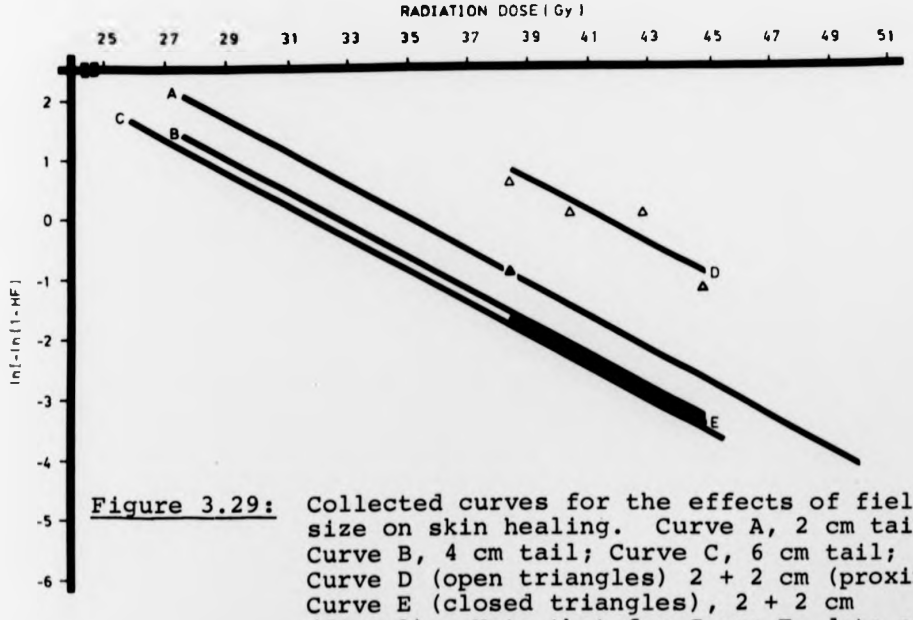


Figure 3.29: Collected curves for the effects of field-size on skin healing. Curve A, 2 cm tail; Curve B, 4 cm tail; Curve C, 6 cm tail; Curve D (open triangles) 2 + 2 cm (proximal) Curve E (closed triangles), 2 + 2 cm (distal). Note that for Curve E, data at 0% and 100% healing are used in the fitting but are not plotted. Hence the fitted line does not go through the single plotted point.

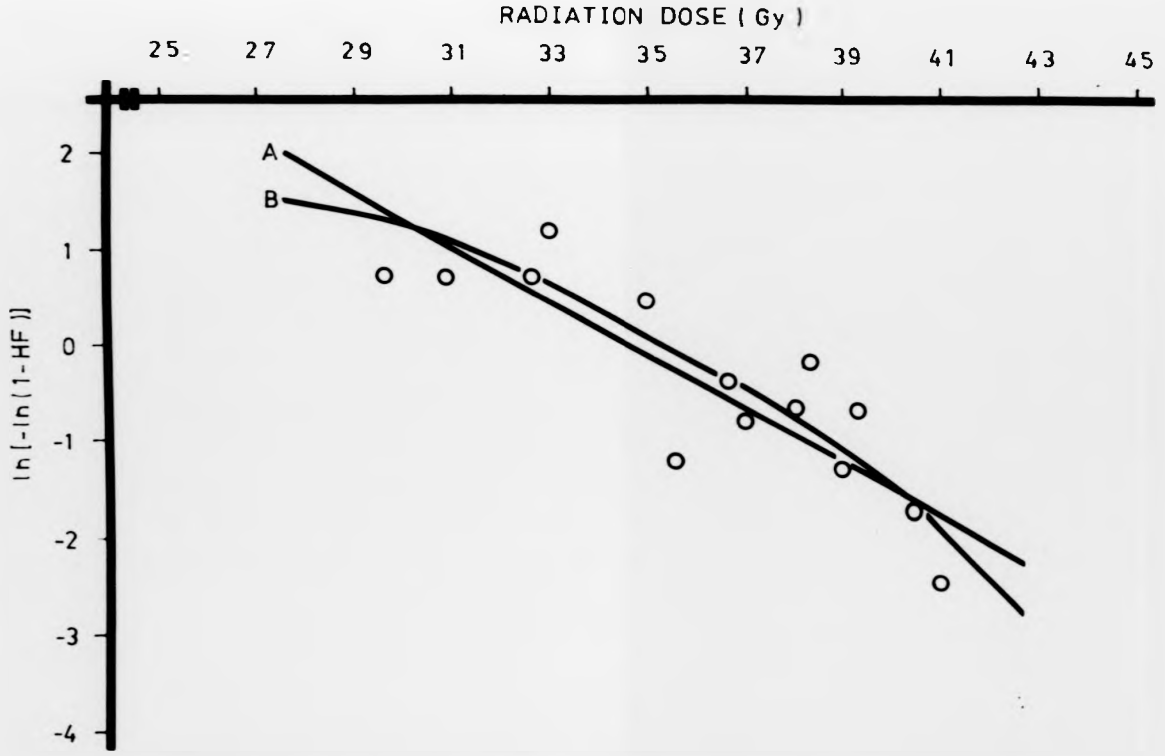


Figure 3.31: Effects of field-size on skin healing. Curve A represents the 2 cm data fitted using the DLOGS program. Curve B represents the same data fitted using the PROBIT program.

TABLE 3.17: Comparison of Survival Parameters Fitted Using DLOGS and PROBIT Programs. Field Size Experiment: Healing End Point

DATA SET	LD_{37} (cGy)	D_0 (cGy)	X^2	D.F.	X^2/D.F.
Pooled with common D_0					
DLOGS					
2 cm	3496 ± 72	360±41	207	66	3.14
4 cm	3236 ± 84				
6 cm	3211 ± 78				
PROBIT	LD_{50} (cGy)*				
2 cm	3684 ± 67	387±42**	183	66	2.77
4 cm	3440 ± 73				
6 cm	3419 ± 68				

* Unlike "DLOGS", the "PROBIT" program used LD_{50}.

** Probit width = approximately 1.2 x D_0 (see Methods Section, 2.5).

experiment. The radiation source was the PANTAK X-ray unit, with an extended f.s.d of 70 cm to achieve a more uniform dose distribution between the 2 and 4 cm lengths of tail. Total radiation doses ranging from 52 Gy up to 68 Gy were used. They were subdivided into 12 equal fractions, and the interval between fractions was kept at 8 hours. In the main experiment all three assay techniques were used, namely microcolonies, macrocolonies and healing. Four dose groups were used for the microcolony studies. They were irradiated with 52 and 58 Gy using either 2 cm or 4 cm tail. At higher doses (i.e. 60, 64 and 68 Gy) the macrocolony technique was employed, and 6 dose groups were used. Five dose groups ranging from 52 Gy to 68 Gy were used for the healing studies. Only 2 extra groups (i.e. 52 and 58 Gy) were used for this study as the healing data after 60, 64 and 68 Gy could be obtained from the macrocolony groups. All microcolony, macrocolony and healing data were analysed using the DLOGS program. The results are described separately as follows:

a). Microcolonies and Macrocolonies

The detailed analysis is given in Table 3.18. Both microcolony and macrocolony data were pooled to give a longer range of dose in the dose response relationship. D_0 values were 347 ± 22 cGy for 2 cm tail and 348 ± 43 cGy for 4 cm tail. When they were pooled a common D_0 of 348 ± 25 cGy was obtained. The analysis showed that there was no significant difference between 2 and 4 cm tail after they were irradiated with 12 small fractions, therefore, a common curve was drawn through the data for 2 and 4 cm tails (Figure 3.32). A further experiment using a single dose of 32 Gy gave the following numbers of macrocolonies per cm^2, 2.64 ± 0.47 (2 cm) and 1.3 ± 0.23 (4 cm).

TABLE 3.18: The 12 Fractions Field-size Experiment Using 2 and 4 cm Lengths of X-Irradiated Tail : Microcolonies and Macrocolonies

DATA SET	NE (NO. OF COL./CM^2)	D_0 (cGy)	X^2	D.F.	X^2/DF	F RATIO	SIGNIFICANCE
2 cm	$1.7 \pm 0.7 \times 10^6$	347 ± 22	5.3	4	1.33		
4 cm	$1.2 \pm 1.1 \times 10^9$	348 ± 43	51.3	4	12.83		
2 cm and 4 cm pooled	$1.3 \pm 0.7 \times 10^9$	348 ± 25	70	11	6.36	0.61	P > 0.05

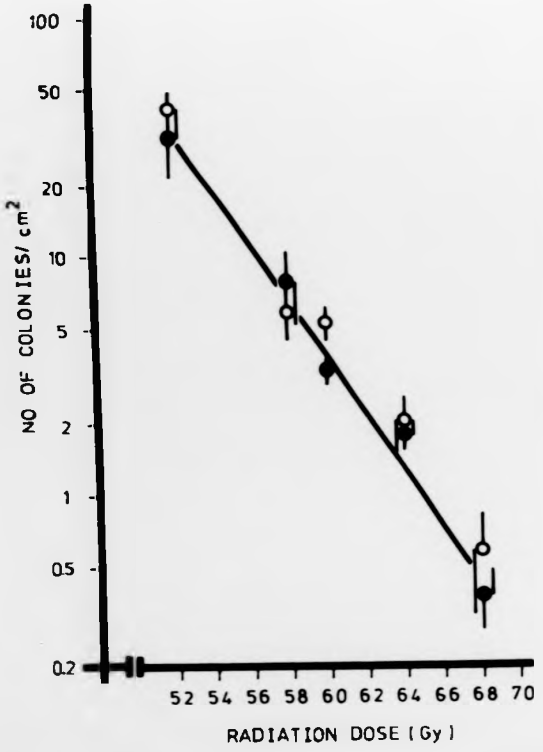


Figure 3.32: Effects of field-size on the survival of microcolony and macrocolony-forming cells (12 fractions). ○, 2 cm tail; ●, 4 cm tail.

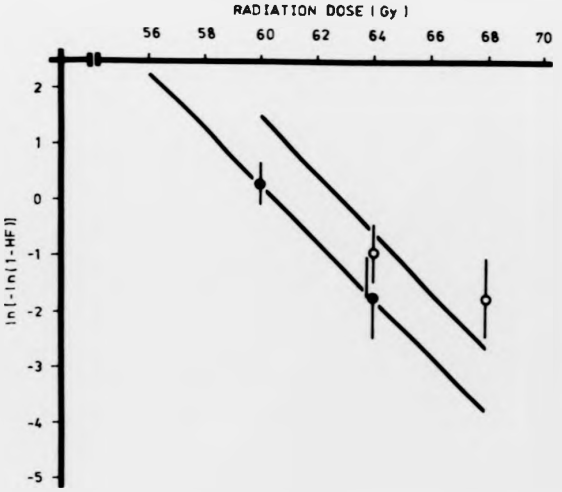


Figure 3.33: Effects of field-size on skin healing (12 fractions) ○, 2 cm tail; ●, 4 cm tail.

b). Healing Data

The analysis is detailed in Table 3.19. D_0 values were 215 ± 44 cGy for 2 cm tail and 182 ± 19 cGy for 4 cm tail when fitted separately. The difference between the D_0 value was of borderline significance at the 5% level, and the common D_0 was 201 ± 25 cGy. There was about a 4% dose reduction when comparing the LD_{37} values for the 4 cm and the 2 cm tail. The response curves were drawn using a common D_0, and they are presented in Figure 3.33. In the separate experiment using a single dose of 32 Gy (see previous section) the fractions of tails healed were 0.42 (2 cm) and 0 (4 cm).

TABLE 3.19: The Effect of Field Size on the Skin Receiving 12 Fractions: Healing

	DATA SET	LD_{37} (cGy)	NE	D_0 (cGy)	X^2	D.F.	X^2/D.F.	F RATIO	SIGNIFICANCE
A	2 cm	6292 ± 53	$5.2\pm0.4\times10^{12}$	215±44	1.8	3	0.6		
B	4 cm	6053 ± 20	$2.6\pm1.2\times10^{14}$	182±19	0.3	3	0.1	5.24^a	$P \sim 0.05$
C	2 & 4 cm pooled with common D_0	6155 ± 45	$3.2\pm1.1\times10^{11}$	232±43	7.6	9	0.84		
D	2 cm	6295 ± 39	$4.1\pm1.1\times10^{13}$	201±25	2.4	7	0.34	0.86^b	$P > 0.05$
	4 cm	6048 ± 40	$1.2\pm0.6\times10^{13}$						

[a] Comparison of group C with the sum of groups A and B (separated D_0's).
[b] Comparison of group D (common D_0) with the sum of groups A and B (separate D_0's).

3.3. RESIDUAL INJURY

3.3.1. Threshold-dose Residual Injury Experiment

There were three experiments performed in the investigation of threshold radiation doses which induced residual injury, measured using test doses at 6-9 weeks after the priming treatment. The microcolony (either using vincristine treatment or tritiated thymidine labelling methods), macrocolony, and skin healing techniques were used in this study. In the case of the microcolony technique (vincristine treated), the tails were irradiated using a range of priming doses from 10 Gy up to 25 Gy. The animals were left for 9 weeks to allow the skin to recover completely. Then a fixed dose of 21.25 Gy test dose was given. A similar experiment was also performed using skin samples labelled with ^{3}H-TdR. In this case, more single doses were used ranging from 10 Gy up to 25 Gy priming doses. Also, two groups of mice received fractionated priming doses and three groups received Adriamycin in combination with single doses of radiation in this experiment. Another experiment was performed using the macrocolony and healing techniques. The animals were irradiated with 10, 20 and 25 Gy priming doses. At 6 weeks after the priming irradiation, two varying test doses were given to both the aged controls and the previously irradiated skin. The time for macrocolony counting was around 21-30 days after the test dose. The healing was scored in the 9th week after the test dose.

a). Microcolonies (Vincristine Treated)

A total of 96 mice with 12 mice per dose group was used. They were irradiated with different priming doses ranging from 10 Gy up to 25 Gy, 9 weeks before a fixed test dose of 21.25 Gy was given. A group of 12 mice was tested using 15 mg/kg Adriamycin 30 minutes before 21.25 Gy.

This was designed to study any additional effect on the epidermis due to the Adriamycin administration. This experiment was carried out using vincristine to accumulate cells in mitosis as described in the Material and Methods Section. The detailed irradiation regimens and their results are given in Table 3.20. The results are presented in terms of number of colonies per cm^2 with a standard error. Significant reductions in colony numbers were seen for priming doses of 16.25 Gy and above. In order to compare the data with dose-reductions reported in the literature it was decided also to calculate the ratio of doses for equivalent levels of survival. In the present situation a fixed test dose was used, and the dose-ratio was calculated using a control microcolony curve obtained previously which is shown in Figure 3.8. Assuming that the slope for each curve is the same as the control, parallel curves can be drawn through the individual survival levels (i.e. number of colonies per cm^2). Then a specified level of survival can be chosen (at zero priming dose) and the dose reductions for the response of the pretreated skin which give this same level of survival can be calculated. Using this measurement about 5% of the dose was remembered in skin pretreated with 17.5 Gy up to 25 Gy. No gradual increase of residual injury with increasing priming dose was found within this range. 15 mg/kg Adriamycin injected 30 minutes before irradiation did not show a significant effect in the killing of clonogenic cells in the irradiated epidermis.

b). Microcolonies (^{3}H-TdR Labelled)

A total of 168 mice was divided into 14 groups with 12 mice per dose group. They were irradiated either with various priming doses ranging from 10 Gy to 25 Gy single doses or with 34 Gy or 39 Gy in 12 fractions

(i.e. 2.83 Gy or 3.25 Gy per fraction with an interval of 8 hours). Two groups of mice received pretreatment using the combination of 15 mg/kg Adriamycin with 15 Gy and 21.25 Gy single dose. The detailed treatment regimens and their results are given in Table 3.20. The results are presented in terms of number of colonies per cm^2 with standard errors. In order to assess the degree of residual injury after various pretreatments, exactly the same exercise was performed as that for the vincristine treated groups. For single doses about 4 to 7% of the residual injury was measured after doses between 17.5 Gy and 25 Gy. This is similar to the results obtained from the vincristine treated samples. The 34 Gy/12F or 39 Gy/12F groups received the test radiation dose 9 weeks after the last fraction. The percentage of residual injury for the 34 Gy/12F group was very similar to that for the single dose of 16.25 Gy, whereas for the 39 Gy/12F the percentage was similar to that for single doses above 20 Gy. Administration of 15 mg/kg Adriamycin 30 minutes prior to the test radiation dose of 21.25 Gy showed an enhanced killing effect on the clonogenic cells compared to irradiation alone. Two groups of mice received treatment either with 15 mg/kg Adriamycin + 30 minutes + 15 Gy or 15 mg/kg Adriamycin + 30 minutes + 21.25 Gy, followed by a test dose of 21.25 Gy 9 weeks later. About 5 % of residual injury was measured in both cases.

A comparison was made of the threshold dose for the skin and the gross appearance of the skin pigment and hairs 9 weeks after different priming treatments. The arbitrary scale in relation to the radiation dose is shown in Table 3.21. A photograph (Plate 3.1) is also shown here for the comparison of the state of the skin pigment and hairs versus dose.

TABLE 3.20: Response to Test Doses of Control Skin or Skin Receiving One Previous Treatment : Microcolonies

PRIMING DOSE	TREATMENT METHOD / TIME INTERVAL	TEST DOSE	VINCRISTINE NO. OF COL./CM^2	VINCRISTINE DOSE RATIO*	^{3}H-TdR NO. OF COL./CM^2	^{3}H-TdR DOSE RATIO
Aged Control		2125 cGy	50 ± 7.1	1.0	93 ± 8.9	1.0
1000 cGy	+ 9 weeks +	2125 cGy	54 ± 9.4	1.01		
1250 cGy	+ 9 weeks +	2125 cGy			94 ± 21	1.0
1500 cGy	+ 9 weeks +	2125 cGy	52 ± 8.3	1.0	78 ± 9.3	0.99
1625 cGy	+ 9 weeks +	2125 cGy			65 ± 14	0.97
1750 cGy	+ 9 weeks +	2125 cGy	32 ± 3.8	0.95	57 ± 9.2	0.96
2000 cGy	+ 9 weeks +	2125 cGy	31 ± 8.0	0.95	46 ± 11	0.94
2125 cGy	+ 9 weeks +	2125 cGy			58 ± 27	0.96
2250 cGy	+ 9 weeks +	2125 cGy	33 ± 3.9	0.96	45 ± 6.8	0.93
2500 cGy	+ 9 weeks +	2125 cGy	35 ± 7.9	0.96	44 ± 2.8	0.93
3400 cGy/12F	+ 9 weeks +	2125 cGy			64 ± 7.8	0.97
3900 cGy/12F	+ 9 weeks +	2125 cGy			41 ± 7.3	0.93
15 mg/kg ADR	+ 30 min. +	2125 cGy	44 ± 14	0.99	53 ± 11	0.95
15 mg/kg ADR	+ 30 min. +	1500 cGy				
	+ 9 weeks +	2125 cGy			60 ± 12	0.96
15 mg/kg ADR	+ 30 min. +	2125 cGy				
	+ 9 weeks +	2125 cGy			55 ± 7.3	0.95

* Dose ratio : The ratio of doses to produce the same number of colonies in the control and the pre-irradiated skin.

TABLE 3.21: Gross Appearance of Skin Pigment and Hairs at 9 Weeks Following Different Priming Treatment

PRIMING DOSE (cGy)	GENERAL APPEARANCE OF SKIN PIGMENT AND HAIRS
250	No evidence of loss of pigment and hair.
500	No evidence of loss of pigment and hair.
750	Few patches of lightly depigmented area and light hair loss.
1000	About 50% pigment and hair lost.
1250	About 70% pigment and hair lost.
1500	About 80% pigment and hair lost.
1750	More than 90% pigment and hair lost.
2000	Nearly 100% depigmentation and hair lost.
2250	100% depigmentation and hair lost. No evidence of new hair growth.
2500	100% depigmentation and hair lost. Necrosis in 8% of tails.

Plate 3.1: Gross appearance of skin pigment and hairs at 9 weeks following different priming doses as indicated.

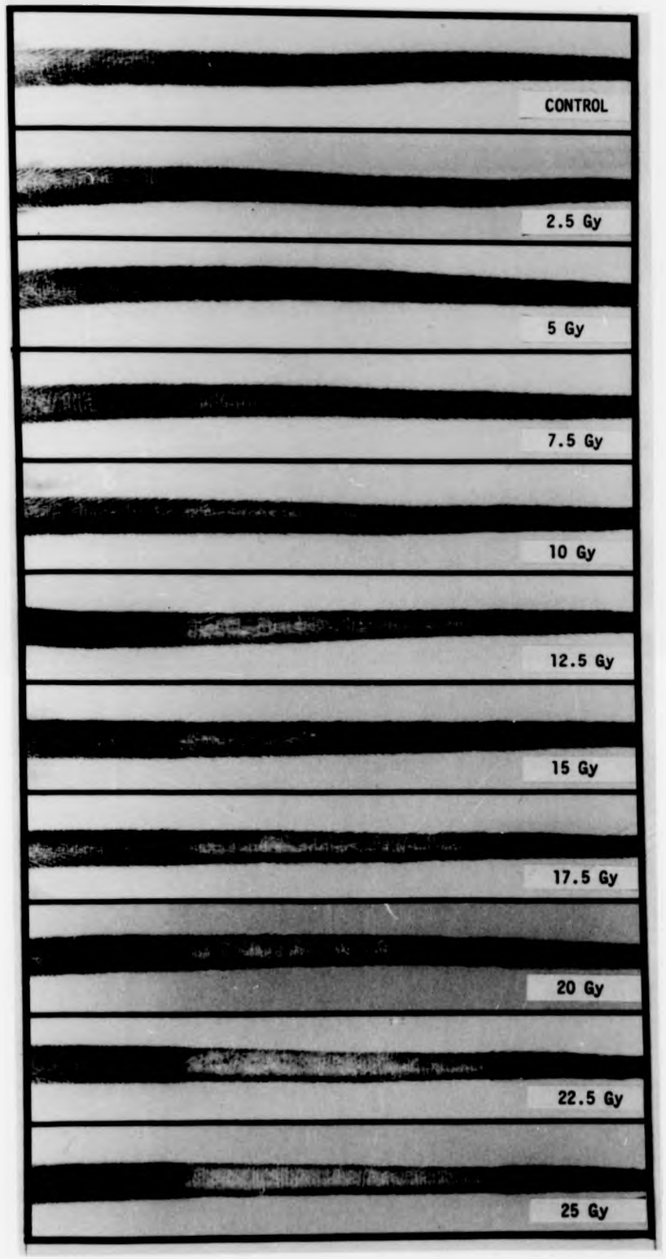
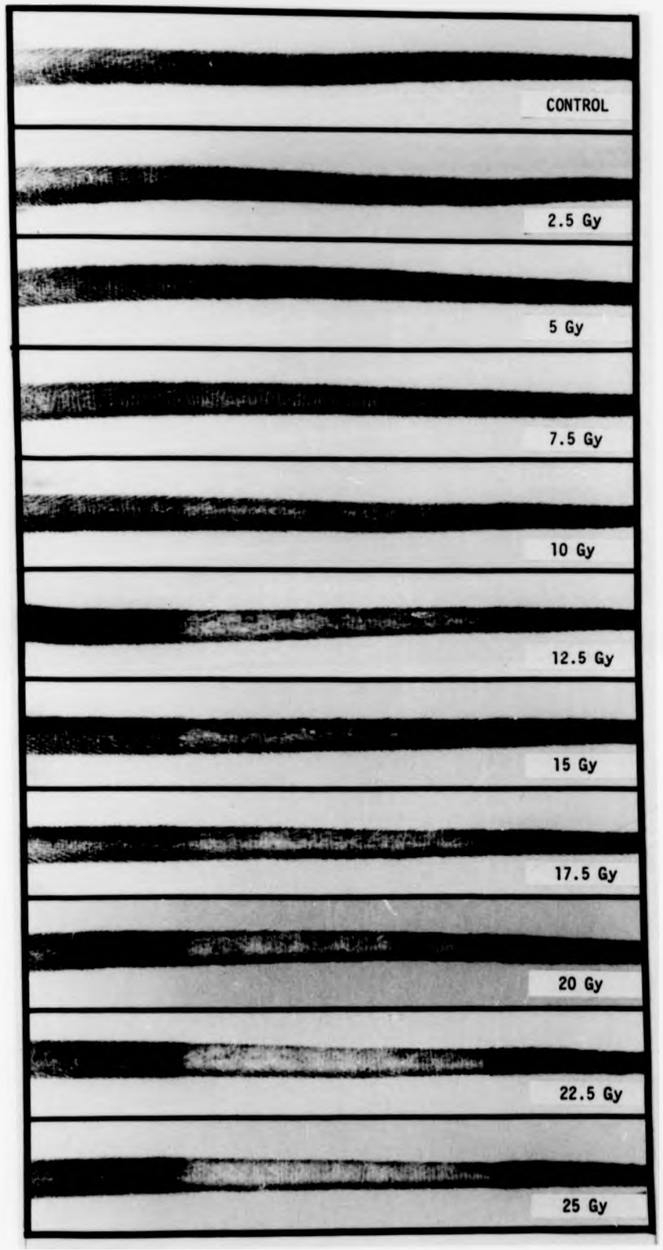


Plate 3.1: Gross appearance of skin pigment and hairs at 9 weeks following different priming doses as indicated.



c). Macrocolonies

A total of 96 mice with 12 mice per dose group was tested. They were irradiated with 10, 20 and 25 Gy 6 weeks before the test doses were given. The test doses were varied from about 25 Gy for the groups receiving priming doses of 20 and 25 Gy to 30 Gy for both the aged control or skin having received 10 Gy previously. The macrocolony data were analysed using the DLOGS computer program. The detailed irradiation regimens used and the data analysis are given in Table 3.22. D_0 values ranging from 430±74 cGy to 593±55 cGy were obtained from the aged control and the pretreated skin. When these data were pooled they were shown to be significantly different (data A, Table 3.22). The D_0 values were not significantly different and a common D_0 of 500 ± 32 cGy could be fitted (data C, Table 3.22). However, in order to calculate the degrees of residual injury for the different pretreatments, it was necessary to plot the curves using the fitted parameters (Figure 3.34). In the fitted curves, the data for skin receiving 25 Gy pretreatment were pooled with those from the 20 Gy group. This was done because there was only one dose point for the 25 Gy group and the number of colonies per cm^2 was very similar to that in the 20 Gy group (X^2/DF = 0.22). The degree of residual injury was obtained by comparing the ratio of the doses necessary to produce a specified level of injury in the pretreated skin and in the control skin. A figure of 2.7 colonies per cm^2 was therefore chosen as the specified level of injury. A direct comparison can be made only at this level of injury because the level of survival common to the three curves was restricted to values near 2.7 colonies per cm^2. When the doses were read from the fitted curves at the level of 2.7 colonies per cm^2 they were 29.9 Gy for the age control, 29.3 Gy for the skin receiving 10 Gy pretreatment and 25.5 Gy for skin

TABLE 3.22: Response to Test Dose of Control Skin or Skin Receiving One Previous Irradiation: Macrocolony

	DATA SET	NE (NO. OF COL./TAIL)	D_0 (cGy)	X^2	D.F.	X^2/DF	F RATIO	SIGNIFICANCE
A	Aged control	$7.5 \pm 2.3 \times 10^2$	593 ± 55	0.25	1	0.25		
	10 Gy + 6 weeks + test doses	$1.2 \pm 0.3 \times 10^3$	528 ± 38	2.1	1	2.1		
	20 & 25 Gy + 6 weeks + test doses	$1.8 \pm 1.3 \times 10^3$	430 ± 74	0.66	3	0.22	21	P < 0.05
B	Pooled	$5.6 \pm 1.2 \times 10^2$	582 ± 43	53.8	9	5.98		
	Pooled with common D_0							
C	Aged control	$1.8 \pm 0.4 \times 10^3$						
	10 Gy + 6 weeks + test doses	$1.7 \pm 0.4 \times 10^3$	500 ± 32	1.53	7	0.22	-1.23^a	P > 0.05
	20 & 25 + 6 weeks + test doses	$7.8 \pm 1.8 \times 10^2$						

[a] Comparison of group C (common D_0) with the sum of group B (separate D_0's).

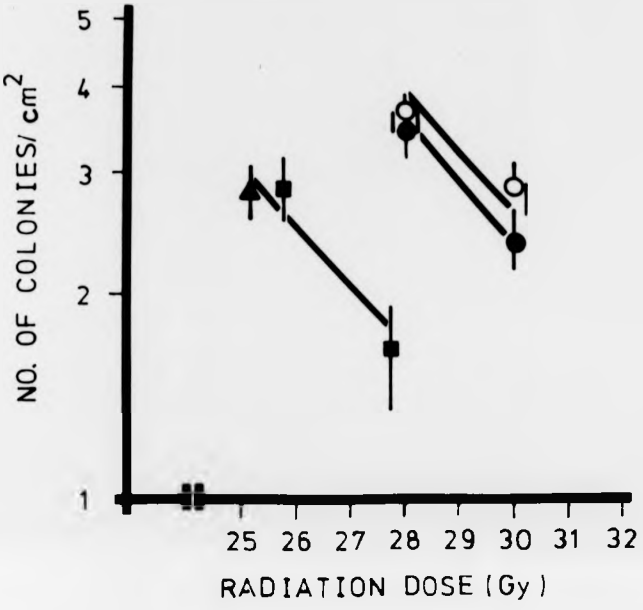


Figure 3.34: Effects of priming treatment on the survival of macrocolony-forming cells to test doses. ○, aged controls; ●, skin receiving 10 Gy pretreatment; ■, skin receiving 20 Gy pretreatment; ▲, skin receiving 25 Gy pretreatment.

receiving 20 and 25 Gy pretreatment. The ratio of the doses to reach this level of 2.7 colonies per cm^2 compared with the age control was 1.0 for age control, 0.98 for skin receiving 10 Gy pretreatment and 0.85 for skin receiving 20 and 25 Gy pretreatment. The ratios for the skin receiving 20 and 25 Gy pretreatment were significantly different from the 10 Gy pretreatment and the aged controls, but the latter two were not significantly different from each other.

d). Skin Healing

Exactly the same treatments and data analysis were used as for the macrocolony data. The only difference is that the healing data was obtained at 9 weeks after test doses were given, rather than about 21 to 28 days as for macrocolonies. The detailed irradiation regimens used and data analysis are given in Table 3.23. D_0 values ranging from 99 ± 11 cGy to 242 ± 87 cGy were obtained when they were fitted separately. The separate sets of data were significantly different (data A, Table 3.33), but the data could be fitted with a common D_0 of 143 ± 23 cGy (significance level $P \sim 0.05$). The residual injury was quantified by the ratio of values of LD_{37} between the pretreated skin and the age control skin. The ratio was 1.0 for the age control, 0.99 for skin receiving 10 Gy pretreatment and 0.87 for skin receiving 20 and 25 Gy pretreatment. The ratio for the skin receiving 20 and 25 Gy pretreatment were significantly different from the 10 Gy pretreatment and the aged control. This result was consistent with the result obtained using the macrocolony technique.

3.3.2. Response to Re-irradiation of Mouse Tails After Single and Fractionated Priming Doses

This type of experiment was designed to study the question - does

TABLE 3.23: Response to Test Dose of Control Skin or Skin Receiving One Previous Irradiation: Healing

	DATA SET	LD_{37} (cGy)	NE	D_0 (cGy)	X^2	D.F.	X^2/D.F.	F RATIO	SIGNIFICANCE
A	Age control	2932 ± 65	1.9×10^5	242 ± 87	0.8	1	0.8		
	10 Gy + 6 weeks + test doses	2876 ± 23	1.5×10^7	174 ± 29	0.2	1	0.2		
	20 Gy & 25 Gy + 6 weeks + test doses	2501 ± 11	8.4×10^{10}	99 ± 11	0.95	4	0.2	87	P < 0.05
B	Pooled	2638 ± 95	5.2×10^3	309 ± 142	31.0	7	4.4		
C	Pooled with common D_0								
	Age control	2897 ± 37	5.9×10^8						
	10 Gy + 6 weeks + test doses	2870 ± 36	4.9×10^8	143 ± 23	6.3	8	0.79	6.45[a]	P ~ 0.05
	20 Gy & 25 Gy + 6 weeks + test doses	2509 ± 24	4.0×10^7						

[a] Comparison of group C (Common D_0) with the sum of group A (separate D_0's).

fractionation have any effect on modifying the degree of residual injury when skin is treated to tolerance (i.e. >95% healed)? Two separate experiments were carried out. The first experiment was performed using 25 Gy single dose and 41 Gy given in 4 fractions. The second experiment used 39 Gy given in 4 fractions, 48 Gy in 8 fractions, 57 Gy in 15 fractions and 60.8 Gy in 30 fractions. In the case of 39 or 41 Gy in 4 fractions, 48 Gy in 8 fractions and 57 Gy in 15 fractions, the fractions were given daily; whereas for 60.8 Gy in 30 fractions, 2 fractions per day were given, with an interval of 6 hours. The number of mice used and the percentage of tails healed 6 weeks after different priming treatments are listed below:

PRIMING DOSE/FRACTION	NO. OF TAILS IRRADIATED	NO. OF TAILS HEALED	% OF TAILS HEALED
25 Gy/ 1F	48	47	97.9
41 Gy/ 4F	48	32	66.7
39 Gy/ 4F	72	70	97.2
48 Gy/ 8F	72	70	97.2
57 Gy/15F	96	90	93.8
60.8 Gy/30F	96	94	97.9

In the case of 41 Gy in 4 fractions, there was an exceptionally low percentage of tails healed, but the rest of the groups were close to tolerance (i.e. 94 to 98% skin healed). 6 weeks after the priming treatment those tails which had fully re-epithelialised (i.e. $\leq$ 0.75 score) were selected and irradiated with graded test doses. In the meanwhile 17 groups of 12 aged control mice were also irradiated with similar test doses. They were divided into 3 aged control groups, each group irradiated at the same time as 1 pre-irradiated group (i.e. 1F and 4F, 4F and 8F, and 15F and 30F). Two assay techniques were used in this

study, namely macrocolonies and healing. The results were analysed using the DLOGS computer program. The data are described separately as follows:

a). Macrocolonies

The different radiation regimens and data analysis are detailed in Table 3.24. D_0 values for the aged controls ranged from 503 ± 90 cGy to 627 ± 58 cGy. There was no significant difference between the aged controls irradiated on different occasions (data A, Table 3.24). A best fit line with D_0 of 580 ± 57 cGy was drawn through the pooled data and is shown in Figure 3.35. D_0 values for the skin pre-irradiated with different doses and fractionations ranged from 249 ± 39 cGy to 425 ± 67 cGy. There was a significant difference between these groups (data C, Table 3.24). As listed above, 41 Gy in 4 fractions gave only 67% tails healed, whereas 39 Gy in 4 fractions gave about 97% tails healed. The response to re-irradiation of these two groups was not significantly different, and a common curve with a D_0 of 249 ± 37 cGy could be fitted to the pooled data (data G, Table 3.24). The data concerning the responses of all pre-irradiated groups could be fitted with a common D_0 of 314 ± 27 cGy (data H, Table 3.24). Analysis showed that the D_0 for the pre-irradiated groups was different from that for the pooled age controls (data I, Table 3.24). Best fit lines are shown in Figures 3.36, 3.37, 3.38, 3.39, and 3.40 for the data fitted with separate D_0's. For the direct comparison of the relationship between the response of the aged controls and the pre-irradiated groups, 5 curves for the pre-irradiated groups, fitted with a common D_0, and 2 curves for the aged controls are shown in Figure 3.41. The 2 curves for the aged controls refer to the aged controls in this experiment or all of the age controls from Figure 3.12. When the pre-irradiated groups were each

TABLE 3.24: Response to the Test Doses of Control Skin or Skin Receiving Various Fractionated Tolerance Doses: Macrocolonies

DATA SET	NE (NO. OF COL./TAIL)	D_0 (cGy)	X^2	D.F.	X^2/DF	F RATIO	SIGNIFICANCE
A Aged control 1 & 4F	$5.4 \pm 1.5 \times 10^2$	627 ± 58	7	2	3.5		
4 & 8F	$1.4 \pm 1.2 \times 10^3$	511 ± 103	14	8	1.75		
15 & 30F	$1.4 \pm 1.0 \times 10^3$	503 ± 90	5.1	5	1.02	1.72	P > 0.05
B Aged controls pooled	$6.9 \pm 2.4 \times 10^2$	580 ± 57	32	17	1.88		
C Irradiated 25 Gy/1F	$1.7 \pm 1.2 \times 10^3$	425 ± 67	2.2	3	0.73		
41 & 39 Gy/4F	$1 \pm 1.2 \times 10^5$	249 ± 39	42.5	9	4.72		
48 Gy/8F	$2.5 \pm 1.9 \times 10^3$	397 ± 62	5	5	1.0		
57 Gy/15F	$8.0 \pm 9.6 \times 10^3$	347 ± 74	6.6	5	1.32		
60.8 Gy/30F	$1.4 \pm 1.3 \times 10^4$	336 ± 54	6.9	5	1.38	5.85	P < 0.05
D Irradiated pooled	$1.7 \pm 1.1 \times 10^4$	311 ± 34	173.1	35	4.95		
E 41 Gy/4F	$7.6 \pm 1.2 \times 10^2$	440 ± 19	0.5	2	0.25		
F 39 Gy/4F	$5.6 \pm 7.0 \times 10^5$	218 ± 32	24.7	5	4.94		
G 41 Gy & 39 Gy/4F pooled	$1 \pm 1.1 \times 10^5$	249 ± 37	42.5	9	4.72	2.4^a	P > 0.05
Pooled with common D_0							

TABLE 3.24: (CONTD.)

	DATA SET	NE (NO. OF COL./TAIL)	D_0 (cGy)	X^2	D.F.	X^2/DF	F RATIO	SIGNIFICANCE
H	25 Gy/1F	$1.5 \pm 0.8 \times 10^4$						
	41 & 39 Gy/4F	$1.0 \pm 0.5 \times 10^4$						
	48 Gy/8F	$1.5 \pm 0.7 \times 10^4$	314 ± 27	78.6	31	2.54	1.65[b]	$P > 0.05$
	57 Gy/15F	$1.8 \pm 1 \times 10^4$						
	60.8 Gy/30F	$2.6 \pm 1.3 \times 10^4$						
I	Aged controls pooled	$7.7 \pm 3.5 \times 10^3$						
	25 Gy/1F	$2.7 \pm 1.1 \times 10^3$						
	41 & 39 Gy/4F	$1.6 \pm 0.7 \times 10^3$						
	48 Gy/8F	$2.5 \pm 1.1 \times 10^3$	395 ± 35	147	49	3	4.8[c]	$P < 0.05$
	57 Gy/15F	$3.0 \pm 1.3 \times 10^3$						
	60.8 Gy/30F	$4.2 \pm 1.8 \times 10^3$						
J	All aged controls pooled	$4.0 \pm 1.2 \times 10^3$						
	25 Gy/1F	$1.5 \pm 0.4 \times 10^3$						
	41 & 39 Gy/4F	$8.6 \pm 2.3 \times 10^2$						
	48 Gy/8F	$1.4 \pm 0.4 \times 10^3$	434 ± 27	200.8	87	2.3	4.29[d]	$P < 0.05$
	57 Gy/15F	$1.6 \pm 0.4 \times 10^3$						
	60.8 Gy/30F	$2.2 \pm 0.6 \times 10^3$						

TABLE 3.24: (CONTD.)

* Pooled data for all aged controls were taken from Table 3.5.

a = Comparison of group G with the sum of groups E and F (separate D_0's).

b = Comparison of group H (common D_0) with the sum of group C (separate D_0's).

c = Comparison of group I (common D_0) with the sum of groups B and C (separate D_0's).

d = Comparison of group J (common D_0) with the sum of groups in Table 3.5 and C in this table (separate D_0's).

e = When $P < 0.05$ indicates that the fit of the pooled data is significantly different from the separate fitting of the individual data.
When $P > 0.05$ indicates that the fit of the pooled data is not significantly different from the separate fitting of the individual data.

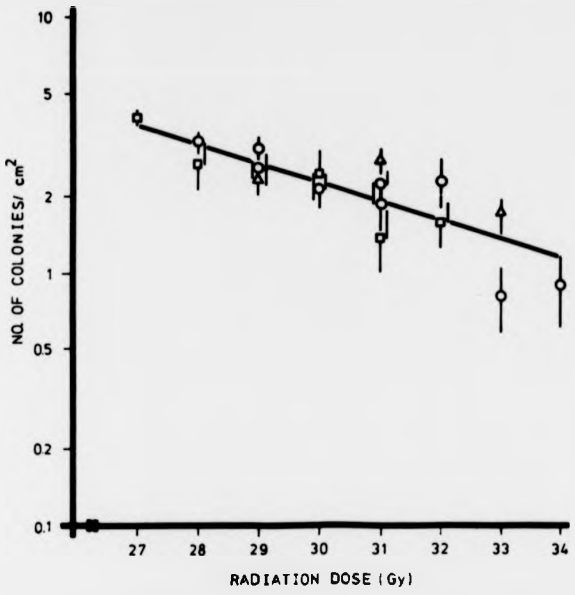


Figure 3.35: Response to the test doses of control skin (macrocolonies). △ , aged controls in single priming dose experiment; ○, aged controls in 4 and 8 fraction priming dose experiment; □ , aged controls in 15 and 30 fraction priming dose experiment.

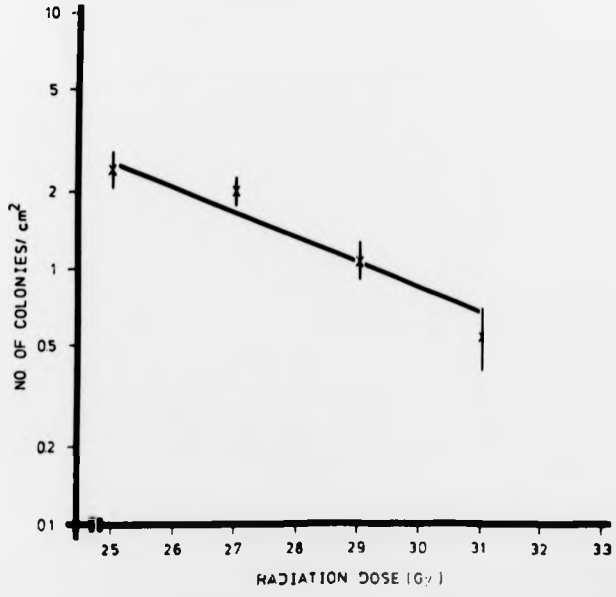


Figure 3.36: Response to the test doses of skin which received 25 Gy single dose in the previous treatment: Macrocolonies.

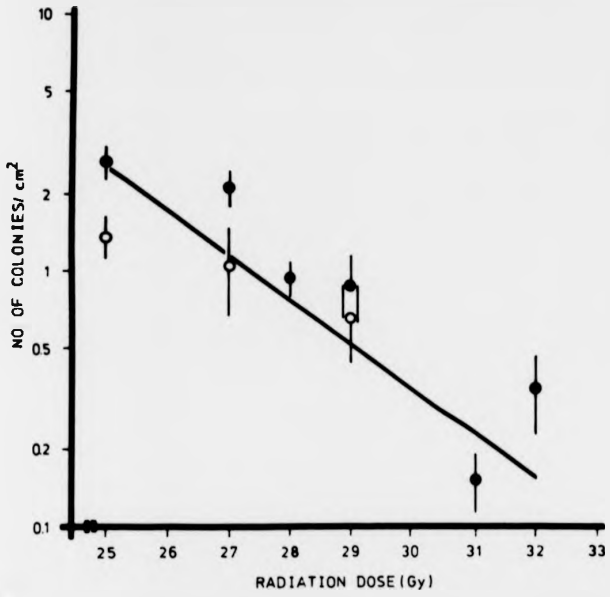


Figure 3.37: Response to the test doses of skin which received 41 and 39 Gy/4F in the previous treatment: Macrocolonies ○ , 41 Gy/4F; ●, 39 Gy/4F.

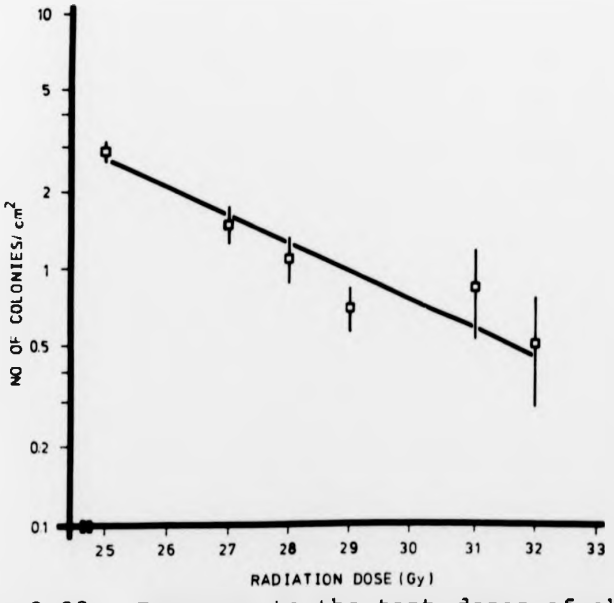


Figure 3.38: Response to the test doses of skin which received 48 Gy/8F in the previous treatment: Macrocolonies.

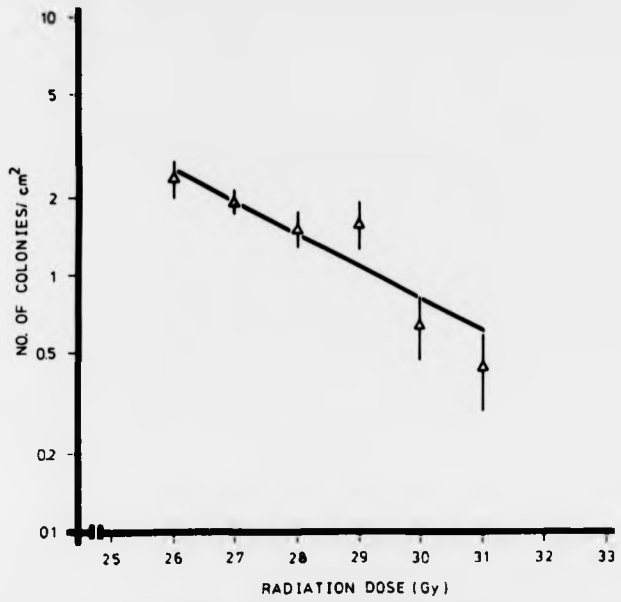


Figure 3.39: Response to the test doses of skin which received 57 Gy/15F in the previous treatment: Macrocolonies.

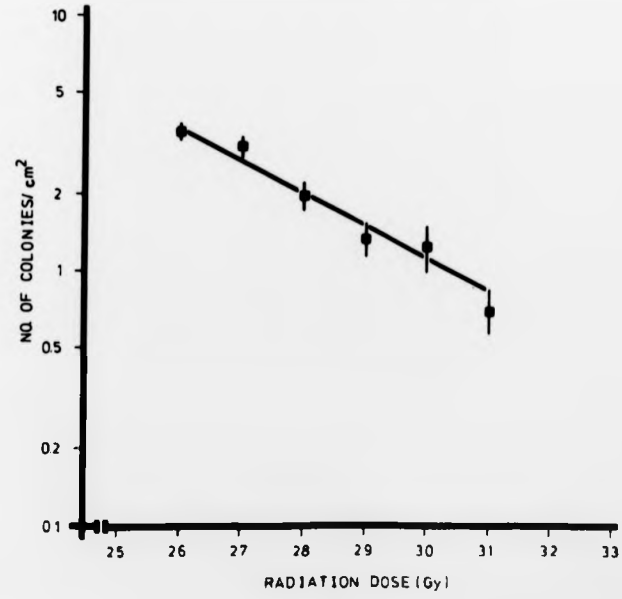


Figure 3.40: Response to the test doses of skin which received 60.8 Gy/30F in the previous treatment: Macrocolonies.

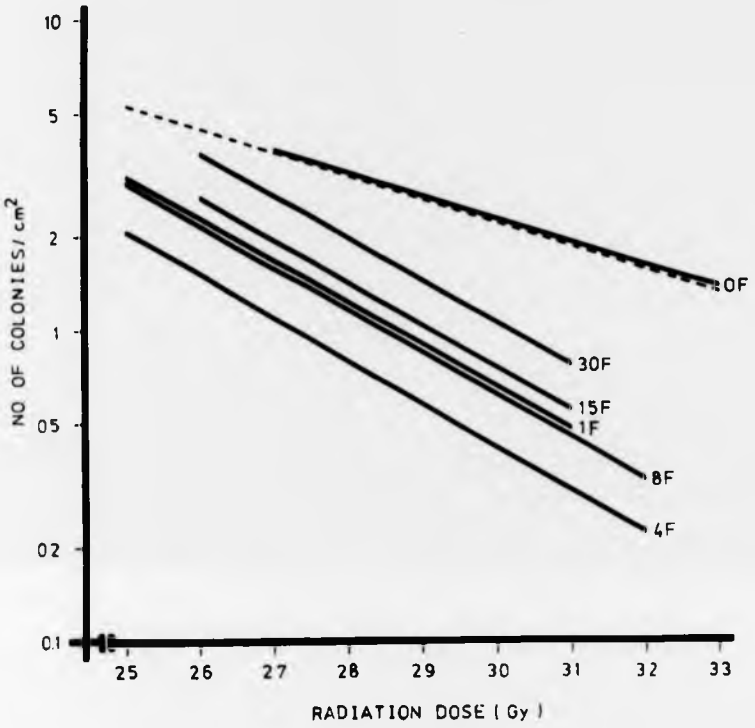


Figure 3.41: Response to the test doses of control skin and skin receiving previous fractionated tolerance doses: Macrocolonies. Solid line (top curve) represents aged controls in this experiment (OF); Dashed line (2nd curve) represents aged controls in all the experiments. 5 solid lines at the bottom represents each number of fractions used in the previous irradiation.

fitted with a common D_0 there was a trend toward a higher number of colonies per cm^2 for groups where there was a higher number of fractions i.e. a smaller dose per fraction in the priming treatment. If a specified level of injury is set at 1 colony per cm^2, then the dose ratios to a baseline of 1.0 for 60.8 Gy/30F were 0.97, 0.95, 0.94, 0.91 for 57 Gy/15F, 25 Gy/1F, 48 Gy/8F and 41 and 39 Gy/4F respectively. The single dose group (25 Gy/1F) was intermediate between 15 and 8 fractions. The aged controls (solid line) in this experiment responded very similarly to all the aged controls (dashed line, taken from Figure 3.12).

b). Skin Healing

The detailed data analysis is shown in Table 3.25. In the aged control groups, the D_0 values ranged from 238 ± 67 cGy to 421 ± 652 cGy and they were not significantly different (data A, Table 3.25). A single curve could be fitted through all the data (Figure 3.42). A very large error in the single dose aged control was probably due to the small sample size as it only used 3 doses, whereas the 4 and 8 fraction or 15 and 30 fraction groups used more than 6 doses. D_0 values for the skin pre-irradiated with various priming doses and fractionations ranged from 105 ± 18 cGy to 157 ± 59 cGy. They were not significantly different from each other (data C, Table 3.25), although the F ratio is not far from reaching significance. The reason for this could be due to the more scattered data for the healing compared with the colony data. There was a significant difference between the aged control data and the data for pre-irradiated tails (data E, Table 3.25). In order to compare the dose reductions among the pre-irradiated groups an analysis was done using common D_0 values but different LD_{37}s. The 41 Gy/4F and 39 Gy/4F

TABLE 3.25: Response to the Test Doses of Control Skin or Skin Previously Receiving Various Fractionated Doses: Healing

	DATA SET	LD_{37} (cGy)	NE	D_0 (cGy)	X^2	D.F.	X^2/D.F.	F RATIO	SIGNIFICANCE
A	Age control								
	1F	2654 ± 656	$5.5\pm29.1\times10^2$	421 ± 652	3.9	1	3.9		
	4 & 8F	2686 ± 92	$8.1\pm12.0\times10^4$	238 ± 67	5.0	7	0.7		
	15 & 30F	2571 ± 125	$2.4\pm 3.3\times10^3$	330 ± 118	2.5	4	0.63	0.76	P > 0.05
B	Age control pooled	2610 ± 92	$3.5\pm 3.5\times10^3$	320 ± 77	14.3	16	0.89		
C	Irradiated								
	25 Gy/1F	2409 ± 27	$8.6\pm11.0\times10^9$	105 ± 18	0.2	2	0.1		
	41 & 39 Gy/4F	2317 ± 114	$2.5\pm 6.6\times10^6$	157 ± 59	7.3	7	1.04		
	48 Gy/8F	2408 ± 53	$1.0\pm 2.0\times10^9$	116 ± 31	2.0	4	0.5		
	57 Gy/15F	2453 ± 46	$3.7\pm 5.2\times10^7$	141 ± 32	3.0	5	0.6		
	60.8 Gy/30F	2569 ± 57	$2.5\pm 3.9\times10^8$	133 ± 51	16.8	5	3.36	3.06	P > 0.05
D	Irradiated pooled	2430 ± 43	$1.6\pm 1.9\times10^7$	147 ± 29	60.7	31	1.96		
	Age controls pooled	2610 ± 92	$3.5\pm 3.5\times10^3$	320 ± 77	14.3	16	0.89		
	Irradiated pooled	2430 ± 43	$1.6\pm 1.9\times10^7$	147 ± 29	60.7	31	1.96	15.7^a	P < 0.05
E	Age controls pooled + irradiated pooled	2255 ± 138	$4.5\pm 4.6\times10^2$	369 ± 92	125.4	49	2.56		

TABLE 3.25: (CONTD.)

	DATA SET	LD_{37} (cGy)	NE	D_0 (cGy)	X^2	D.F.	X^2/D.F.	F RATIO	SIGNIFICANCE
	Pooled with common D_0, different LD_{37}								
F	25 Gy/1F	2370 ± 70	$4.9 \pm 5.2 \times 10^7$						
	41 & 39 Gy/4F	2356 ± 58	$4.4 \pm 4.9 \times 10^7$						
	48 Gy/8F	2383 ± 65	$5.4 \pm 5.9 \times 10^7$	134 ± 20	30.3	27	1.12	0.18[b]	P > 0.05
	57 Gy/15F	2460 ± 45	$9.6 \pm 9.0 \times 10^7$						
	60.8 Gy/30F	2569 ± 32	$2.2 \pm 1.3 \times 10^8$						
G	Age controls pooled	2732 ± 39	$1.1 \pm 0.8 \times 10^6$						
	25 Gy/1F	2270 ± 108	$1.1 \pm 0.9 \times 10^5$						
	41 & 39 Gy/4F	2247 ± 88	$9.6 \pm 8.5 \times 10^4$						
	48 Gy/8F	2275 ± 102	$1.1 \pm 1.0 \times 10^5$	196 ± 26	55.6	44	1.26	2.23[c]	P > 0.05
	57 Gy/15F	2384 ± 71	$1.9 \pm 1.5 \times 10^5$						
	60.8 Gy/30F	2533 ± 49	$4.1 \pm 2.3 \times 10^5$						
H	All age controls pooled	2766 ± 26	$2.4 \pm 1.0 \times 10^5$						
	25 Gy/1F	2222 ± 116	$2.1 \pm 1.2 \times 10^4$						
	41 & 39 Gy/4F	2194 ± 92	$1.9 \pm 1.1 \times 10^4$						
	48 Gy/8F	2222 ± 108	$2.1 \pm 1.2 \times 10^4$	223 ± 21	99.4	81	1.23	1.74[d]	P > 0.05

TABLE 3.25: (CONTD.)

	DATA SET	LD_{37} (cGy)	NE	D_0 (cGy)	X^2	D.F.	X^2/D.F.	F RATIO	SIGNIFICANCE
	57 Gy/15F	2345 ± 74	$3.7 \pm 1.9 \times 10^4$						
	60.8 Gy/30F	2513 ± 53	$7.8 \pm 2.9 \times 10^4$						
I	41 Gy/4F	2022 ± 593	$5.6 \pm 36.3 \times 10^3$	234 ± 235	0.7	1	0.7		
J	39 Gy/4F	2406 ± 63	$5.8 \pm 11.1 \times 10^7$	135 ± 41	3.8	4	0.95	1.56^e	P > 0.05
K	41 Gy & 31 Gy/4F pooled	2317 ± 113	$2.5 \pm 6.8 \times 10^6$	157 ± 60	7.3	7	1.04		

[a] Comparison of group E with the sum of groups B and D (separate D_0's).
[b] Comparison of group F (common D_0) with the sum of group C (separate D_0's).
[c] Comparison of group G (common D_0) with the sum of groups B and C (separate D_0's).
[d] Comparison of group H (common D_0) with the sum of groups in table 3.6 and group C in this table (separate D_0).
[e] Comparison of group K (pooled) with the sum of groups I and J (separate D_0's).

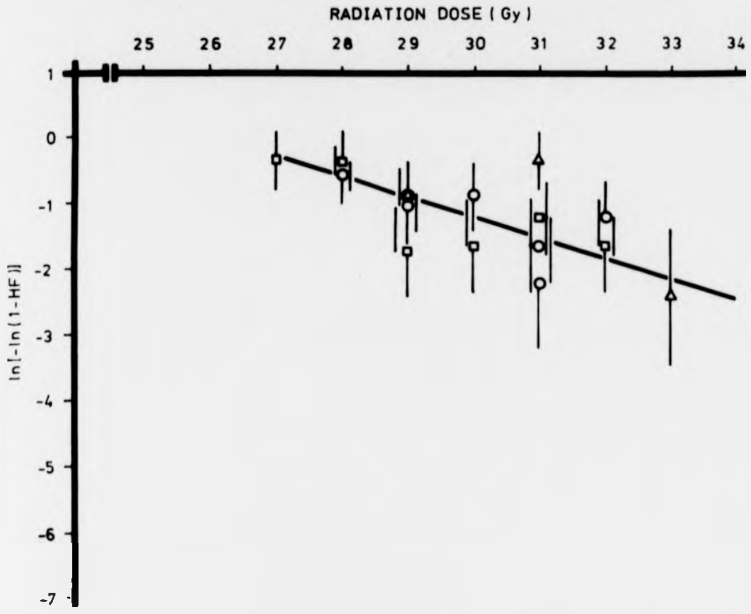


Figure 3.42: Response to the test doses of control skin (healing). △, aged controls from single priming dose; ○, aged controls from 4 & 8F priming doses. □, aged controls from 15 & 30F priming doses.

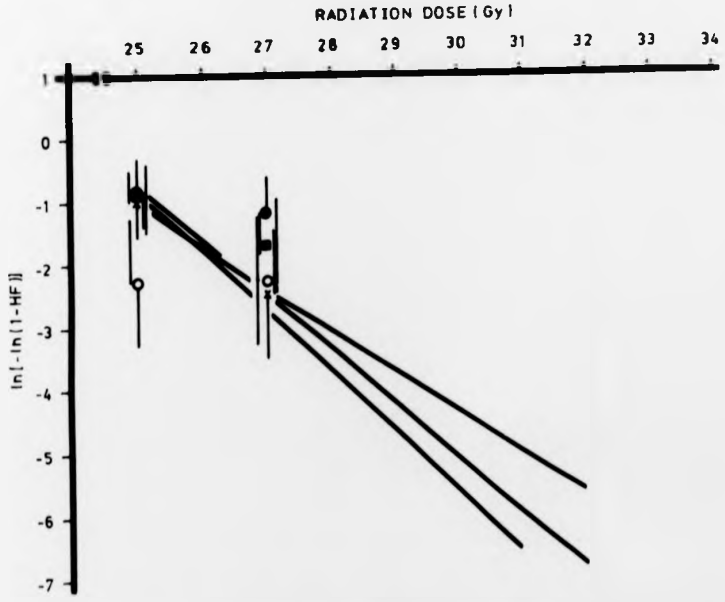


Figure 3.43: Response to the test doses of skin receiving 25 Gy/1F (X), 41 Gy/4F (○), 39 Gy/4F (●) and 48 Gy/8F (■) previous treatment (healing).

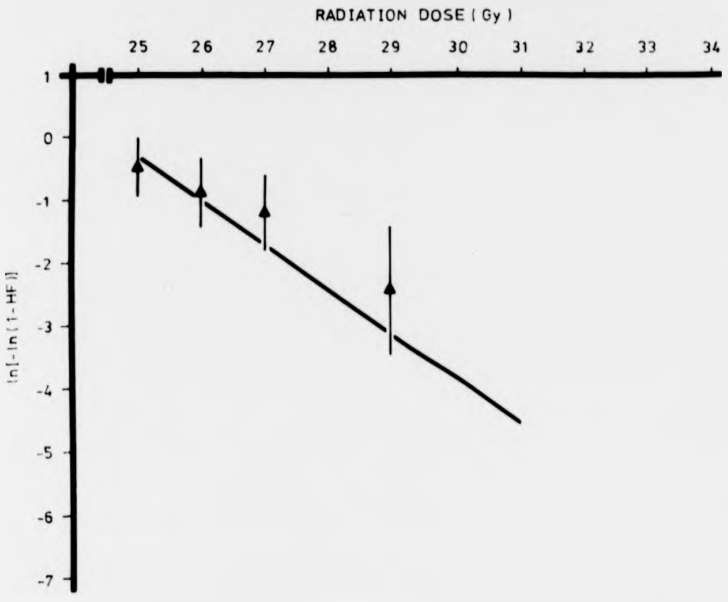


Figure 3.44: Response to the test dose of skin receiving 57 Gy/15F previous treatment (healing).

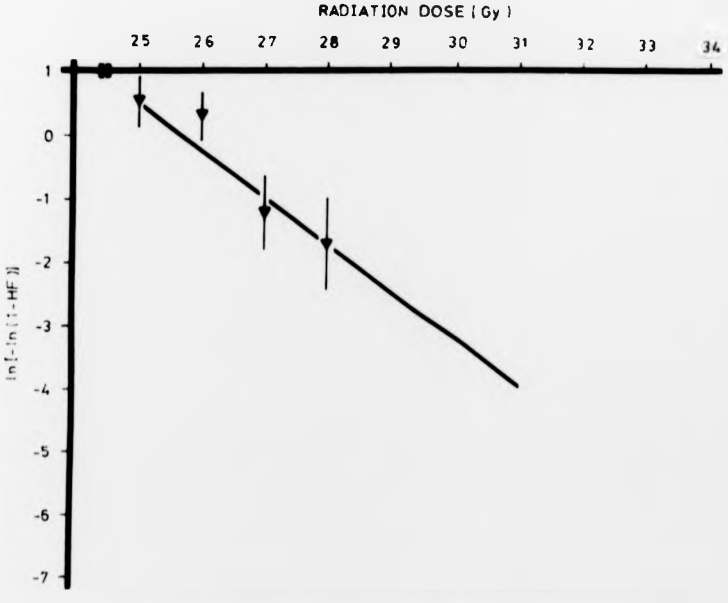


Figure 3.45: Response to the test doses of skin receiving 60.8 Gy/30F previous treatment (healing).

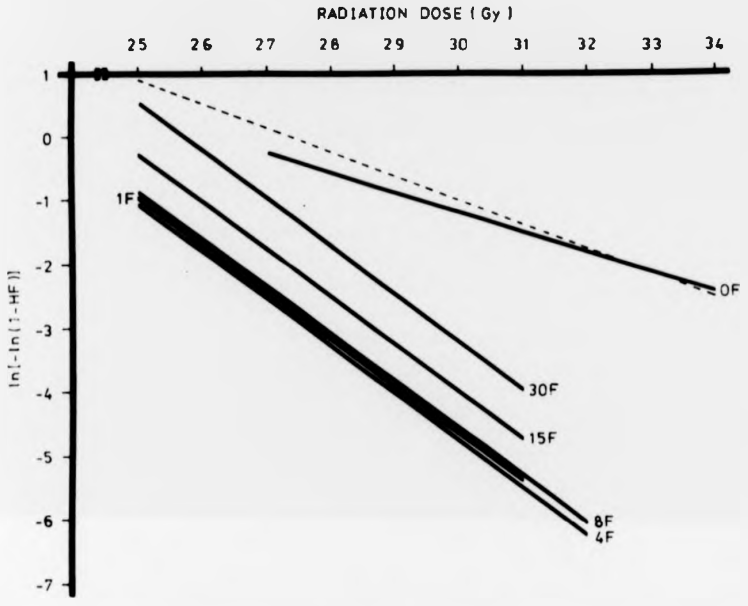


Figure 3.46: Response to the test doses of control skin and skin receiving fractionated tolerance doses (healing). Dashed line (top curve) represents all aged controls; solid line (2nd curve) represents aged controls in this experiment (OF); 5 solid lines at the bottom represent each number of fractions used in the priming irradiations.

groups were not significantly different from each other, and they were pooled (data K, Table 3.25). Best fit lines are drawn through the data fitted with separate D_0's, they are shown in Figures 3.43, 3.44 and 3.45. Note that with most of the curves for healing no observations but only a fitted line was drawn at higher doses. This is due to all tails necrosing when the doses reach to a certain level or else not a sufficient number of tails was used. In both cases the range of the fitted line represents the range of dose used. In Figure 3.46, combined curves were drawn from fitted values using a common D_0 of 134 ± 20 cGy for the pre-irradiated groups and a Do of 320 ± 77 cGy for the pooled aged controls. The curve for all the aged controls taken from Figure 3.13 was also drawn in this figure (dotted line). When comparing LD_{37} values to the 60.8 Gy/30F group as a baseline the dose ratios were 0.94, 0.90, 0.90 and 0.89 for 57 Gy/15F, 48 Gy/8F, 25 Gy/1F and 41 and 39 Gy/4F groups respectively. The single dose (25 Gy/1F) group in this case is still very close to the 48 Gy/8F group (Figure 3.46) but it was intermediate between 8 and 4 fractions. The dose ratio was 0.93 when comparing the LD_{37} for the 60.8 cGy/30F group to the aged controls using a common D_0. In short, data analysed using both macrocolony and healing techniques demonstrated that there was a common trend toward a lower level of residual injury when the number of fractions increased or the dose per fraction decreased. Also, about 7 - 18% residual injury was measured if one compared the healing data using a common D_0 for both aged controls and the pre-irradiated groups.

3.3.3. Response to Test Dose of Aged Control Skin or Skin Receiving 3 Previous Tolerance Doses

Three experiments were performed. Each experiment used 4 to 6 dose

groups for both aged controls and tails receiving 3 tolerance doses previously, with 12 mice per dose group. 3 tolerance doses were given in the schedule 25 Gy + 6 weeks + 22.5 Gy + 6 weeks + 20.25 Gy + 6 weeks + a range of test doses. 25 Gy was chosen as the 1st tolerance dose based on the results obtained by Hendry (1976). It produced less than 5% tail necrosis 6 weeks after the irradiation. Tolerance of the skin was reduced by a factor of about 10% after each dose (Hendry, 1978), and therefore a further 10% dose reduction was allowed for each of the second and the third tolerance doses. In the main experiment there were 4 types of assay technique used: namely, gross skin reaction, microcolony, macrocolony and healing (or necrosis). In this section only the gross skin reaction, macrocolony and the healing data will be described, and the microcolony data will be shown in the following section.

a). Gross Skin Reaction

The pattern of the skin reaction between aged controls and skin irradiated 3 times previously is illustrated in Figure 3.47. Reactions in the pretreated skin occurred about 2-6 days earlier than with controls. The peak reaction was reached at about 22 days for both controls and skin treated 3 times previously. This indicates that the pretreated skin reacts more quickly but the reaction process takes longer. The degrees of skin reaction were dose dependent in both cases. The end point of skin healing was qualitatively different in the two cases. For instance, at 25 Gy all the aged controls healed completely, whereas all the skin irradiated 3 times previously showed a complete necrosis of the tails.

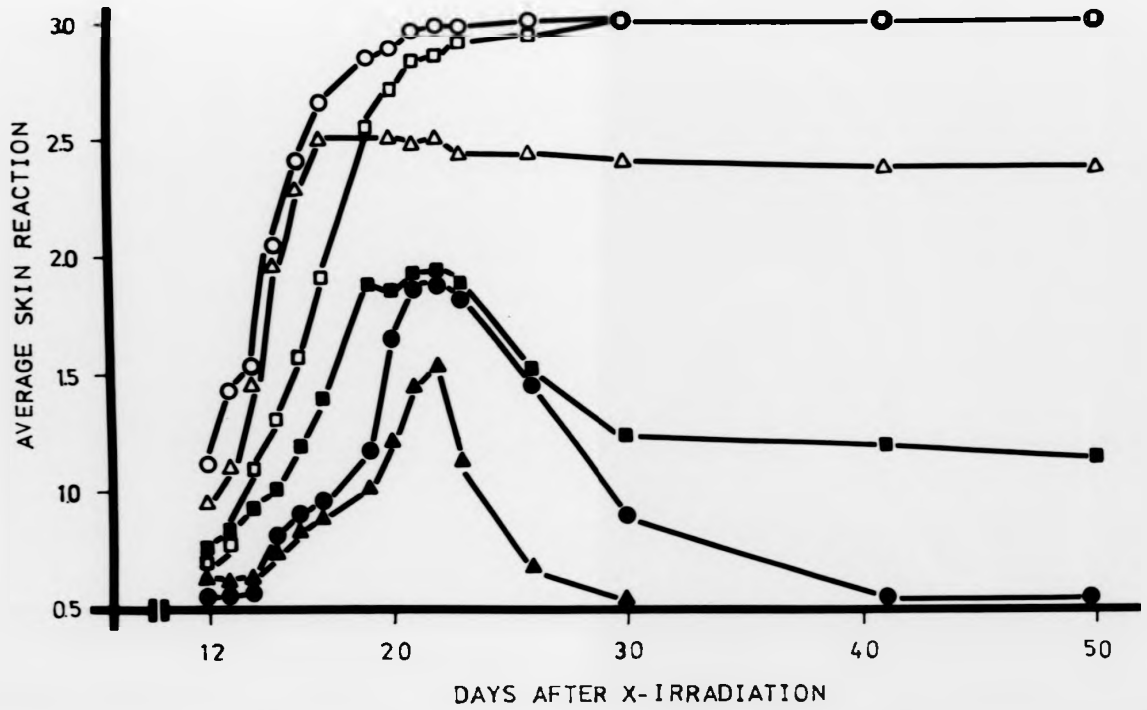


Figure 3.47: Skin reaction scores plotted against time. Open symbols △, ○, □ represents skin irradiated 3 times previously with test doses of 23, 25 and 27 Gy respectively; Closed symbols ▲, ●, ■ represent aged controls irradiated with test doses of 23, 25 and 27 Gy respectively.

b). Macrocolonies

Pooled dose response curves for the number of colonies per cm^2 as a function of the test dose are shown in Figure 3.48. The mice received 3 previous tolerance doses (bottom curve in Figure 3.48) or they were aged controls used in these experiments or in all of the experiments taken together (top curves in Figure 3.48). Data from repeat experiments at each dose level were pooled. Best fit lines are drawn. The different irradiation regimens used and the data analysis are detailed in Table 3.26. The D_0 values ranged from 314 ± 14 to 399 ± 96 cGy for the aged controls, and a single curve could be fitted through all of the data with D_0 of 368 ± 43 cGy (data B, Table 3.26). The groups receiving 3 previous tolerance doses showed D_0 values ranging from 329 ± 46 to 424 ± 23 cGy, and these were significantly different (data C, Table 3.26). However, this could be due to the exceptionally low value of X^2 per degree of freedom at about 0.42 in these experiments (it should be around 1-1.5). The D_0 values for the aged controls and the tails receiving 3 previous tolerance doses are significantly different (data D, Table 3.26). The same finding was also applied when all the aged controls and the tails receiving 3 previous tolerance doses were pooled (data F, Table 3.26). Comparison between aged controls and tails receiving 3 previous tolerance doses using common D_0 of 421 ± 44 cGy showed a significant difference (data G, Table 3.26). Their N values were 5 ± 3 x 10^3 colonies per tail for the aged controls and 1 ± 0.5 x 10^3 colonies per tail for tails receiving 3 previous tolerance doses. When comparison was made between all the aged controls and tails receiving 3 previous tolerance doses with a common D_0, a common D_0 of 478 ± 17 cGy could be used (data H, Table 3.26). The N values were 2 ± 0.3 x 10^3 colonies per tail for all age controls and 5 ± 0.6 x 10^2

TABLE 3.26: Response to Test Dose of Control Skin or Skin Receiving 3 Previous Tolerance Doses: Macrocolony

	DATA SET	NE (NO. OF COL./TAIL)	D_0 (cGy)	X^2	D.F.	X^2/DF	F RATIO	SIGNIFICANCE
A	Aged control							
	1st exp.	$6 \pm 2 \times 10^4$	314 ± 14	0.3	4	0.08		
	2nd exp.	$1 \pm 1 \times 10^4$	396 ± 75	4.3	4	1.08		
	3rd exp.	$7 \pm 9 \times 10^3$	399 ± 96	2.1	2	1.05	3.66	P > 0.05
B	Aged controls pooled	$2 \pm 1 \times 10^4$	368 ± 43	16.5	14	1.18		
C	Irradiated							
	3TD* 1st exp.	$8 \pm 6 \times 10^3$	329 ± 46	0.8	3	0.27		
	2nd exp.	$2 \pm 1 \times 10^3$	388 ± 52	3.0	5	0.6		
	3rd exp.	$7 \pm 2 \times 10^2$	424 ± 23	0.4	2	0.2	11.5	P < 0.05
D	3TD pooled	$7 \pm 2 \times 10^2$	449 ± 36	18.7	13	1.44		
	Aged control pooled	$2 \pm 1 \times 10^4$	368 ± 43	16.5	14	1.18		
	3TD pooled	$7 \pm 2 \times 10^2$	449 ± 36	18.7	13	1.44	164[a]	P < 0.05
E	Aged control pooled + 3TD pooled	$4 \pm 0.6 \times 10^2$	588 ± 38	463.1	29	16		
	All aged controls** pooled	$7 \pm 1 \times 10^2$	575 ± 22	9.6	55	1.75		
	3TD pooled	$7 \pm 2 \times 10^2$	449 ± 36	18.7	13	1.44	145[b]	P < 0.05
F	All aged controls + 3TD pooled	$4 \pm 0.4 \times 10^2$	816 ± 23	605.9	70	8.66		

TABLE 3.26: (CONTD.)

	DATA SET	NE (NO. OF COL./TAIL)	D_0 (cGy)	X^2	D.F.	X^2/DF	F RATIO	SIGNIFICANCE
	Pooled with common D_0: different LD_{37}							
G	Aged control pooled	$5 \pm 3 \times 10^3$	421 ± 44	37	28	1.32	434[c]	$P < 0.05$
	3TD pooled	$1 \pm 0.5 \times 10^3$						
H	All aged controls pooled	$2 \pm 0.3 \times 10^3$	478 ± 17	107	69	1.55	-4.6[d]	$P > 0.05$
	3TD pooled	$5 \pm 0.6 \times 10^2$						

* 3TD = Tails receiving 3 previous tolerance doses.
** All aged controls = These data taken from Table 3.5.
a = Comparison of group E (pooled) with the sum of groups B and D (separate D_0's).
b = Comparison of group F (pooled) with the sum of groups in Table 3.5 and D in this table (separate D_0's).
c = Comparison of group G (common D_0) with the sum of groups B and D (separated D_0's).
d = Comparison of group H (common D_0) with the sum of groups in Table 3.5 and D in this table.

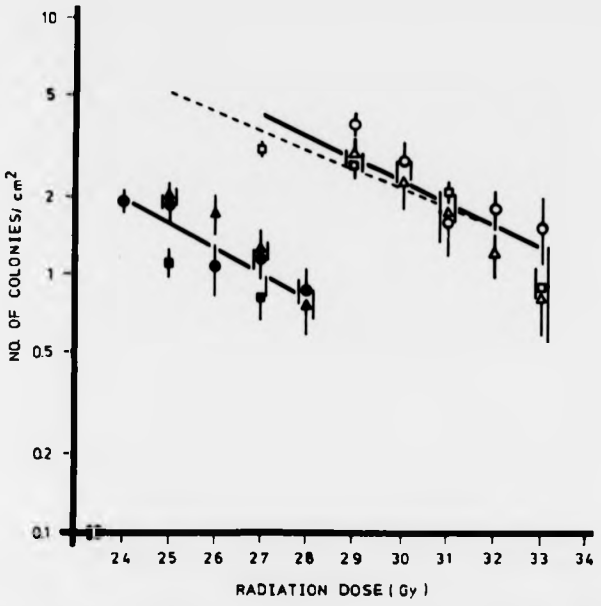


Figure 3.48: The number of colonies per cm^2 as a function of test dose. Open symbols △, ○ and □ (top curve) represents aged controls from the 1st, 2nd and 3rd experiments respectively. Dotted line (2nd curve) represents pooled curve from all aged controls. Closed symbols ▲, ● and ■ (bottom curve) represent the 1st, 2nd and 3rd experiment for skin receiving 3 pretreatments.

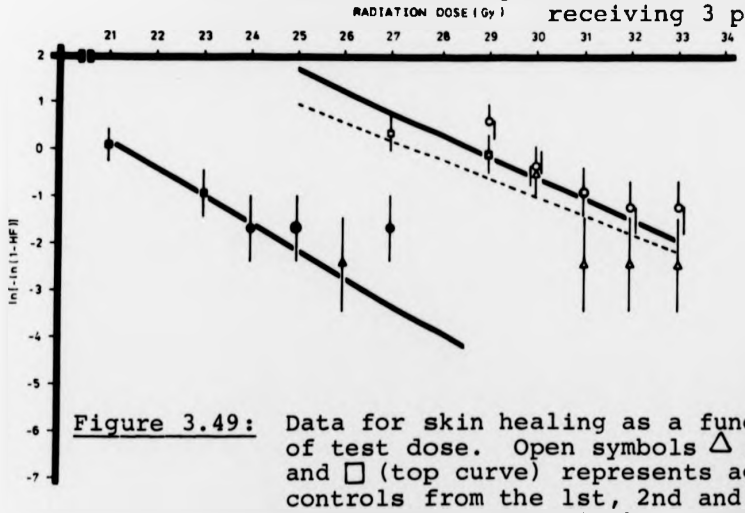


Figure 3.49: Data for skin healing as a function of test dose. Open symbols △, ○ and □ (top curve) represents aged controls from the 1st, 2nd and 3rd experiments respectively. Dotted line (2nd curve) represents pooled curve from all aged controls. Closed symbols ▲, ● and ■ (bottom curve) represents the 1st, 2nd and 3rd experiment for skin receiving 3 pre-treatments.

colonies per tail for skin receiving 3 previous tolerance doses. When a specified level of survival was chosen at 1.5 colonies per cm^2, there was about 22% of residual injury measured.

c). Skin Healing

The data analysis is detailed in Table 3.27. The D_0 values ranged from 135 ± 31 cGy to 209 ± 40 cGy. The data from the 3 experiments were not significantly different and a single curve could be fitted with D_0 of 218 ± 33 cGy (data A, Table 3.27). The skin receiving 3 pretreatments gave D_0 values ranging from 72 ± 23 cGy to 242 ± 17 cGy, and one curve could be fitted through the data from all 3 experiments with a common D_0 of 172 ± 30 cGy (data C, Table 3.27). There was a significant difference between the response of the aged controls and tails receiving 3 pretreatments (data E, Table 3.27). However, a common D_0 of 201 ± 23 cGy could be fitted through both sets of data (data F, Table 3.27) also see Figure 3.49. The tolerance of skin treated 3 times previously was reduced by about 27% when the LD_{37} values were compared either with the aged controls used in these experiments or all the aged controls taken from Table 3.~~13~~ 6.

d). Microcolonies

A total of 16 dose groups with 12 mice per group was used in 2 experiments. The schedule for mice receiving 3 previous treatments was 25 Gy + 9 weeks + 22.5 Gy + 9 weeks ; 20.25 Gy + 9 weeks + a range of test doses. The test doses ranged from 17.5 Gy up to 30 Gy for the skin receiving 3 previous treatments and 20 Gy up to 32 Gy for the aged control skin. One split-dose experiment was also performed using skin receiving 3 previous treatments, they were irradiated with two equal doses of 17.5 Gy separated by an interval of 24 hours. The data were

TABLE 3.27: Response to Test Doses of Control Skin or Skin Receiving 3 Previous Tolerance Doses: Healing

	DATA SET	LD_{37} (cGy)	NE	D_0 (cGy)	X^2	D.F.	X^2/D.F.	F RATIO	SIGNIFICANCE
A	Age control								
	1st experiment	2888 ± 35	$2.0 \pm 2.6 \times 10^9$	135 ± 31	1.8	3	0.6		
	2nd experiment	2971 ± 31	$3.8 \pm 2.3 \times 10^6$	196 ± 42	1.4	3	0.47		
	3rd experiment	2822 ± 41	$7.2 \pm 0.6 \times 10^5$	209 ± 40	3.0	4	0.75	2.82	P > 0.05
B	Age control pooled	2866 ± 32	$5.0 \pm 2.7 \times 10^5$	218 ± 33	13.2	14	0.94		
C	Irradiated								
	3TD 1st experiment	2385 ± 51	$2.3 \pm 8.4 \times 10^{14}$	72 ± 23	0.5	2	0.25		
	2nd experiment	2012 ± 379	$3.8 \pm 15.6 \times 10^3$	244 ± 17	4.4	4	1.1		
	3rd experiment	2126 ± 38	$2.5 \pm 2.6 \times 10^7$	125 ± 35	1.7	2	0.85	1.33	P > 0.05
D	3TD pooled	2123 ± 52	$2.2 \pm 2.1 \times 10^5$	172 ± 30	1.1	12	0.92		
	Age control pooled	2866 ± 32	$5.0 \pm 2.7 \times 10^5$	218 ± 33	13.2	14	0.94		
	3TD pooled	2123 ± 52	$2.2 \pm 2.1 \times 10^5$	172 ± 30	11.0	12	0.92	64.5[a]	P < 0.05
E	Age controls pooled + 3TD pooled	1845 ± 716	7.9 ± 11.9	890 ± 635	144.1	28	5.15		
	Pooled with common D_0 different LD_{37}								
F	Age control pooled	2874 ± 28	$1.6 \pm 0.7 \times 10^6$	201 ± 23	24	27	0.89	0.22[b]	P > 0.05
	3TD pooled	2085 ± 53	$3.2 \pm 2.0 \times 10^4$						

TABLE 3.27: (CONTD.)

	DATA SET	LD_{37} (cGy)	NE	D_0 (cGy)	X^2	D.F.	X^2/D.F.	F RATIO	SIGNIFICANCE
G	All age controls pooled	2750 ± 28	8.1± 3.3x10^4	243 ± 23	72	66	1.09	0.73[c]	P > 0.05
	3TD pooled	2021 ± 65	4.1± 1.9x10^3						

[a] Comparison of group E with the sum of groups B and D (separate D_0's).
[b] Comparison of group F (common D_0) with the sum of groups B and D (separate D_0's).
[c] Comparison of group G (common D_0) with the sum of groups in Table 3.6 and D in this table (separate D_0's).
[d] When P < 0.05 indicates that the fit of the pooled data is significantly different from the separate fitting of the individual data. When P > 0.05 indicates that the fit of the pooled data is not significantly different from the separate fitting of the individual data.

analysed using the DLOGS computer program. The detailed analysis is shown in Table 3.28. D_0 values were 219 ± 10 cGy for the aged controls and 235 ± 3 cGy for the skin receiving 3 previous treatments. The two sets of data were significantly different (data A, Table 3.28), however, both sets of data could be fitted with a common D_0 of 227 ± 6 cGy (data C, Table 3.28). Best fit lines were drawn from data fitted with a common D_0 and they are shown in Figure 3.50. When a specified level of survival at 10 colonies per cm^2 was chosen, there was about 10% residual injury measured. In the split-dose experiment the survival was 6.69 ± 0.98 colonies per cm^2 after 17.5 Gy + 24 hours + 17.5 Gy. This observation is shown as a solid triangle in Figure 3.50. When the number of colony-forming cells at zero dose was calculated using single and split-dose information as described in Section 3.1.1 II, the value was 8404 colonies per cm^2.

TABLE 3.28: Response to Test Doses of Control Skin and Skin Receiving 3 Previous Treatments: Microcolonies

DOSE SET	NE (NO. OF COL./TAIL)	D_0 (cGy)	X^2	D.F.	X^2/DF	F RATIO	SIGNIFICANCE
A Aged control	$4.5 \pm 1.5 \times 10^6$	219 ± 10	63.8	9	7.1		
3 tolerance doses	$7.0 \pm 0.5 \times 10^5$	235 ± 3	3.7	8	0.5		
B Aged control and 3 tolerance doses pooled	$1.8 \pm 1.1 \times 10^5$	295 ± 38	2082	19	109.6	252	$P < 0.05$
Pooled with common D_0							
C Aged control	$3.3 \pm 0.6 \times 10^6$	227 ± 5.9	72.9	18	4.1	1.4^a	$P > 0.05$
3 tolerance doses	$9.6 \pm 1.5 \times 10^5$						

[a] = Comparison of group C (common D_0) with the sum of group A (separate D_0's).

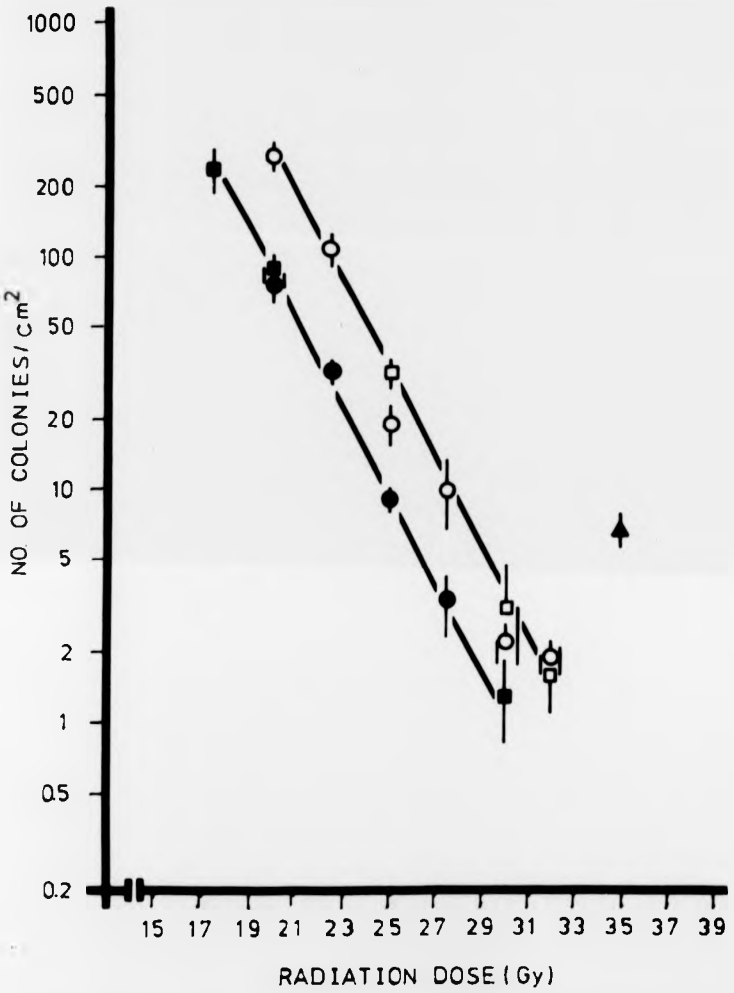


Figure 3.50: Response of the aged controls and skin receiving 3 previous tolerance doses: Microcolonies. Symbols ○ and □ represents the aged controls for the 1st and the 2nd experiments: Symbols ● and ■ represents the 1st and the 2nd experiment for the skin receiving 3 previous tolerance doses; Symbol ▲ is the datum for the split dose experiment.

SECTION FOUR

DISCUSSION

4.1. SKIN RESPONSES

4.1.1. Gross Skin Reaction

One of the most obvious consequences of an acute exposure to ionizing radiation is a reddening of the skin. This is the first manifestation of radiation damage to the skin and is part of a sequence of changes, visible to the naked eye, called the erythema reaction or, in a broader sense, the skin reaction. Although in the present studies we were more interested in the survival of micro- or macrocolony forming cells and in the healing of the skin. The levels of skin reaction were scored using an arbitrary scoring scale as described in Table 2.1. The reactions were studied in an attempt to correlate the gross response of the skin and the survival of colony forming cells in tail epidermis, in conditions such as single doses, after plucking, field size, and skin irradiated previously. In this section only the single dose results are discussed, and the reaction of the skin after plucking, with different field sizes and using skin irradiated previously, will be discussed under the appropriate headings. Neither the time of onset nor the time of the peak reaction was influenced by dose in a clear-cut fashion, except after high doses i.e. > 32 Gy. It appeared as if the peak reaction was delayed, but in fact it represents the progression of necrosis or healing rather than the peak reaction. The rate at which the severity of reaction increased was largely independent of dose: once the reaction began, the severity increased at the same rate for all doses. However, the percentage of tails which healed was dose-dependent.

In terms of the time of onset, the present value of 12 days is similar to some results using mouse tail skin of 12 days (Al-Barwari and Potten, 1979) or mouse ear skin - 12 days (Law *et al*, 1977). Also, it is similar to pig skin (10-12 days, Fowler *et al*, 1963). For comparison

TABLE 4.1: Mouse Skin Reaction Results

MOUSE STRAIN	SITE	RADIATION	DOSE (Gy)	TIME OF ONSET (d)	TIME OF PEAK (d)	TIME TO HEAL (d)	REFERENCE
CFLP	Ear	250 kV X	30	12	28	>50	Law et al (1977)
hr/hr	Dorsum	300 kV X	20	5	13	>30	Al-Barwari and Potten (1979)
			16	5	12	26	Al-Barwari and Potten (1979)
	Tail		35	12	20	>30	Al-Barwari and Potten (1979)
	Dorsum	10 kV X	27	5	12	30	Al-Barwari and Potten (1979)
SAS/TO	Feet	250 kV X	33	7	21	>30	Hegazy and Fowler (1973)
			29	7	18	30	Hegazy and Fowler (1973)
	Plucked dorsum		20.5	6	12	30	Hegazy and Fowler (1973)
SAS/4	Leg	15 MeV electrons	25	7.5	17.5	>30	Fowler et al (1965)
BDF_1	Tail	300 kV X	28	12	26	36	Present data
SAS/4	Leg	250 kV X	29	8	17	>30	Denekamp et al (1966)
		6 MeV neutrons	13	8	15	>30	

Modified from Potten (1985).

TABLE 4.2: Skin Reaction Results for Rat, Pig and Man

SPECIES	SITE	RADIATION	DOSE (Gy)	TIME OF ONSET (d)	TIME OF PEAK(d)	TIME TO HEAL(d)	REFERENCE
Rat	Flank	150 kV X	36	-5	-17	>40	van den Brenk (1966)
	Feet	250 kV X	18-40	-7	14-21	35-50	Field et al (1967)
		6 MeV neutrons	12-19	-7	14-21	35-50	Field et al (1967)
	Tail	14.7 MeV neutrons	22.5	12	35	>50	Hendry et al (1976)
Pig	Flank	8 MeV X	17.5-22	10-12	20+	-	Fowler et al (1963)
	Shoulder	250 kV X	14.5-28	17.5	24.9	36	Archambeau et al (1968)
	Back	250 kV X	16-31	20.8	28.5	38	Archambeau et al (1968)
Man		8 MeV X	12.5	8	30+	-	Field et al (1976)
		7.5 MeV neutrons	4,5	8	30	-	Field et al (1976)

Modified from Potten (1985).

purposes two tables presenting data from mouse and other species are given in Table 4.1 and Table 4.2 respectively. Regarding the time taken for the first sign of a reaction to appear, a variation of about a factor of about 1.5 to 2.0 was found between different sites and species. For instance, the reactions appear most quickly in the thin skin on the back of the mouse and most slowly in the thicker skin on the tail. However, as described by Potten (1981) the time of onset, or peak, in the skin reaction does not correlate well with epidermal thickness, or other cell kinetic parameters such as transit time through the layers or average cell cycle time. In contrast, the appearance of the first signs of skin reaction correlates reasonably well with the time taken to pass through the first transit cell cycle alone (T1) or the first transit cycle plus the basal layer post-mitotic maturation time (T1 + Pm). In this case, the (T1 + Pm) for the tail skin is 14.2 days which is similar to the time of onset of reaction of 12 days observed in the present studies.

In some cases the time of the peak reaction is not affected by dose, whilst in others it does appear so. The time of peak reaction in the present results was 26 days which is similar to the total transit time plus post-mitotic maturing time, i.e. T1 + T2 + T3 + Pm = 27.7 days for the tail skin (Potten, 1981). Similar results were also obtained using mouse tail skin of 20 days (Al-Barwari and Potten, 1979), or ear skin of 28 days (Law et al, 1977) and results found in other species such as the pig of > 20 days (Fowler et al, 1963) of 25-28 days (Archambeau et al, 1968), and human skin of 30 days (Field et al, 1976), see Table 4.1 and Table 4.2.

The times taken to heal in all species are generally similar and within 30-40 days after irradiation, except in the rat tail and in the mouse ear skin which take more than 50 days to heal (Hendry et al, 1976; Law et al, 1977). Rat feet irradiated either with X-rays or neutrons take 35-50 days to heal (Field et al, 1967).

4.1.2. The Radiosensitivity of Microcolony- and Macrocolony-Forming Cells

4.1.2.1. Sampling Criteria

In the present technique, samples are taken for scoring microcolonies when the skin reaction has reached a specified level, i.e. erythema or severe erythema. This was found to be necessary because there was a large discrepancy in both the time of the skin reaction and the development of microcolonies after irradiation among different mice. However, similar colony size distributions were found among samples showing the same skin reaction taken at various times after a given dose (Table 3.2). Hence there was a close relationship between the development of gross skin reactions and the growth of microcolonies. If the rate of cell loss from the epidermis determines the time of the skin reaction, the differences between animals suggests that the rate of cell loss among individual mice may not be constant. Most data in the literature show that cell loss in the basal layer after irradiation is dose-independent and it continues at a steady rate ranging from about 4% to about 8% a day in different species (De Rey and Klein-Szanto, 1972; Etoh et al, 1975; Etoh et al, 1977; Al-Barwari, 1978; Archambeau et al, 1979; Potten and Hendry, 1983). It should be noted that all these authors quoted the percentage of cell loss using the average value from several observations and not the individual values for different animals.

A variation in the latency time for the reaction or the appearance of colonies after irradiation may also occur in other tissues. Magli et al (1982) illustrated that in the spleen some of the macrocolonies seen at 8 days disappeared by 10 days. At the same time, some colonies were visible at 10 days or later which were not seen at 8 days. All microcolony techniques use a fixed time for tissue sampling. For example, in the microcolony assay in the mouse small intestine (Withers, 1970) the samples were taken on day 3 to 3½ after irradiation. Whereas for assay the macrocolonies in mouse small intestine, a fixed assay time of 13 days was used by Withers and Elkind (1968, 1969). In the epidermis a fixed assay time of 3 days after irradiation was used by Al-Barwari and Potten (1976) and Keech (1982) assayed the epidermal microcolonies at 13 days after irradiation. In the tail epidermis the variation in sampling time is large, and probably larger than required for other tissues. Nonetheless, this is one aspect of any technique which must be satisfactorily worked out. If a fixed assay time is employed, the colonies in some samples could be too large so that there was coalesence of colonies, or the colonies may be too small to be scored at the fixed time of assay. Both of these effects will result in greater variability in the data.

4.1.2.2. Microcolony Definition

Microcolonies were defined by using vincristine treatment to show mitotic figures or by ^{3}H-TdR to label S-phase cells in the colonies (see Section 3.1.1.b). The mitotic index in the vincristine treated samples ranged from as low as 2% to as high as 97% (Table 3.1), whereas the labelling index in the ^{3}H-TdR treated samples was in a much narrower range between 70% and 94% (Table 3.1). The reasons for this difference

are (a) the mitotic index from 2% to 97% represents the lowest and highest values obtained from 240 individual colony samples which includes colonies as small as 10 cells, and as large as 292 cells. These colonies were taken from samples in pilot experiments. In later experiments using the erythema criteria for sampling, the colony size was between 32 and 228 cells per colony. In this case the range of mitotic index for the individual colonies was from about 5% to about 50% (Chen and Hendry, 1986a). (b) the colonies used for the labelling index studies were also in the narrower range of size, ranging from 32 to 228 cells.

It should also be noted that the difference in either mitotic index or labelling index among colonies is primarily due to the size of colonies measured. Usually the larger the colony the lower the mitotic or labelling index, particularly in the centre of the colony (Plate 2.10). However, this was not always the case as some larger colonies had a high mitotic index, particularly at high doses where the number of colonies per cm^2 was very low. In order to re-epithelialise the epidermis there is a great demand for the surviving clonogenic cells to proliferate and differentiate continuously. These cells are dividing and spreading easily because of the low number of colony forming cells per unit area. Although these colonies can be large, most of the cells may be cycling, so that their mitotic indices are relatively high when compared with similar size colonies at higher numbers per cm^2 after lower doses, where proliferation and spreading are more limited due to colony confluence.

The definition of a surviving colony used by Al-Barwari and Potten (1976) was based on counting a group of 17 or more associated labelled nuclei (32 or more total nuclei) 3 days after irradiation. Apart from

the major difference in the time of sampling (i.e. 3 days versus 12-18 days in the present work), the definition of a surviving colony is basically the same as in the present studies. Concerning colony identification, it is easy to identify colonies in our system because they are more typical with unpigmented actively dividing cells surrounded by pigment which formed a clear boundary around and between colonies (Figure 2.8a). This is not usually the case in the system developed by Al-Barwari and Potten (1976), and the appearance of satellite clones or clones associated with hair follicles makes identification of primary colonies difficult.

The clone-size distribution was found to be broader in the observations of Al-Barwari and Potten (1976) than in the present work. By day 3 (the normal assay time) after irradiation, the number of labelled cells per clone varied from 3 to 54 cells, with about 3 peak frequencies in the clone-size distribution. This is slightly different from our present observations where the clone-size distribution was confined to a range of colony sizes starting at 32 cells and reaching 250 cells. This showed 1 peak frequency in the clone-size distribution (Figure 3.6). The main difference between these two assay systems is due to the use of a fixed assay time in the former case to assay samples which normally have an extremely wide variability in individual colony development.

4.1.2.3. Survival Curves

Survival curves were derived from at least two experiments and the results were shown to be reproducible.

In both microcolony and macrocolony survival there are limits to the range of measurements. For instance, it is not possible to measure

numbers of microcolonies more than about 900 colonies per cm^2 or more than about 5 colonies per cm^2 for the macrocolonies because of the coalescence of colonies. Also it is difficult to measure samples which have less than about 0.5 colonies per cm^2 for either microcolony of macrocolony technique, because it needs a lot of samples to get an accurate value. This limits the range of doses that can be used in both assay systems.

4.1.2.3.1. Radiosensitivity

The comparison of microcolony and macrocolony survival curves (Figure 4.1) demonstrates that microcolonies containing more than 32 cells, i.e. where surviving colony-forming cells completed at least five divisions after irradiation, all develop into macrocolonies. This is the case at high doses, as the numbers of macrocolonies are within the range of number of microcolonies (Fig. 4.1). At the lower doses the macrocolony count is lower than expected because of the greater probability of several microcolonies surviving in the area covered by a single macrocolony. This accounts for the difference between the apparent sensitivity of macrocolony-forming cells and the sensitivity of microcolony-forming cells, by a factor of 4.1/2.7 = 1.5 (data D & E in Figure 4.1 and Table 4.3).

A comparison of survival curves for colony-forming cells in mouse skin epidermis reported in the literature is shown in Figure 4.1. Curves A, B and D refer to macrocolonies, and C and E to microcolonies. Also, curves A, B and C refer to dorsal epidermis, and D and E to tail epidermis. The curves of tail epidermis are displaced to higher doses compared to dorsum by about 10 Gy in the high-dose range. At lower doses the curve for tail converges towards the macrocolony curves for

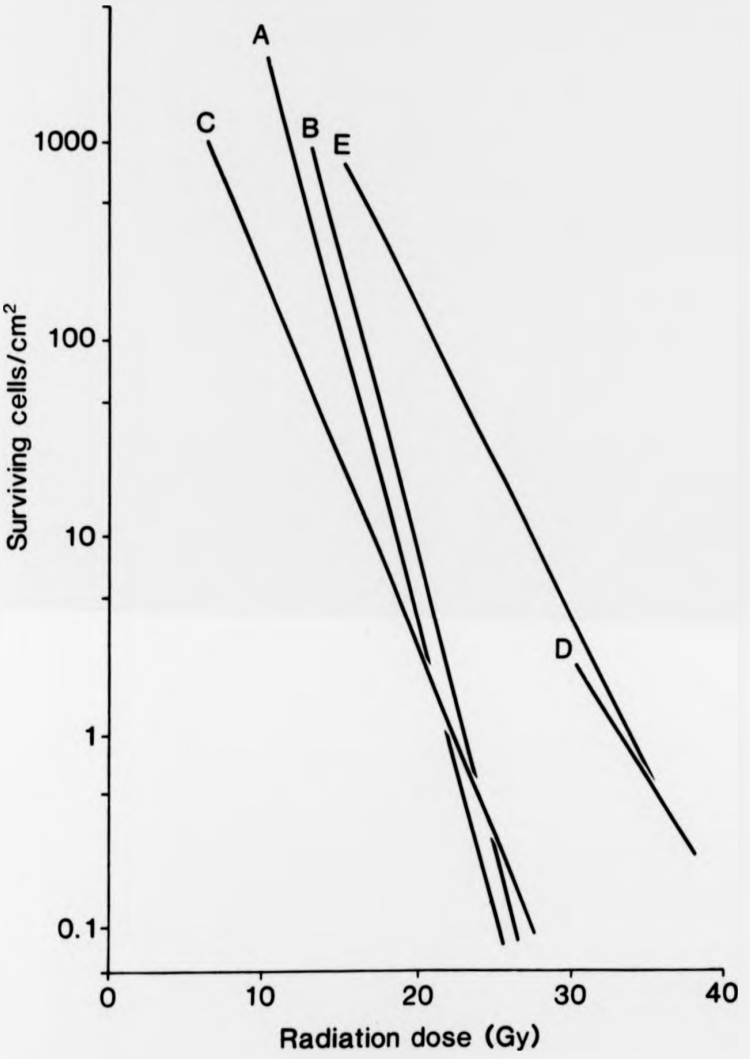


Figure 4.1: Comparison of survival curves. A, macrocolonies on dorsum (Withers, 1967b). B, macrocolonies on dorsum (Emery et al, 1970). C, microcolonies on dorsum (Al-Barwari and Potten, 1976). D, macrocolonies on tail (Hendry, 1984). E, microcolonies on tail (present data).

TABLE 4.3: Survival Parameters of Epidermal Colony-Forming Units to Single Doses

SITE	DOSE TO GIVE ONE COLONY/ cm^2 (Gy)	MAXIMUM TEST AREA (cm^2)	D_0 (Gy)	n/cm^2	CFU/cm^2	REFERENCE
Macrolonies						
Dorsum (plucked 1-12h before irradiation)	20	1.13	1.37 (1.26-1.50)*	$1.39x10^6$	9×10^4	Withers, 1967 a,b
Dorsum (plucked 20-24h before irradiation)	22	2.85	1.35 (1.20-1.55)*	$1.35x10^7$	2×10^5	Emery et al, 1970
Tail (unplucked)	34	1.7	4.10±0.58+	$6.57x10^3$	-	Chen & Hendry, 1986a**
Tail (unplucked)	34.5	1.7	5. 8±0.22+	$7.08x10^2$	-	Present data***
Tail (unplucked)	34	1.4	3.45±0.36+	$2.03x10^4$	3030	Hendry, 1984
Microcolonies						
Dorsum (plucked)	22	Whole Body	2.33±0.11+	$1.23x10^4$	-	Al-Barwari & Potten, 1976
Dorsum (unplucked)	-	-	4.4	$4.6 x10^3$	-	Keech, 1982
Tail (unplucked)	33	1.7	2.70±0.12+	$2.24x10^5$	6×10^4	Present data

* 95% confidence limits.
** survival parameters obtained from experiments using 12 week old mice.
*** survival parameters obtained when all aged controls were pooled.
\+ standard error.

dorsum, but not towards the microcolony curve. It should be noted that the macrocolony technique for dorsum does not involve a correction for colony coalescence as is required with the present technique and hence the comparison of these curves remains valid. Also, very few colonies were associated with hair follicle openings in the present work, in contrast to microcolonies in dorsum (Al-Barwari and Potten, 1976), and possibly also with macrocolonies in dorsum (discussed by Potten, 1985). Reported survival parameters for epidermal colony-forming units are shown in Table 4.3. The reason for the higher D_0 value for colony-forming cells in tail epidermis (D_0 approx. 2.7 Gy) or microcolonies in dorsal epidermis (2.3-4.4 Gy) compared with macrocolony-forming cells in dorsal epidermis (D_0 approx. 1.35 Gy) remains unknown. There are some variables which may partly explain the difference. For instance, a. plucking; b. strain difference; c. anaesthesia; d. structural and kinetic differences among skin sites; e. different scoring techniques used. Each of these assumptions will be discussed separately as follows.

(a). Plucking - It can be seen from Table 4.3 that apart from Keech (1982), most of the work on the dorsal skin (e.g. Withers, 1967a,b; Emery et al, 1970; Al-Barwari and Potten, 1976) used a plucking technique. In the tail skin experiments all the animals were unplucked except one experiment which was particularly designed to look at the effect of plucking described in Section 3.1.3. When skin was plucked 18 hours before irradiation the sensitivity was increased when compared with the unplucked skin by a factor of about 1.3. Keech (1982) used the same microcolony assay technique as developed by Al-Barwari and Potten (1976). The former used unplucked skin and the latter used skin plucked

20 hours before irradiation. The D_0 value was 4.4 Gy for the unplucked skin and 2.33 Gy for the skin plucked 20 hours before irradiation. Also, at 12 hours post-plucking the uptake of ^{3}H-TdR was increased and there was a peak at 36 hours (Hamilton and Potten, 1972). This transition of basal cells from G_0 into the cycle may increase the radiosensitivity, because of the shorter time in G_0 for repair of potential lethal damage. It has shown that plucking increased the radiosensitivity of hair follicles (Griem et al, 1973; Griem et al, 1979; Hendry et al, 1980).

(b). Strain difference - Different strains of mice have been used in different experiments by different authors. For example, ALBINO mice were used by Withers (1967a,b), DBA-2 mice by Al-Barwari and Potten (1976) and BDF1 mice were used in the present studies. In the present study one experiment was designed to look at the sensitivity difference between BDF_1 and BALB/C using the macrocolony and healing techniques. 30 Gy was given to 12 mice in each group. The number of colonies per cm^2 was 2.69 ± 0.38 for BDF_1 mice and 0.96 ± 0.29 for BALB/C mice. The fraction of tails healed was 3% for BDF_1 mice and 0% for BALB/C mice. Although only little data were obtained, these results suggest that the strain difference might play a role in the difference in radiosensitivity of the epidermis. Closely similar skin reactions have been reported when SAS/4 and C3H male mice were compared (Denekamp and Fowler, 1966). On the contrary Pearson and Steel (1983) showed that the radiosensitivity differed in terms of early skin damage and late skin contraction in C_{57} and DBA mice (also the sensitivity to early lung damage (pneumonitis) and intestinal damage in these two strains were different).

(c). Anaesthesia - All of the dorsal skin studies used anaesthesia during the irradiation. This is unlike the present experiments where the animals are unanaesthetised during irradiation. The effects of anaesthesia on the response of dorsal skin were reported by Withers (1967c). He found that there was neither protection nor sensitisation. On the contrary, Hendry (1978) demonstrated for skin necrosis that there was a marked sensitisation effect in the target cells of the tail skin when 60 mg/kg Nembutal was given before irradiation with the animals breathing air. Hornsey et al (1977) also demonstrated that for skin reactions using Nembutal, anaesthesia increased the sensitivity by a factor of about 1.2. This is probably associated with the peripheral dilatation characteristic of barbiturate anaesthetics, which leads to improved oxygenation of skin. Suit et al (1983) also reported that in anaesthetised conditions (sodium pentobarbitol 50 mg/kg body weight) at 25 and 35°C, anaesthesia reduced RD_{50} (radiation dose which on average produced a specified peak reaction in half of the irradiated subjects) by 4-5%.

(d). Structural and kinetic differences among skin sites - The structural and cell kinetic parameters between dorsal and tail skin were reviewed by Potten, Hendry and Al-Barwari (1983). The main differences are: (1) tail skin has an exceptionally high number of about 32 corneocyte layers compared with about 6 for the dorsum. (2) the number of basal cells per mm^2 in the tail is > 20,000 whereas in dorsal skin it is about 15,000. (3) the average labelling index is 4.5% for the tail skin and 9.1% for the dorsal skin, with the length of S phase being 6.4 hours and 12.6 hours for the tail and dorsal skin respectively. This indicates that the fraction of cells in cycle is about the same in the two sites.

In the literature the latency period for the first sign of a skin reaction to appear varies from about 6 days for the dorsal skin to about 12 days for the tail skin (reviewed by Potten, 1985). This longer latency period in the tail skin may allow more PLD repair to take place in cells before they are induced into cycle which may have the effect of reducing the sensitivity.

Other studies, concerning oxygenation, have been described by Hendry et al (1978, 1980). In air, the tail epidermis is naturally homogeneously hypoxic. However, the high D_0 in the tail cannot be ascribed solely to hypoxia, because even when warmed to 37°C, when hypoxia is markedly reduced, the cells are still more resistant than in other sites.

(e). Different scoring techniques used - It has not been proved whether the nodules are true clones derived from surviving skin epidermal cells or from clumps of follicle cells. It should be noted that the D_0 value obtained for skin colonies of about 1.35 Gy (Withers, 1967a; 1967b; Emery et al, 1970; Denekamp et al, 1974) are similar to some of the follicle survival curves with D_0 values between 1.35 and 1.7 Gy (Griem et al, 1973, 1979). In the present microcolony studies, most of the colonies were not associated with hair follicles.

Whatever the uncertainties are, there are altogether seven published examples of survival curves using Withers' macrocolony technique (reviewed by Potten, 1985). Unfortunately, because the type of radiation, the level of oxygenation of the skin, and the proliferative status of the skin (time after plucking) were all different among these seven examples, comparisons are difficult. However, the curves do all

have a roughly parallel slope with D_0 values ranging from 1.12 Gy to 1.5 Gy for low LET radiations. The overall average D_0 value is about 1.35 Gy which is very different from those data obtained using microcolony techniques in the dorsum with D_0 values of 2.33 Gy (Al-Barwari and Potten, 1976) and 4.4 Gy (Keech, 1982). In the tail also higher values of D_0 are found, namely D_0 = 2.7 Gy (microcolonies in the present studies) and macrocolonies giving D_0 values of 3.5 Gy (Hendry, 1984) to 4.1 Gy (Chen and Hendry, 1986) and 5.8 Gy (present data).

4.1.2.3.2. Comparison of the Radiosensitivity of Microcolony-Forming Cells with Values in the Literature

The microcolony technique developed by Al-Barwari and Potten (1976) was used by Keech (1982). The D_0 was about 2.4 Gy in the former studies and 4.4 Gy in the latter. The same technique was used in both studies except that in the former the skin was plucked before irradiation and the reason for the difference in sensitivity is not clear. In the present studies using a newly developed technique, the D_0 value of 2.7 Gy is much closer to the D_0 value obtained by Al-Barwari and Potten (1976). In their studies they described many complicating factors which detract from the practical usefulness of the technique. The major difficulty with their technique is one of identification of the colonies for the following reasons:

a). Small clusters of labelled cells were sometimes seen in unirradiated plucked skin.

b). The colonies formed satellite colonies which made the colony boundaries difficult to identify.

c). There was a good indication that many of the colonies were associated with, and possibly arose from, the hair follicle canals.

These problems with the identification of colonies were largely overcome by the present technique. For example, the colonies observed were well-defined by high cell density with a clear boundary from the pigmented background cells. There were very few (i.e. < 1%) colonies closely associated with hair follicles. Apart from the problems which were associated with the identification of colonies in their system, the extremely wide variability in colony development in the individual animal is another major problem using the microcolony technique. Instead of assaying samples at a fixed time after irradiation the skin was sampled in the present work according to a specified level of skin reaction. This proved to be very useful to assay samples with wide variability in a more accurate way. In other words it reduces the discrepancies between individual animals a great deal. Also this new microcolony technique was found to be reproducible.

The similar D_0 values between the present result and Al-Barwari and Potten (1976) does not necessarily mean that the two results have reached general agreement in terms of radiosensitivity of microcolony-forming cells in the epidermis. There may be an effect of plucking on sensitivity, and the position of the colony-forming cells i.e. interfollicular or perifollicular may influence the sensitivity. Regarding the effect of plucking, it will be discussed in Section 4.2.2. This mechanical damage to the hair follicle induces faster regeneration in the hair follicle epithelium, and also there is removal of surface keratinised layers from the epidermis, therefore, it causes stimulation of the basal cells. In Al-Barwari and Potten's experiments, the hair was plucked 20 hours before irradiation and samples were taken 3 days after irradiation. This is a very short time compared with 13-14 days used by Keech (1982) and the present approach (12-18 days), both using

unplucked skin. If one takes into account the reported cell doubling time of about 25 hours (Al-Barwari and Potten, 1976) or 20-24 hours reported by Hegazy and Fowler (1973), and also if a survivor must contain more than 32 cells, then one should find that in order to complete 5 cell divisions (colonies reaching 32 cells) it needs about 5 days. This time scale is even longer than the total interval of 4 days between plucking and sampling. Therefore, it could be either that the doubling time of the basal cells was much shorter than 25 hours or else these colonies were derived from other cell types having shorter doubling times, perhaps associated with the follicles. Using the same technique, Keech (1982) sampled the skin at day 13-14, which is about 10 days different from plucked skin (Al-Barwari and Potten, 1976). Whether the plucking is the major course of this difference has not yet been determined. The present studies using plucking of tail-skin suggest that when plucking was performed there was about 7 days less to reach a specified size of colony. If this also applies for dorsal epidermis then the time for the development of microcolonies (> 32 cells) in plucked skin should be no less than 6 days (i.e. 13 minus 7). It is noteworthy that microcolonies developed at 13-14 days in the unplucked dorsal skin reported by Keech, and this is very similar to the unplucked tail-skin epidermis where most of the colonies developed at around this same time.

4.1.2.3.3. Extrapolation Number

Regarding the estimation of the number of CFU per cm^2, it was calculated by reducing the extrapolation number by the recovery factor measured in split-dose experiments (e.g. Potten and Hendry, 1973). This factor was 3.8 at 24 hours in the present studies, being the extrapolation number on the second-dose survival curve (Figure 3.8). A factor of 4.8 was

measured in Figure 3.9, and if 6 hours or 10 hours had been chosen instead, the factor might have been respectively lower or higher by at most a factor of 2. The factor of 3.8 is lower than the factors of 15 to 18 (Withers, 1967a) and about 70 (Emergy et al, 1970) calculated using dorsum. However, because of the lesser sensitivity of tail epidermal CFU, the corresponding values of (D_2-D_1) are more similar than the values of recovery factor, namely 3.6 Gy (present data), 3.75 Gy (Withers, 1967a) and 5.7 Gy (Emery et al, 1970). The present data provide values of about 8×10^4 CFU per cm^2 using the observed numbers of colonies at the two dose levels, and 6×10^4 CFU per cm^2 using numbers of colonies at these doses given by the curves fitted through the data for all doses. These values are slightly lower than, but of the same order as, the values reported by Withers (1967b) and Emery et al (1970). Using a value of about 2×10^6 basal cells per cm^2 of tail epidermis (Potten, 1983), a fraction of about 0.03-0.04 would be clonogenic, i.e. CFU. This would represent a lower limit to the fraction if a radioresistant proportion of cells is being assayed. Also a fraction of 0.03 to 0.04 would correspond to 4½ to 5 divisions of transit cells (i.e. $½^5 \sim 0.03$) in the basal layer. This would be an upper limit to the number of transit cell divisions if the proportion of CFU in the basal layer is truly higher. It is noteworthy that 3 divisions of transit cells were deduced for tail epidermis from an analysis of latency periods and cell kinetics (Potten, 1981).

In the dorsum, the extrapolation number on the single-dose curve was $123/mm^2$ (Al-Barwari and Potten, 1976). As the number of hair follicles was about $50/mm^2$, of EPU was about $1400/mm^2$ and of basal cells was about $15{,}000/mm^2$ (Potten and Hendry, 1983), the number of microcolony-forming cells was closest to the number of hair follicles (allowing for an

extrapolation number per cell > 1). This is unlike the present findings with the tail, where the approximate numbers/mm^2 to be compared are 40 (hair follicles), 600 (CFU), 1300 (EPU) (Potten, 1983), 2 x 10^6 (basal cells). Hence in this site the numbers of CFU are closest to the estimated numbers of EPU.

Summary of Conclusions about the Present Microcolony and Macrocolony Techniques

(1). The microcolony technique is labour intensive but not technically difficult to execute, and the colonies are easy to identify.

(2). The microcolony technique can assay the epidermal clonogenic cell response to radiation over a much wider dose range than can the present macrocolony technique. This enables measurement of the radiosensitivity of the epidermal clonogenic cells to be made more accurately.

(3). There is very little chance of confluence of small microcolonies forming a large microcolony. This suggests that the microcolony assay is more suitable for the measurements of clonogenic cell survival than the macrocolony technique described here which requires a correction for colony confluence after low doses.

(4). Sampling epidermal microcolonies using a skin reaction criterion provides a new technique to overcome problems of assaying samples with an extremely wide variability in the appearance times of colonies. This reduces the individual variation and sampling error greatly. It may be possible to apply this approach to colony techniques in other types of tissue.

(5). The present microcolonies seem unlikely to be associated with hair follicles.

(6). The microcolony technique can be used to calculate more accurately than the macrocolony technique, the proportion of clonogenic stem cells

in the basal layer. A proportion of about 0.03 was deduced for tail epidermis.

(7). The technique has been used to show for the first time that all microcolonies ($\geq$ 32 cells) in epidermis develop into macrocolonies.

(8). The microcolony technique can be used to study the effects of radiation on various parts of the skin. Epidermal clonogenic cell survival could be measured in small animals such as the mouse or large animals such as the pig, and it may prove to be a better method than those which have been used previously.

(9). The dose-response relationships for the target cells for epidermal healing and the cells which form microcolonies show more similar values of sensitivity than reported previously.

4.1.2.3.4. Comparison of the Present Mouse Data with Data in Other Species

Comparison of survival curves for epidermal colony-forming units in other species, such as pig skin and human skin irradiated either in vivo or in vitro have been reviewed by Potten (1985). These data together with the data obtained in the present studies are shown in Table 4.4. It is interesting that some of these results are very similar to either the microcolony or macrocolony data in the present studies. For example, the present microcolony data resulted in a D_0 of 2.7 Gy which is the same as for the microcolony data in pig skin when small colonies are defined by runs of 20-100 cells in histological sections (Archambeau *et al*, 1979). This similarity can be discussed in more detail as follows:

First, in the studies by Archambeau *et al* (1979), various D_0 values were obtained depending on the criteria used. For instance, when small

TABLE 4.4: Survival Parameters for Epidermal Colony-Forming Units

RADIATION QUALITY	SKIN STATE OR SITE	OXYGENATION	D_0[a] (Gy)	EXTRAPOLATION NUMBER[a] ($cells/mm^2$)	REFERENCE
Macroscopic colonies					
29 kV X-rays	1-12h post-plucking	Air	1.35	14000	Withers (1967a)
150 kV X-rays	1-12h post-plucking	Air	1.34	27000	Withers (1967b)
150 kV X-rays	1-12h post-plucking	Oxygen	1.12	27000	
250 kV X-rays	19h post-plucking	Air	1.35	130000	Emery et al (1970)
1 MeV electrons	20-24h post-plucking	Air	1.50	35000	Denekamp et al (1974)
8 MeV neutrons	16h post-plucking	Air	1.09	2200	Denekamp et al (1971)
28 MeV helium ions	Hairless mice	Air	0.95	300000	Leith et al (1971)
Microscopic colonies					
290 kV X-rays	(20h post-plucking) (3d post-irradiation)	Oxygen	2.33	123	Al-Barwari and Potten (1976)
X-rays	13-14d post-irradiation		4.4	46	Keech (1982)
300 kV X-rays	12-19d post-irradiation	Air	2.7	2300	Chen & Hendry (1986)
Macroscopic colonies in humans in vivo					
5.7 MeV X-rays 4 hourly fractionation	Chest wall	-	4.9[c] 5.4[d]	-	Arcangeli et al (1980)
Macroscopic colonies in mouse tail					
300 kVp X-rays	Mouse tail	Air	3.5	200	Hendry (1983)
300 kVp X-rays	Mouse tail	Air	4.1		Chen & Hendry (1986)
300 kV X-rays	Mouse tail	Air	5.8		Present data

TABLE 4.4: (contd.)

RADIATION QUALITY	SKIN STATE OR SITE	OXYGENATION	D_0[a] (Gy)	EXTRAPOLATION[a] NUMBER (cells/mm^2)	REFERENCE
Human colonies in culture					
^{137}Cs γ-rays	Human keratocytes in culture	-	0.74	10	Dover & Potten (1983)
Pig skin microcolonies					
300 kVp X-rays	Various sites		2.7	-	Archambeau et al (1979)
Very large colonies			16.2		Archambeau et al (1979)
Average of all colonies			3.4		Archambeau et al (1979)

[a] The values vary depending on how the curves have been fitted e.g. the two curves in Withers (1967b) have been fitted with a common extrapolation number. Slightly different values would be obtained if they were fitted independently.
[b] Various sized colonies were assayed separately or together.
[c] D_0 value calculated using Poisson formula $SN = (\frac{-\log_e f}{A})$
[d] D_0 value calculated directly from the average number of colonies per area.
* Most of these data taken from Potten (1985).

colonies were defined by runs of 20-100 cells in sections, a portion of a survival curve was obtained having a D_0 of 2.7 Gy. Colonies of 100-300 cells per run provided a curve with a D_0 of 5.7 Gy and colonies with more than 300 cells per run gave a curve with a D_0 of 16.2 Gy. The difference in their approach from others makes comparisons with the mouse results difficult. For example, it is impossible to say whether the small 'islands' (or runs) of healthy-looking cells seen in sections, represent a section through the middle of roughly circular colonies or sections that skim the edge of large or irregularly shaped colonies. This makes it difficult to relate these data to our present data where colonies were counted per unit area. If the similarities in both cases are considered, namely (a) irradiation of the same area of skin with the same energy of X-rays (300 kVp), (b) unplucked skin, and (c) the probability of coalescence of small colonies forming large colonies as in the present macrocolony studies, it is therefore not surprising that the radiosensitivity is decreased when a criterion of a larger colony size is used. Hence it is more reasonable to use the small colonies (i.e. 20-100 cells per section of the island) as a criterion to measure the radiosensitivity of colony-forming cells in pig skin epidermis. In this case the D_0 value of 2.7 Gy (Archambeau et al, 1979) is the same as the value in tail epidermis using the present microcolony technique. Also, there are other assumptions which are a problem when using the sectioning technique (a) it was assumed that no single colony has been sectioned or counted more than once i.e. skimming the edges of irregularly shaped colonies or sectioning large colonies more than once in different sections and (b) it was assumed that no small colonies were left out due to the section interval being too large.

Colonies developing from human epidermal cells have been assayed in vitro as well as in vivo. The in vitro data reported by Dover and Potten (1983) have give a D_0 value of 0.74 Gy. The reason for the low D_0 value is not known. There is only one data set using human skin in situ (Arcangeli et al, 1980). This made use of the fact that two patients after radical mastectomy received a series of small doses of varying size (1.5 to 2.5 Gy per fraction) to the chest wall, at four-hourly intervals for 16 to 18 days, so accumulating considerable total dose (63-71.5 Gy). Observations on different strips of skin receiving slightly different total doses revealed the presence of nodules which were assumed to represent macrocolonies. From the number of these colonies, a very short range of the dose-response curve could be constructed. This indicated a D_0 of 5.4 ± 1.2 Gy for these fractionated doses. When no Poisson correction was made for colony coalescence which had a greater effect after the lower doses, the D_0 calculated from the fraction of areas without colonies which corrects for coalescence, was 4.9 ± 1.5 Gy.

It was considered (Arcangeli et al, 1980) that the value of 4.9 Gy was compatible with the value expected for single doses, namely 1.35 Gy as determined in mouse dorsum (Withers, 1967). This was because of the size of dose per fraction, and the expected D_0 at the mean dose per fraction calculated from the mouse data. In the present studies using 12 fractions in 4 days where the mean dose per fraction was about 5 Gy, the D_0 was about 3.5 Gy. This is less than the value of 4.9 Gy above, and this is expected because of the larger dose per fraction in the present series.

4.1.3. Skin Healing

The present skin healing data pooled from all aged groups have shown a D_0 of 2.6 ± 0.3 Gy with HD_{50} approximately 28.3 Gy. These values are similar to the reported D_0 value of 2.8 ± 0.5 Gy and HD_{50} value of 30 ± 0.4 Gy (Hendry, 1984). Using the same length of tail and the same irradiation condition. The deduced D_0 of target cells for epidermal healing 2.6 ± 0.3 Gy is now much closer to the measured D_0 for colony-forming cells of 2.7 ± 0.2 Gy (Chen and Hendry, 1986) than the D_0 of 3.5 ± 0.4 Gy reported previously for macrocolony-forming units (Hendry, 1984). This close relationship between epidermal healing and microcolonies indicates that the epidermal clonogenic cells respond the same as the target cells for epidermal healing, and they may be the same cells. Also, the microcolony or the healing technique can be used to study the cellular basis of epidermal response in the tail in an accurate, quantitative way. The healing technique requires larger numbers of samples in order to get an accurate value of sensitivity. This close relationship between epidermal healing and colony-forming cells in the tail epidermis is in disagreement with the data obtained in the pig skin where a lack of correlation between basal cell survival and gross skin response was found (Shymko *et al*, 1984). The reasons for a lack of correlation between basal cell survival and gross skin response could be because the authors did not consider the likely possibility of a proliferative hierarchy in the basal layer, and particularly because of the inherent difficulties in interpreting the counts of regenerating islands in sections of epidermis (Hendry and Potten, 1985).

A direct comparison of the HD_{50} value with values reported for other regions of unplucked mouse skin such as the ear or the foot is not easy because of the different scoring systems used. In the ear skin the

maximum skin reaction is a crust over all the outer and the inner surfaces (Law et al, 1977). In the mouse foot system it is the severe necrosis of the whole area, possibly with bleeding (Hegazy and Fowler, 1973). However, the dose to produce an average skin reaction (e.g. total moist desquamation) used in the tail skin scoring system can be compared directly with the data obtained using the ear or foot skin. In this instance, it is about 30 Gy for the ear skin (anaesthetised mice, ear at 25°C or 37°C; Law et al, 1977), 33 Gy for the foot skin (anaesthetised mice, presumed at room temperature, Hegazy and Fowler, 1973) and 30 Gy for the tail skin (Hendry, 1984 and the present data). Hence, there does not appear to be a large difference in sensitivity of skin in these different sites.

Regarding the time course of healing in these three regions of mouse skin, the tail skin in the present studies is similar to that of the foot skin (Hegazy and Fowler, 1973) with > 30 days required to heal fully. The ear skin takes a longer time to heal e.g. > 50 days (Law et al, 1977).

4.2. MODIFICATION OF RESPONSES

4.2.1. Effects of Age

The effect of age was analysed using data accumulated from 5 separate experiments which comprised the aged controls for most of the residual injury experiments. Their age varied from 12 weeks up to 39 weeks at the time when the test dose was given. In terms of the number of macrocolonies per cm^2 and HD_{50} there was no significant change among different age groups. Using the microcolony technique there was an apparent change in sensitivity from D_0 = 270 ± 12 cGy for 12 week old mice to D_0 = 219 ± 10 cGy for 39 week old mice. Whether this difference in sensitivity represents an intrinsic radiosensitivity difference or is

due to the use of slightly different procedures at the 2 times (i.e. Vincristine was used at 12 weeks, ^{3}H-TdR was used at 39 weeks) is not clear. In theory this slightly different sampling treatment shouldn't change the sensitivity significantly because the same criterion for defining a surviving colony was used in both methods i.e. > 32 cells. Using the macrocolony and healing techniques, no significant difference in sensitivity was detected between the mice at different ages. This is expected because of the poor resolution of both techniques in the detection of small changes in sensitivity. This is largely due to the more limited range of dose over which measurements were made, and the greater scatter in the data because of fewer colonies counted or smaller samples in the case of the healing data. Using mouse tail skin, Hendry (1978) also showed that there was no significant difference in ND_{50} between the mice at different ages. However, using the mouse foot, Denekamp (1975) demonstrated that the dose to produce an average skin reaction of 1.5 (8-30 days) was increased at ages below or above 18 weeks.

In other species, Hamlet and Hopewell (1982) using rat feet have shown a clear change in sensitivity with age. Animals at 14 weeks of age were more sensitive than at either 7 weeks or 52 weeks. The 52 week old animals were the most resistant in terms of the skin reaction. In pig skin studies there was no evidence of an age effect in the dermal vascular response in 3-12 month old animals (Hopewell and Young, 1982). This was further confirmed by Simmonds and Hopewell (1984-85). In human skin, Rubin and Casarett (1968) stated that there was no observed difference in the acute response of the skin of young or adult patients to radiotherapy. Other studies have been concerned with the repair of radiation-induced DNA damage as a function of age in rat dorsal

epidermis (Sargent and Burns, 1985). Using animals with ages of 28, 100, 200 and 400 days respectively, they demonstrated that the rate of repair of radiation-induced DNA damage in proliferating rat epidermal cells diminished progressively with increasing age of the animal.

If we take the microcolony results for the comparison with other data reported in the literature, the present data appear to be in disagreement with the results obtained with rat feet (Hamlet and Hopewell, 1982); pig skin (Hopewell and Young, 1982) and human skin (Rubin and Casarett, 1968). However, the present results are consistent with the progressive loss of the capacity to repair DNA damage with increasing age (Sargent and Burns, 1985), being correlated with the increase of sensitivity in the skin. The reasons for the differences in the literature are not clear, but they may be related to (a) the different assay techniques used (i.e. skin reaction versus microcolony), (b) different tissues being investigated (i.e. pig dermal vascular tissue versus epidermis) or (c) true differences between species.

4.2.2. Effect of Plucking

Mechanical stripping of the skin using sellotape removes several cornified layers but also causes some structural damage and cell killing in the basal layer (Hennings and Elgjo, 1970; Christophers, 1972; Hamilton and Potten, 1972; Potten and Allen, 1975; Bertsch *et al*, 1976). This effect, in combination with X-rays, was clearly revealed by Hegazy and Fowler (1973). In their experiments, plucking the hair (a process which also involves the removal of some cornified layers) influenced the time of the peak reaction, and the healing of the skin. The maximum reaction occurred on day 20 after irradiating normal skin as

opposed to day 12 for plucked skin. This was in accord with the cell kinetics of the tissue in the two situations. Al-Barwari (1978) has shown that stripping the dorsal skin of hairless mice with sticky tape resulted in an earlier appearance and earlier peak of the X-ray reactions. Also, the stripped skin took longer to heal. The present results show that the gross response, in terms of the appearance of the skin reaction, the time of the peak value, and healing, are all in general agreement with these parameters reported by other workers using plucked skin (e.g. Hegazy and Fowler, 1973b; Al-Barwari, 1978). The present results show that the latency period was about 6-8 days for skin plucked 18 hours before, or immediately after irradiation and about 12 days for unplucked skin.

There is a small but significant difference in the number of colonies per cm^2 between unplucked skin and skin plucked 18 hours before, or immediately after, irradiation, by a factor of about 1.07 in dose. Also, the difference between plucking 18 hours before and plucking immediately after irradiation is small both in terms of skin reaction and macrocolonies. The difference seen in the macrocolony results showed that there was a sensitivity change as judged by eye. The healing data showed no significant difference between unplucked skin and skin plucked 18 hours before, or immediately after, irradiation. This is probably due more to the scatter of the healing data and to fewer observations being made.

All the evidence found so far shows that plucking induces an earlier appearance of the skin reaction, enhances the severity of the skin reaction, reduces the number of surviving clonogenic cells and prolongs the phase of skin healing. According to Fowler and Denekamp (1976),

loss of keratin cannot provide the mechanism for increase proliferation, but actual depletion of the basal cells alone achieves this. Hamilton and Potten (1972) reported that plucking removes not only hair but also some 13-21% of the epidermal cells. This causes an increase in the proliferation of epidermal cells at 12 hours post-plucking, and 24 hours after plucking there is a substantial increase in terms of labelling index to three times the value for unplucked skin. In normal skin most basal cells are in the G_0 phase (Hamilton and Potten, 1972); after plucking these cells are called into cycle. If these cells were injured by radiation then they will express their damage at mitosis. When skin was irradiated more than 12 hours after plucking, some of the G_0 cells will by then be active in the cell cycle and they may demonstrate less repair of potentially lethal damage (PLD), so that survival may be decreased. Similarly, when the skin was plucked immediately after irradiation, less PLD repair also will occur and this would decrease the survival, as indeed was found in the present studies.

Although the initial time course of the skin reactions is almost independent of dose (Potten and Hendry, 1983), there is a small shortening of the latency period after high doses (see Section 3.1.2.a). This could be due to a lower survival level of stem cells (all performing the normal number of transit divisions), or alternatively, there being fewer divisions of transit cells. Further, if these effects were also produced, and more markedly by plucking which resulted in less PLD repair in both stem and transit cells, then the numbers of new mature cells produced after irradiation would be decreased. This would lead to a shortening of the time to denude the epithelium. Also, the sellotape used for plucking removes some keratinised layers from the surface of the skin. This effect will also contribute to a shortening

of the time to denude the epithelium. The latter is not the sole explanation for the shortening, because other methods of plucking which do not involve removal of surface layers, still shorten the latency period (Hegazy and Fowler, 1973). There is also the possibility that the rate of cell loss is increased after plucking.

It is concluded that the earlier appearance and greater skin reactions caused by plucking before or after irradiation may be due to fewer new mature cells being produced after irradiation and changes in the rate of cell loss.

4.2.3. Effect of Adriamycin

In one experiment using either the macrocolony or the healing technique there was no enhancement of skin damage, namely when 10 mg/kg adriamycin was administered intraperitoneally 24 hours after graded doses from 28 Gy to 33 Gy (Section 3.1.4). Another two experiments using the microcolony assay technique, on the other hand, have shown that there is an enhanced level of clonogenic cell killing when 15 mg/kg adriamycin was given 30 minutes before the fixed dose of 21.25 Gy was delivered. When we compare the effect of adriamycin combined with radiation, with the effect of radiation alone, the ratio of doses required to produce the same number of colonies was 0.99 for the vincristine treated group and 0.95 for the ^{3}H-TdR labelled group. The latter value was significant.

The effect of 10 mg/kg adriamycin given 24 hours after irradiation is in accordance with the data reported by Redpath and Colman (1979) where various treatment schedules were used. These comprised a single dose of adriamycin administered either 24 hours or 2 hours before, or 24 hours

after, a single dose of 6 MeV photons. Also, there was split dose experiments with the drug being administered either 24 hours or 2 hours before the first radiation dose, and a five-daily-fraction experiment with drug administered 2 days before the first and third doses. None of their experiments showed any significant enhancement of the severity of the radiation-induced skin reaction, or alteration of the kinetics of appearance. Using the same scoring system and the mouse foot, Maase (1984) reported no effect of administration of 8 mg/kg adriamycin at 24 hours after irradiation. The observed enhancement of the clonogenic cell killing in the present studies, when 15 mg/kg adriamycin was given 30 minutes before irradiation, is in disagreement with the observation by Redpath et al (1978) and Redpath and Colman (1979), where the administration of 10 mg/kg adriamycin 0.5-2 hours before irradiation did not show any enhancement of skin reaction. On the contrary Maase (1984) found that 8 mg/kg adriamycin given 15 minutes before irradiation enhanced the radiation-induced skin reactions, the DEF (Dose Effect Factor) being 1.09.

$$DEF = \frac{DD_{50} \text{ (or } MRD_{50}\text{) for radiation alone}}{DD_{50} \text{ (or } MRD_{50}\text{) for radiation + drug}}$$

Where DD_{50} is the desquamation dose 50, and MRD_{50} is the Median Response Dose 50.

This finding using the skin reaction scoring technique is closer to our result obtained using the clonogenic assay technique. The enhancement of the skin damage in the present case and in the studies by Maase (1984) demonstrated that the effect of adriamycin on the skin appeared when it is given before, or immediately after irradiation. This suggests that the effect of adriamycin is to reduce the capacity to

repair or to accumulate sublethal radiation damage. The latter mechanism corresponds with in vitro studies showing that adriamycin reduces the accumulation but not the repair of sublethal damage (Belli and Piro, 1977; Hellmann and Hannon, 1976), although this was not demonstrated in the intestine (Moore and Broadbent, 1980). The radiation-modifying effect in both cases is consistent with clinical experience (Aristizabal et al, 1977; Cassay et al, 1975; Greco et al, 1976; Phillips and Fu, 1976). Based on this similarity, the mechanism for the adriamycin effect on the skin is in favour of preventing the repair or the accumulation of sublethal radiation damage. But one cannot rule out completely the other factors which might contribute to this observed enhancement of the skin damage, for instance, anaesthesia and skin temperature during irradiation may affect the degree of the enhanced response. In the tail system the skin was warmed up to 37°C which may have increased the cellular uptake of adriamycin and consequently enhanced the cell killing effect rather than just an inhibition of repair or accumulation of sublethal radiation damage. In the experiments described by Redpath, the skin was not pre-warmed. Also it is not clear whether it is because the adriamycin dose used in the present experiment is higher (15 mg/kg) than those used in the mouse foot experiments (8-10 mg/kg), or the rise of skin temperature to 37°C in the tail skin has significantly resulted in a greater killing of clonogenic cells by the drug, or because of the assay system (the microcolony test) which we used is more sensitive than the skin scoring system.

4.3. EFFECTS OF FIELD SIZE

The field size effect was studied using lengths ranging from 2 cm to 6 cm. Owing to the conical shape of tail, the area of epidermis studied

was not proportional to the length of tail (see Table 3.11). Also, the area for colony counting was different from the area irradiated, because of leaving 0.5 cm at either end to avoid problems of cell migration and dose inhomogeneity at the edges of the radiation beam. In this case, the shortest length that could be scored was 1 cm for 2 cm tail and the longest length was 5 cm for the 6 cm tail. The 6 cm length gave almost the maximum possible area that could be used because the area of the narrow distal part of the tail is so small.

The divergence between the microcolony curve for 2 cm (1.3 cm^2) and 4 cm (3.2 cm^2) tails demonstrated that the increase in dose for equivalent effect ranged from about 1% at 100 colonies per cm^2 to about 10% at 3 colonies per cm^2, when 2 cm tails were compared with 4 cm tails. Hence there was a change in sensitivity. A similar increase in dose at low levels of survival was seen with macrocolonies. For instance, about 11% dose increase was found at 3 colonies per cm^2 when 2 cm tails were compared with 4 cm tails. However, in this case the range of survival which could be measured was small and hence the dose dependence was not easily detectable. A similar increase in dose for the 2 cm tail compared to 4 cm was also seen using skin reactions and the healing end point. Therefore, all four endpoints showed approximately the same increase in dose required with the small field size.

When 2 cm lengths of 2 cm separated by 1 cm were irradiated on the same tail, the proportion of healing in the proximal portion was greater than in the distal portion. This contrasts with the macrocolony data where the levels of survival were very similar in both portions. This indicates that the healing response of the distal irradiated portion is affected by the irradiation of a proximal part of the tail. A more

detailed analysis of macrocolony numbers along the tail showed that there was a tendency for fewer colonies to appear in the distal region of the irradiated 6 cm length (Table 3.15). This is unlikely to be due to dose difference because the beam flatness was measured and found to be uniform to within ± 1.5% (see Section 2.1.2). The numbers of macrocolonies were similar for the 4 cm and 6 cm tail. This is partly due to the lesser proportional difference in irradiated areas (3.2 to 4.2 cm^2) compared to the difference between the 2 cm and 4 cm lengths (from 1.3 to 3.2 cm^2). Also, it may be due partly to a reduction in vascular function at the distal part of the irradiated tail.

When 12 fractions were used there was no difference between the levels of survival using both microcolony and macrocolony techniques and both 2 cm and 4 cm lengths. The healing data showed only borderline significance for the difference in response of the two lengths. The skin reaction data showed that there was no significant difference in terms of the time of appearance and the peak of the skin reaction. Hence, the field size effect tended to disappear with fractionated as opposed to single doses. This is discussed below for the colony data. Using 12 fractions the D_0 value was 3.47 Gy using a mean dose per fraction of about 5 Gy. This is higher than the single dose D_0 of 2.7 Gy as expected, because the single doses were ranging around 30 Gy where sensitivity is greater.

Field-size effects have been studied extensively in pig skin. Hopewell and Young (1982) found that there was no difference in the radiation response of skin with field sizes between 16 cm^2 (4 x 4 cm) and 64 cm^2 (16 x 4 cm). This absence of field-size effect applied both to the early epithelial lesion and to later dermal changes after single doses

of X-rays. In more recent studies, smaller circular areas of pig skin of 1, 5, 11 and 22.5 mm diameter fields were irradiated using strontium-90 (^{90}Sr) plaques, and a distinct area effect was observed for the acute epithelial reaction (Peel et al, 1984). Using larger areas of pig skin irradiated with 22.5 mm and 40 mm diameter ^{90}Sr plaques, no difference in the peak reaction was observed (Hopewell et al, 1985). These results indicate that the field-size effect is present only when irradiated areas < 22.5 mm diameter (< 4 cm^2) were used, as proposed by Peel et al (1984). The results obtained by Peel et al (1984) using 1 mm diameter field showed a large field-size effect, due to the migration of cells from the periphery of the irradiated area. When 11 mm diameter (1 cm^2) and 22.5 mm diameter (4 cm^2) fields were irradiated with doses ranging from about 30 Gy up to about 100 Gy, they showed a significant field-size effect. In this case the contribution of cell migration to the irradiated field could be neglected. Our present studies using 2 cm (1.3 cm^2), 4 cm (3.2 cm^2) and 6 cm (4.2 cm^2) tails with doses greater than 32 Gy also showed a significant field-size effect. This is compatible with their finding both in terms of the gross skin reaction and the survival of the number of micro- or macrocolonies.

It is generally considered that target cell survival is independent of field-size, as described by Hopewell and Young (1983). Our data is in disagreement with this assumption. It is possible that colony-forming cell survival can be modified by secondary response such as effects on the microvasculature (Chen and Hendry, 1986; Hauser et al, 1983 and Shymko et al, 1985).

There are two pieces of evidence in the literature which support the idea that the field-size effect is due primarily to damage of the vasculature which secondarily affects epidermal cell survival. First, there was a study using both Strontium-90 and Thulium-170 sources to irradiate pig skin with field diameters ranging from 1 mm to 22.5 mm (Peel et al, 1982). A very large field size effect was observed with very small diameter source (i.e. 1 mm for ^{90}Sr or 2 mm for ^{170}Th), which as described previously was due to the contribution of cell migration from the edge of the field. But when the source diameter was above 5 mm their results showed that the lower energy beta source (Thulium-170) produced only an epidermal reaction whereas the higher energy beta source (Strontium-90) produced both epidermal and dermal reactions. Because for the large field sizes the epidermal responses following ^{90}Sr irradiation were greater than following ^{170}Th irradiation but this was not seen with the smaller field sizes. This was interpreted as due to the greater influence of the dermal response on the epidermal reaction following the ^{90}Sr irraiation. Also, there was a significant field-size effect among 5 mm, 11 mm and 22.5 mm diameter fields only when the ^{90}Sr source was used. No field size effect was observed among 5 mm, 9 mm and 19 mm diameter fields using the ^{170}Th source. These results suggest that (a) more vasculature damage due to the use of higher energy beta source such as ^{90}Sr could affect not only the late dermal damage, but also the early epidermal response, (b) the larger area of vasculature damage using the larger field size resulted in a detectable field size effect in the acute phase.

Secondly, using the vascular network model, Hauser et al (1983) were able to predict that the tolerance dose for basal cell survival should depend both on direct basal cell killing and on damage to the

circulatory system (microvasculature). In this case, cell survival should decrease with increasing field size. Therefore, both reports discussed support the idea that the effect of field size is influenced by the larger volume of microvasculature damaged when a larger field size is used.

In order to compare our present data with data using human skin, two examples were taken from Hopewell and Young (1982), and are shown below in Table .

TABLE 4.5: "Skin Tolerance" and Field Sizes

Ellis (1942)

	FIELD SIZE (cm)			
Treatment	6x4 (S)	8x10	15x20 (L)	L/S%
Single dose	2000	1450	1100	55%
3 week	5000	3750	2900	58%
5 week	5800	4350	3350	58%

Paterson (1963)

Treatment	7x5 (S)	8x10	15x20 (L)	L/S%
Single dose	2000	1700	-	-
3 week	5250	4500	3000	57%
5 week	6000	5000	3500	58%

From Table 4.5 given above, it is clearly shown that an even greater field size effect than in the present studies was observed both in terms of single and fractionated doses. The tolerance dose decreases with increasing field size. In the single dose data, Paterson (1963) showed

about 18% less tolerance dose when the field size increased from 7x5 cm to 8x10 cm, whereas about 38% reduction in skin tolerance was reported when the field size was increased from 6x4 cm to 8x10 cm (Ellis, 1942). These human skin data are in disagreement with pig skin using similar field sizes (Hopewell and Young, 1982) - discussed below.

For human skin, the field size effect has been described by the equation:

$$\text{Dose} = K\ (\text{area})^{-0.16} \quad \text{(Von Essen, 1968)}$$

If this relationship is applied in the present work, a change in area from 1.3 to 3.2 cm^2 should change the isoeffect dose by 16%. This is greater than the observed change of 7-11%. However, if the equation $K\ (\text{area})^{-0.16}$ is changed to $K\ (\text{area})^{-0.11}$, then this formula could be applied to the tail skin system using the data for 2 cm and 4 cm lengths. For 2 cm versus 6 cm the exponent would be lower at -0.085.

The microcolony technique is capable of assessing epidermal injury over the largest range of dose from about 20 Gy up to about 44 Gy, compared with the other techniques. It was demonstrated that the sensitivity assessed using the microcolony assay increased when the area increased. This increase was not detected when the other assays were used, due to the more limited range of dose over which measurements were made, and the greater scatter in the data because of fewer colonies counted or smaller number of samples in the case of the healing data. This change in sensitivity when the area is increased is compatible with the analysis made by Shymko *et al* (1985) where they deduced from published data that the overall sensitivity to radiation increased with increasing field size. The increasing sensitivity of large field sizes could stem from the fact that as field size increases the irradiated part of the

vessel supplying any particular surviving cell is longer and hence more at risk for radiation damage. In the simplest case the probability of inactivating any particular vascular network increases with the linear dimension of the irradiated field and the consequent damage to tissue within the field increases accordingly (Shymko et al, 1985).

The lack of field size effect after 12 fractions is similar to that discussed by Shymko et al (1985). They postulated that radiation responses in large fields change more rapidly with changing dose per fraction than in small fields. This means that when low doses per fraction are used, this results in less damage to the vasculature. Hence the sensitivity for different field sizes may not be changed much using low doses per fraction and consequently there is a lack of the field size effect. This lack of field size effect after 12 fractions is in agreement with a tendency for the response of large fields to approach that of small fields for highly fractionated exposures (small dose per fraction), as analysed and discussed by Shymko et al (1985).

The difference in response of pig and human skin when similar field size were used was discussed by Hopewell (1982). He indicated that the difference was due to the different specified levels of damage being used. For instance, in pig skin an iso-effect dose was used to compare the effect in different field sizes whereas in the human skin the skin tolerance was used to compare the doses in the specific field sizes. The skin tolerance is not iso-effective i.e. more severe reactions would be acceptable in small fields and hence higher doses could be given.

Conclusions on the Effects of Field Size

(1). This is the first approach using colony techniques to study the effects of field size.

(2). The field size effect shown in the present system in terms of gross skin response was reflected by similar changes in the survival of colony-forming cells.

(3). It is likely that the initial injury is modified by subsequent effects which are mediated through the irradiated environment of the cells and these other effects depend on the field size.

(4). This is the first experimental result showing that the field size effect may be caused by a difference in sensitivity of the cells, which may be indirectly influenced by the degree of microvasculature damage.

(5). Fractionated doses using small dose per fraction show little field size effect. This could possibly be due to more sparing of microvasculature damage with fractionation.

4.4. RESPONSE TO RETREATMENT

4.4.1. Response to Re-treatment - Threshold Dose

The present studies using 3 assay systems,namely microcolony, macrocolony and healing showed that the smallest single dose where significant residual injury was detected was 16.25 Gy. This is nearly half of the HD_{50} (28 Gy) for the skin healing. It is difficult to compare the present threshold dose data with data in the literature. This is because most published studies have been designed to look at residual injury without specifically intending to measure threshold doses. However, there are few studies where a range of priming doses was used (Table 4.6). Hence a few comparisons can be made. For example, Denekamp (1975) using mouse feet found the test dose had to be reduced by 10% following all priming doses from 10 Gy up to 30 Gy. Field and Law (1976) using rat ears showed that there was no residual

TABLE 4.6: Comparison of the Residual Injury in Various Sites of the Rodent Skin Using Single Doses

SITE	PRIMING DOSE (cGy)	TIME BETWEEN FIRST AND SECOND COURSES (MONTH)	MAXIMUM RESIDUAL INJURY (%)					REFERENCE
			SKIN REACTION	MICROCOLONIES	MACROCOLONIES	HEALING	LATE DEFORMITY	
Foot	1000 2000 3000	5 - 6	~ 10					Denekamp, 1975
Ear (rat)	2000	8	0					Field & Law, 1976
Tail	750 1500 2100 2750	1.5				0 0 8 11		Hendry, 1978
Foot	1500 1750 2000 2500	8	0				0 0 25 50	Raju et al, 1983
Tail	1000 1250 1500 1625 1750 2000 2500	1.5-2		0 0 0 3 4 6 7	0 15 15	0 13 13		Present data

injury after receiving 20 Gy priming dose. In the tail skin Hendry (1978) has shown that the test doses had to be reduced by about 8% when skin was irradiated following a priming dose of 21 Gy. Raju et al (1983) using mouse feet showed that there was no residual injury after 15, 17.5, 20 and 25 Gy pretreatment. When 34 Gy was given in 12 fractions the same degree of residual injury was found as after 16.25 Gy single dose. 34 Gy in 12 fractions is nearly half of the ND_{50} (62 Gy) using 12 fractions with a fraction interval of 8 hours. Using 10 fractions of either 3 Gy or 5 Gy per fraction, Brown and Probert (1975) demonstrated a similar threshold dose of 30 Gy in 10 fractions for the skin reaction endpoint (Table 4.7).

The reduction in the numbers of colony forming cells at 16.25 Gy corresponds to between 80 and 90% loss of pigment and hair. This correlation between the degrees of residual injury (i.e. remembered priming dose) and the disappearance of skin pigment and hair would be of interest in the clinical situation. The clinician could estimate directly the degree of residual injury from the previously irradiated skin of patients, based on the observation of the change in skin pigment and hair. For instance, 10% remembered dose in the present case corresponding to a total loss of pigmentation and hair. This sign might indicate that there is a residual injury.

The combination of 15 mg/kg adriamycin with either 15 Gy or 21.25 Gy did not enhance the degrees of residual injury. This is the first experiment to demonstrate that there is a lack of enhancement in the residual injury when the adriamycin is combined with radiation. This may suggest that 15 mg/kg adriamycin doesn't kill sufficient cells which results in the induction of residual injury.

TABLE 4.7: Comparison of the Residual Injury in Various Sites of Mouse Skin Using Fractionated and Repeated Doses

SITE	PRIMING DOSE (cGy)	TIME BETWEEN FIRST AND SECOND COURSES (MONTH)	MAXIMUM RESIDUAL INJURY (%)					REFERENCE
			SKIN REACTION	MICROCOLONIES	MACROCOLONIES	HEALING	LATE DEFORMITY	
Foot	2 x 1750	5 - 6	~ 10					Denekamp, 1975.
Foot	10 x 400	6					33	Brown & Probert,
	10 x 500						45	1973.
Foot	10 x 300	3 -10	~ 4				21	Brown & Probert,
	10 x 500		~ 11				36	1975.
Tail	3-6 tolerance doses*	4.5- 9				35		Hendry, 1978.
Tail	12 x 283)	2		3				Present data.
	12 x 325)			7				
	4 x 1000)***	1.5				18		
	8 x 600)					17		
	15 x 380)					13		
	30 x 203)					7		
	3 tolerance doses**	~ 6		10	22	27		

* 6 weeks between tolerance doses.
** 9 weeks between tolerance doses.
*** All treated to tolerance.

In the data using ^{3}H-TdR to delineate the colonies, the maximum effect observed using a tolerance priming dose (i.e. 25 Gy) was about 7% in terms of dose reduction. This is close to the value of about 10% reported by Denekamp (1975), Field and Law (1976), Hendry (1978) and Hornsey and Field (1980) using other endpoints.

In terms of threshold doses for residual injury, the macrocolony data are consistent with the microcolony data. There was no residual injury at 10 Gy but there was an effect at 20 Gy. The reductions in the colony number caused by 20 Gy or 25 Gy could be converted into dose reductions and the value was about 15% reduction for skin receiving 20 or 25 Gy priming doses. Similarly, the healing data also showed a dose reduction of about 13% for skin receiving 20 and 25 Gy pretreatment. These results are close to reported data which show generally around 10% dose reduction using various endpoints as noted above (Denekamp, 1975; Brown and Probert, 1975; Field and Law, 1976; Hendry, 1978; Hornsey and Field, 1980).

The present data indicate that the residual injury in the skin expressed as a gross skin reaction followed by healing, is also seen at the level of colony-forming cell survival. Furthermore, the lowest threshold dose to induce a detectable residual injury was 16.25 Gy single dose or 34 Gy in 12 fractionated doses.

4.4.2. Response to Re-treatment - Single and Fractionated Priming Doses

It was demonstrated in the threshold dose residual injury experiment that when a tolerance dose of 25 Gy (dose to produce about 95% of tails healed) was used, there was about 10% residual injury at six weeks after the first treatment (see Section 4.4.1). This was studied further using

fractionated doses ranging from 4 fractions up to 30 fractions. Nearly equal tolerance doses were given in different fractionated schedules. In the present studies both macrocolony and healing techniques were used to measure the amount of residual injury.

These are the first studies to measure survival curves using a range of test doses, to measure any changes in sensitivity of the cells associated with residual injury following fractionated priming doses. Both the macrocolony and the healing data re-confirmed the previous threshold dose results where there was about 10% residual injury for the single dose group. In the fractionated dose groups there was a trend toward a higher survival (i.e. higher numbers of colonies per cm^2 or % healing) for groups where there was a higher number of fractions i.e. smaller dose per fraction in the priming treatment. In this case, the group receiving 60.8 Gy in 30 fractions had the least residual injury and the single fraction group was similar to 4 fractions and 8 fractions.

The difference in the response between aged controls and the pretreated skin is due to a change in the radiosensitivity. This is shown significantly in the macrocolony data where D_0 values of 580 ± 57 cGy for the aged controls (data A, Table 3.24) and 314 ± 27 cGy for all pre-irradiated skin (data H, Table 3.24) were obtained. Similarly, for the healing data D_0 values of 320 ± 77 cGy (data B, Table 3.25) and 147 ± 29 cGy (data D, Table 3.25) were deduced for the aged controls and all of the pre-irradiated skin respectively. However, the radiosensitivity between different fractionated priming schedules was very similar (data C, Table 3.25). The higher survival observed by reducing the size of dose per fraction in the priming treatment shows that there was a

sparing effect with dose fractionation. This effect could possibly be due to the long term sparing of vasculature or connective tissue damage.

There are many reports concerned with the relationship between acute and late skin injury. In the mouse foot studies most of the data agree that late foot deformity appears to be causally related to early skin reaction for a limited range of fraction numbers or overall time. When more than 15 fractions were used or when the overall time exceeded 8 weeks, this relationship did not apply in some experiments (Douglas and Fowler, 1976; Brown and Probert, 1975). In other experiments Denekamp (1975 and 1977) using mouse feet, and Field and Law (1976) using mouse feet and ears, it was found that the relationship between early and late damage remains the same for a wide range of fractionated treatments and re-treatments. Also using mouse hind leg skin, Masuda et al (1980) studied late skin contraction. They found that for lower doses the contraction was less severe and it occurred later.

Experimental studies of early and late radiation response in pig skin (Berry et al, 1974; Withers et al, 1978) indicated that the severity of late response (contraction) could not be adequately judged from early skin reactions when changing to larger doses per fraction. In other words, there was a dissociation between acute reactions and late changes when the fraction size was changed.

When conventional radiotherapy treatment schedules have been altered to fewer fractions of larger doses per fraction, a large increase in late complications has ensued with little or no difference in the severity of the acute responses (Arcangeli et al, 1974; Bates and Peters, 1975; Fletcher et al, 1974; Fletcher and Shukovsky, 1975; Kim et al, 1975;

Kagelnik and Karcher, 1977; Montague, 1968 and Stell and Morrison, 1973). These results suggest that the dose-survival characteristics of target cells for late injuries are different from those of target cells of acutely responding tissues.

The present results are in agreement with most of these results mentioned above. For instance we observed a similar early response (i.e. > 95% healing) using different fraction size and total doses, but the degree of residual injury tested at later time was decreased when the numbers of fractions was increased i.e. dose per fraction was decreased. Also, when a range of test dose was given the dose-survival curves for the pre-irradiated skin were steeper than for the aged control skin.

Other studies using an analysis of the tolerance to re-irradiation of previously irradiated human skin (Hunter and Stewart, 1977) demonstrated that neither acute nor late skin reactions exceeded those expected in similar radical treatments of patients not previously irradiated. This suggests that the tolerance of skin to re-irradiation following a long latent period may approach normal. The reason for the difference between these data and other data in the literature is not known, but it may be related to:

a). The first dose may not have been high enough to induce residual injury, because there were only 2 out of 15 patients with telangiectasia a long time after the 1st treatment.

b). There was a very long latent period before the 2nd treatment started which may allow for more complete slow repair to take place.

c). The skin dose for the 2nd treatment was generally on the low side, therefore no patient had an early skin reaction which exceeded

pigmentation or dry desquamation. This affects the accuracy of the skin reaction scoring. As described by Simmonds and Hopewell (1984-85) in the case of re-irradiation, observations of erythema led to an underestimation of the true extent of the damage.

4.4.3. Response to Re-treatment - 3 Tolerance Doses

Three experiments using gross skin reaction, macrocolony and healing techniques have studied the residual injury of skin after 3 priming doses. The results showed that there was no significant change in radiosensitivity measured using both macrocolony and healing techniques. Also, when a specified level of survival was chosen (i.e. 1.5 colonies per cm^2 for macrocolonies and the LD_{37} value for healing, there was about 22% residual injury with the macrocolony data and about 27% with the healing data. Using the microcolony technique it was confirmed that the radiosensitivity of the aged controls and skin receiving 3 previous treatments was very similar, as also was seen using the macrocolony and the healing techniques. However, when a specified level of survival was chosen at 10 microcolonies per cm^2, there was only about 10% residual injury. The 10% dose reduction measured using microcolonies corresponded with a reduction by a factor of 7 in numbers of microcolony-forming cells per unit area after recovery from the priming doses (i.e. before the test dose was given). This 7-fold reduction was calculated by extrapolating the respective survival curves to zero test dose, and allowing for the shoulder by doing a split-dose experiment. The reduction was not due to the patches of non-repopulated epithelium, as these formed only about 10% of the total area. There are two possible reasons for the reduction: (1) a secondary effect of the heavily-irradiated substratum on the survival of some of the microcolony-forming cells; (2) a true reduced concentration of stem

cells in the heavily-irradiated epidermis, as noted for example in haemopoietic tissue (Hendry et al, 1983). However, there is no direct evidence for this hypothesis.

The dissociation between the amounts of residual injury measured using the microcolony technique (10%) or the macrocolony (22%) and healing (27%) technique could be due to several reasons, as follows.

Firstly, the higher dose remembered using the macrocolony technique when compared with the microcolony technique is probably due to:
(a) an underestimate of the number of macrocolonies in re-treated epidermis. The colonies in the pre-treated skin tended to be much flatter and very ill defined as compared to those in the aged controls where very well defined nodules easily were observed. A typical colony arising in the re-treated epidermis is shown arrowed in panel f in Plate 2.6. The underestimation of the numbers of macrocolonies also was seen when a single priming tolerance dose was given (see last section). However, the underestimation after 1 priming tolerance dose of about 6% (i.e. the difference between 7% residual injury for the microcolonies and 13% for the macrocolonies) is less than the value of 12% after 3 tolerance doses (i.e. the difference between 10% residual injury for the microcolonies and 22% for the macrocolonies). The reason for the smaller differential using a single priming dose is that the delineation of the macrocolonies in the skin re-treated after 1 tolerance dose was closer to that of the macrocolonies found in the aged control skin than in skin receiving 3 previous treatments.
(b) some of the surviving microcolonies may not have been able to grow into macrocolonies due to injury in the supporting underlying tissues, for example, the vasculature. Such injury has been demonstrated by

long term changes in blood flow in the mouse tail with doses of 20 Gy or more (de Ruiter and Van Putten, 1975).

Secondly, the large difference in terms of the percentage residual injury between microcolony data (10%) and healing data (27%) may be due to the situation where in the pretreated skin there were patches covered by very thin epidermis with a very low cell density. In these regions very few colonies were found. Examples of these areas is given in Plate 4.1 and 4.2. More of these areas were noticed after 3 tolerance doses than after 1 tolerance dose, but the relative amounts were not measured. After 3 tolerance doses, the thin regions comprised approximately 10% of the irradiated area. This would not affect the counts of colonies per unit area significantly, but it could have a marked effect on the healing of the skin following a subsequent test dose because the colonies may not be able to re-epithelialise these regions during the acute reaction phase. In the skin receiving 3 tolerance doses the numbers of microcolonies per cm^2 after a test irradiation was reduced by a factor of about 3. This corresponded to only about 10% dose shift for equal effect. Therefore, the 17% more residual injury measured using the healing technique, may be due to either the surviving microcolonies not being able to grow into macrocolonies and eventually re-epithelialising the whole epidermis or because other supporting tissues such as the damaged vasculatures, connective tissues or basement membrane being unable to support the initial growth of microcolonies.

The similar radiosensitivity of the skin pre-irradiated with 3 tolerance doses and the aged controls contrasts with the significant change in radiosensitivity caused by 1 priming treatment and tested 6 weeks later. The possible reasons are as follows: The PLD repair in the dermal

Plate 4.1: Skin receiving 3 previous treatments followed by 16.5 Gy test dose, showing a circular area with very few labelled cells and colonies surrounded by densely labelled cells in many microcolonies. ×40

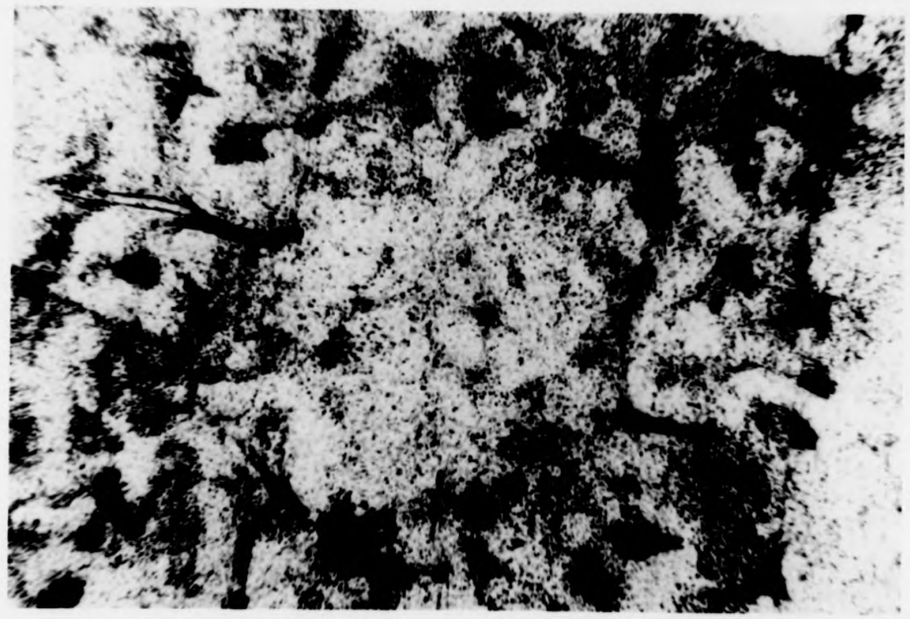


Plate 4.2: Skin receiving 3 previous treatments followed by 16.5 Gy test dose, showing an elliptical area with very few labelled cells and colonies surrounded by densely labelled cells in many microcolonies. ×40

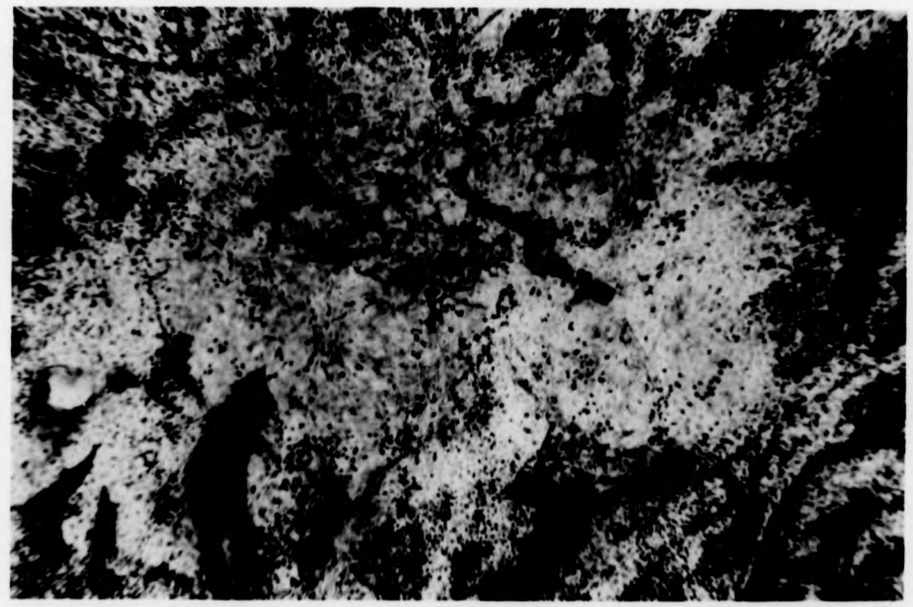


Plate 4.1: Skin receiving 3 previous treatments followed by 16.5 Gy test dose, showing a circular area with very few labelled cells and colonies surrounded by densely labelled cells in many microcolonies. ×40

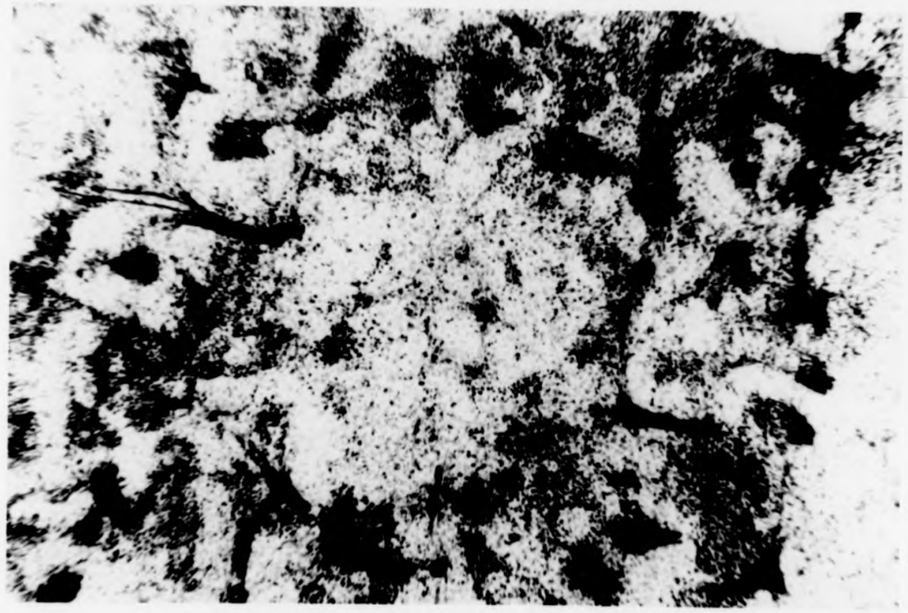
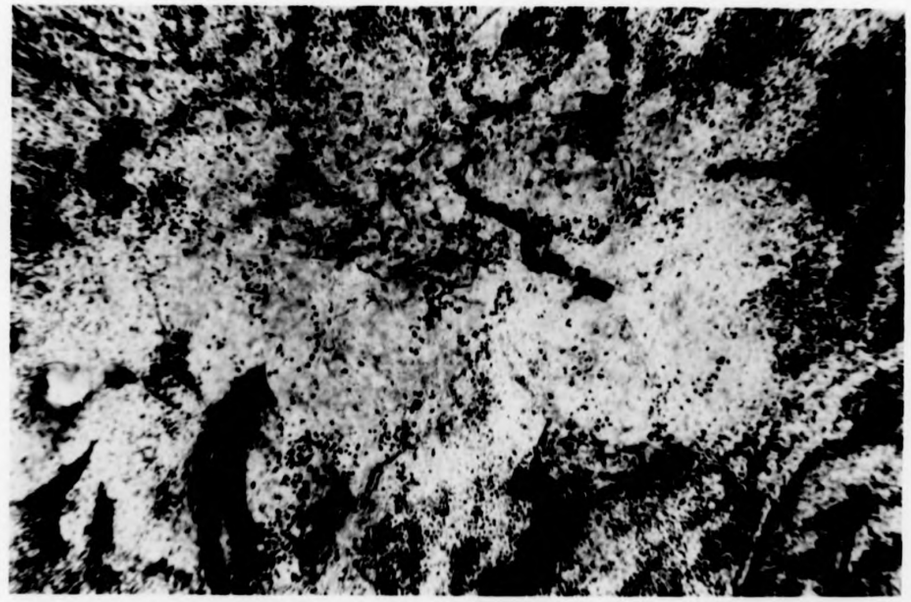


Plate 4.2: Skin receiving 3 previous treatments followed by 16.5 Gy test dose, showing an elliptical area with very few labelled cells and colonies surrounded by densely labelled cells in many microcolonies. ×40



component of the response may not be completed by 6 weeks after the priming dose, so that the sensitivity is greater than it would be if more time was allowed. There was a significant reduction in ND_{10} 6 weeks after the 1st, and after the 2nd and 3rd tolerance doses with 6 weeks between doses (Hendry, 1978). It is possible that there was a further increase in sensitivity at the time of the 2nd dose and after the third doses. Hence, a change in radiosensitivity in both macrocolony and healing data would be expected and this is confirmed by the present studies. There was no further significant reduction in ND_{10} with more tolerance doses given at intervals of 6 weeks (Hendry, 1978). This suggests that there was no further change in radiosensitivity and that there may have been more complete PLD (or slow) repair which decreased sensitivity back to control levels. In the present studies the test dose was given 27 weeks after the first of 3 priming tolerance doses, each tolerance dose separated from the next by a 9 week interval. This time period may allow a more complete recovery from the original damage to the skin. Another possible reason for the same radiosensitivity after 3 tolerance doses when compared with the aged controls may be related to changes in oxygenation, in other words, the increase in sensitivity seen at 6 weeks may be masked by slight hypoxia decreasing sensitivity when irradiated later at 9 weeks. The only measurements of induced hypoxia in pre-irradiated skin showed no effect at 6 weeks after a single priming dose (Hendry, 1977). No observations have been made after 3 priming doses. However, this is unlikely to contribute significantly to the present changes in radiosensitivity because the skin was irradiated at 37°C which largely avoids any problem of hypoxia in skin.

General Conclusions About the Response to Retreatment

(1). The threshold dose for the residual injury is about 16.25 Gy for a single dose and about 34 Gy for 12 fractions with a fraction interval of 8 hours. These doses are about half of the HD_{50} in both cases.

(2). 15 mg/kg adriamycin combined with radiation did not enhance the residual injury in the tail skin warmed up to 37°C.

(3). Different degrees of residual injury were observed at 6 weeks after various fractionated priming treatments, all being iso-effective at the tolerance level. Generally there was a trend that the smaller the dose per fraction, the greater the sparing of residual injury. This may be due to sparing of tissues supporting the epidermis. This would support the theory that hyperfractionation may have the advantage in reducing late effects in normal tissues (Withers et al, 1984).

(4). The reduction in colony number and in the proportion of healed tails when tails were re-irradiated at 6 weeks after 1 tolerance dose, was associated with a change in radiosensitivity. This could be due to a slow and incomplete recovery of the underlying supporting tissues. No data were available to estimate changes in the number of colony-forming cells per unit area induced by a single priming dose.

(5). The lack of significant changes in radiosensitivity after 3 tolerance doses suggests that there is more PLD (or slow) repair when using longer intervals between doses.

(6). In terms of radiosensitivity the present residual injury studies showed that there was a good correlation among the microcolony, macrocolony and healing data.

(7). The residual injury after 3 tolerance doses measured using microcolonies may be due to the secondary influence of the heavily-irradiated substratum on microcolony production, or a true reduction in concentration of microcolony-forming cells in the basal

layer.

(8). The difference in the percentage residual injury between the microcolony and macrocolony data is probably due to the underestimation of the numbers of macrocolonies.

(9). The difference between the microcolony and the healing data may be due to the damage supporting tissues, such as vasculature, connective tissue or basement membrane affecting the healing of the skin, together with the sparsely-repopulated patches (about 10% of the total irradiated area) which may prevent proper re-epithelialisation following another dose.

(10). Residual injury can be minimised if smaller doses per fraction and lower total doses are used together with longer time intervals between the priming and the subsequent treatments.

(11). It has been shown that the residual injury reaches a maximum of 35% dose reduction after 3 tolerance doses delivered with 6 weeks between doses (Hendry, 1978). In the present work the value was about 27% using intervals of 9 weeks, and hence there is a tendency for greater recovery and therefore less residual injury when the interval is prolonged.

4.4.4. Comparison of the Residual Injury in Other Tissues

Regarding the threshold dose for residual injury the present data showed that there was only 3% residual injury observed when the first dose was about half of the HD_{50}. In other studies, quite a high fraction of the ED_{50} has been used as a priming dose with other tissues. There are only a few comparisons that can be made between the threshold doses in different tissues when they are expressed as a fraction of the ED_{50}. One example is shown in Table 4.8 for spinal cord where there was 10% residual injury when the first dose was 50% of ED_{50} (Hornsey *et al*,

TABLE 4.8: Comparison of Residual Injury in Other Tissues

TISSUE AND SPECIES	END POINT	PRIMING DOSE (FRACTION OF DOSE TO GIVE END POINT)	TIME BETWEEN FIRST AND SECOND DOSES	DOSAGE CHANGE* (%)	REFERENCE
Haemopoietic (Mouse)	$LD_{50/30}$	10%	3 weeks	-10	Porteous & Lajtha, 1966
Haemopoietic (Dog)	$LD_{50/30}$	67%	3 weeks	+13	Ainsworth et al, 1966
Haemopoietic (Sheep)	$LD_{50/60}$	67%	1 month	-(22 to 29)	Hanks et al, 1966
Intestine (Mouse)	10 surviving cells per jejunal circumference	78%	2 months) 6 months) 12 months)	+11 +15 + 7	Reynaud & Travis, 1984
		100%	2 months) 6 months) 12 months)	+18 +24 + 7	
Lung (Mouse)	$LD_{50/80}$	76-85%	4 weeks	-(15 to 24)	Field et al, 1976
Spinal Cord (Rat)	White matter necrosis (ED_{50})	80%	8 weeks) 16 weeks)	-42 -26	Van der Kogel, 1979
	Vascular damage (ED_{50})	80%	8 weeks) 16 weeks)	-58 -51	

TABLE 4.8: (Contd.)

TISSUE AND SPECIES	END POINT	PRIMING DOSE (FRACTION OF DOSE TO GIVE END POINT)	TIME BETWEEN FIRST AND SECOND DOSES	DOSAGE CHANGE* (%)	REFERENCE
Spinal Cord (Rat)	ED_{50}/1 year	25% 50% 75%	14 weeks	- 6 -10 -29	Hornsey et al, 1982.

* - indicates less dose, and + indicates more dose, was required to reach the endpoint for samples which had received previous irradiation compared to no previous irradiation.

1982). When the priming dose was reduced to 25% of the ED_{50} there was still 6% residual injury. This indicates that the threshold dose for residual injury in the spinal cord is a lower fraction of the total dose resulting in the endpoint than is the case for the skin.

After higher priming doses from 67 up to 85% of the ED_{50} given as a priming dose, the residual injury ranges between 58% in the spinal cord (Van der Kogel, 1979) to a negative amount of 24% (i.e. induced resistance) in the intestine (Reynaud and Travis, 1984), see Table 4.8. So there are examples not only of residual injury but also examples of induced resistance to subsequent doses. The degrees of residual injury are more marked in the spinal cord than in the lung, bone marrow and skin (Table 4.8). There are two unusual cases where there was induced resistance. With the 3 studies on bone marrow, one showed 13% induced resistance (Ainsworth and Leong, 1966). However, this value was not significant. In the case of the intestine, Reynaud and Travis (1984) showed that the resistance was due to induced hypoxia. This was demonstrated by giving the radiosensitiser misonidazole just before the test radiation, and the survival curve for crypts returned to that of the control showing that there was no inherent induced resistance in the cells. An earlier report using repeated priming doses to the colon (Hamilton, 1979) also showed this induced resistance, but the mechanism was not known at that time.

There was a tendency in the experiments of Reynaud and Travis (1984) for the induced resistance to decrease a long time after irradiation. This may be due to long term recovery in the vasculature or connective tissues, leading to improved oxygenation. In the spinal cord there was evidence of recovery between 8 and 16 weeks after a priming dose

(Van der Kogel, 1979). Also, in the skin there was less residual injury when the intervals between 3 repeated doses were irradiated from 6 weeks (Hendry, 1978) to 9 weeks (present data). Hence, there is evidence for long term recovery in all these three systems namely, intestine, skin and spinal cord.

There are no known data available concerning residual injury in other tissues following fractionated priming doses, apart from skin which was discussed in Section 4.4.2.

4.5. CONCLUSIONS

In contrast to the skin reaction end-point used in most other residual injury studies in the skin, the aim of the present study was to use the existing assay systems in the tail skin such as skin reaction, skin healing/necrosis, macrocolonies plus the newly developed microcolony technique to investigate thoroughly about the degree and the possible cause of the residual injury when the skin was re-irradiated after single, fractionated and repeated irradiations.

The results of this study were as follows:

(1). This new microcolony technique using both mitotic cell arrest or labelling techniques provided a good estimate of the number of surviving clonogenic cells in the tail epidermis. When assaying re-treated skin only the labelling technique was suitable to use because the epidermal architecture and pigmentation were changed after the priming dose. The sampling time in the microcolony studies had to be carefully selected for individual animals using erythema or severe erythema criteria, because of the variation in colony development times between mice.

(2). The difference in radiosensitivity between the present microcolony data and other reported data in the literature using colony techniques could be largely due to plucking, strain difference, site of skin studied, or the different scoring techniques used. The difference in radiosensitivity between the present microcolony and macrocolony data was due to the underestimation of the number of surviving colonies at low doses using the macrocolony technique. The recovery factor among reported data was very similar. About 3% of the basal cells were clonogenic, and all microcolonies ($\geq$ 32 cells) developed into macrocolonies.

(3). The dose-response relationships for the target cells for epidermal

healing and the cells which form microcolonies showed more similar values of sensitivity than reported previously. This suggests that the microcolony-forming cells may be the target cells responsible for skin healing. The correlation between macrocolony data and healing data or microcolony data was less good because of the problem of the coalescence of macrocolonies at lower doses which changed the apparent radiosensitivity very significantly.

(4). In terms of cell sensitivity, there was no significant difference between different ages of mice studied. Plucking of the skin either 18 hours before or immediately after irradiation both showed an increased cell killing effect. Plucking 18 hours before irradiation showed a tendency toward an increase in the radiosensitivity of the cells.

(5). 10 mg/kg adriamycin given 24 hours after irradiation did not show any additional killing effect on the clonogenic cells. However, with 15 mg/kg adriamycin given 30 minutes before irradiation there was a significant increase in cell killing.

(6). There was a significant field-size effect when comparing 2 cm irradiated tail (1.3 cm^2) to 4 cm (3.2 cm^2) or 6 cm 4.2 cm^2). This effect in terms of gross skin response was reflected by similar changes in the survival of colony-forming cells. The field-size effect may be caused by a difference in sensitivity of the cells, which in turn may be influenced indirectly by the degree of stromal damage. When total doses were fractionated into small doses per fraction (i.e. 56-68 Gy/12F) the field-size effect was smaller. This would be the case if there was more sparing of the stroma with fractionation.

(7). The lowest threshold dose where a significant level of residual injury was detected was 16.25 Gy single dose or 34 Gy in 12 fractionated doses. Both doses were nearly half of the HD_{50} for skin healing. A

single dose of 16.25 Gy corresponded to a gross appearance of about 85% loss of pigment and hair. The combination of 15 mg/kg adriamycin with either 15 Gy or 21.25 Gy did not enhance the degrees of residual injury. This might suggest that 15 mg/kg adriamycin does not kill sufficient cells to result in the induction of residual injury.

(8). Using iso-effect first treatments with various fractionated doses, it was shown that there was a trend toward higher cell survival to test doses for groups where there was a higher number of fractions i.e. smaller doses per fraction in the priming treatment. This indicates that the residual injury can be minimised if smaller doses per fraction were used. The difference in the response between aged controls and the pretreated skin is due to a change in cell sensitivity. There was a significant change in survival but not in the sensitivity among different sizes of dose fraction studied (e.g. 1F, 4F, 8F, 15F and 30F).

(9). After three repeated tolerance doses a maximum of 10%, 22% and 27% of residual injury was observed respectively when microcolony, macrocolony and healing techniques were used. There was no significant change in the radiosensitivity between the aged control skin and skin receiving 3 previous tolerance doses. A level of 10% residual injury measured using the microcolony technique indicated that this may be due to the secondary influence of the heavily-irradiated stroma on microcolony production or a true reduction in concentration of microcolony-forming cells in the basal layer. The result of the split-dose experiment suggests that there was a significant reduction in the numbers of microcolony-forming cells per unit area at zero dose. The reason for the difference between microcolony and macrocolony data could be due to the underestimation of surviving colonies when the macrocolony technique was used. The higher residual injury observed

using the skin healing technique may be due to the damage in supporting tissues such as vasculature, connective tissue or basement membrane which formed sparsely-repopulated patches over the epidermis which eventually prevented proper re-epithelialisation following another dose.

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The radiosensitivity of microcolony- and macrocolony-forming cells in mouse tail epidermis

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ABSTRACT

A new microcolony technique is described for measuring the survival of colony-forming cells in mouse tail epidermis. The survival curve is characterised by $D_0 = 2.70 \pm 0.12$ Gy. The number of microcolonies per cm^2 is similar to the number of macrocolonies after high doses, which shows for the first time that all microcolonies (≥ 32 cells) in epidermis develop into macrocolonies. At low doses the number of macrocolonies underestimates the number of colony-forming cells because of coalescence of microcolonies to form macrocolonies. This results in a lower apparent sensitivity of macrocolony-forming cells by a factor of about 1.5. About 3% of basal cells in tail epidermis appear to be capable of colony formation.

Residual epidermal injury in various sites in the mouse after irradiation has been widely studied using techniques involving skin reactions, deformity, or necrosis (*e.g.* Brown & Probert, 1973; Denekamp, 1975; Hendry et al, 1977; Hornsey & Field, 1980), and some cellular defects associated with long-term injury have been described using the mouse tail (Hendry et al, 1982). In order to study any changes in radiosensitivity of colony-forming cells associated with residual injury measured in terms of gross skin response, a microcolony technique has now been developed for tail epidermis. This site is preferred to dorsal skin, which is used for most colony studies, because dorsal skin contracts markedly after irradiation (Hayashi & Suit, 1972) whereas tail skin is overlying the bony tail structure and this will limit the shrinkage. This lack makes re-irradiation of the same region of skin technically simpler and the results easier to interpret. Although a macrocolony technique has been described for tail epidermis (Hendry, 1984), the method produces survival data spanning only one decade and hence has poor resolution regarding changes in radiosensitivity. The microcolony technique, producing data over three decades in survival, is described here in detail for mice receiving radiation for the first time. The data are compared with macrocolony data and with data in the literature concerning epidermal microcolonies and macrocolonies.

MATERIALS AND METHODS

Female $B6D2F_1$ mice at an age of 3 months were used throughout. The tail skin was not plucked either before or after the irradiation. The middle 3 cm of tail was irradiated with 300kVp X-rays (HVL = 2.2 mm Cu) at a dose-rate of 2.0 Gy per min, as described previously (Hendry et al, 1976). The tail holes in the jig were kept at 37°C, near body-core temperature. The unanaesthetised mice were confined in perforated perspex tubes and were positioned 5 min before irradiation, to allow the tails to acclimatise to the 37°C temperature which was maintained during irradiation. Between four and 12 mice were used per dose point, with the larger numbers for the higher doses where colony numbers were low. The mice were inspected daily after irradiation and were killed at various times for counting colonies. The time of assay for each mouse was chosen on the basis of specified levels of skin reaction, and the validity of this approach is described below. The reactions used were erythema, severe erythema, and slight dry desquamation, because it was found that discrete colonies, most containing more than 32 cells, were detected at times corresponding to these levels of reaction. The erythema reactions were difficult to discern in the skin of these black mice, and they were detected by close scrutiny under a lamp.

On the day the mice were to be killed, they were injected intraperitoneally with 0.01 mg (per mouse) of the mitotic inhibitor vincristine sulphate, three times at intervals of 3 h. Three injections were given to accumulate more mitoses in order better to identify the growing colonies. They were killed 3 h after the last injection, and then the hairs were removed from the tails using at least eight applications of Sellotape. Whole-mount preparations of the epidermis were made using a modification of a method used by Mitani and Potten (personal communication). The skin was dissected away from the underlying tissues, put in Carnoy's fixative overnight, and then transferred to 70% ethanol. The samples were hydrolysed in 5N HCl at room temperature (approx. 22°C) before inspection under a dissection microscope. Fine-point forceps were used to remove, very carefully, all the connective tissue and the hair follicles. Then the epidermal sheet was put on a subbed slide and air-dried. The sample was stained for about 90 min with Schiff's reagent, cleared, and mounted for scoring.

Colonies were identified as foci of high cell density with a mitotic index ranging from about 5% to about 50%, and with a lack of melanin (Fig. 1). Very few

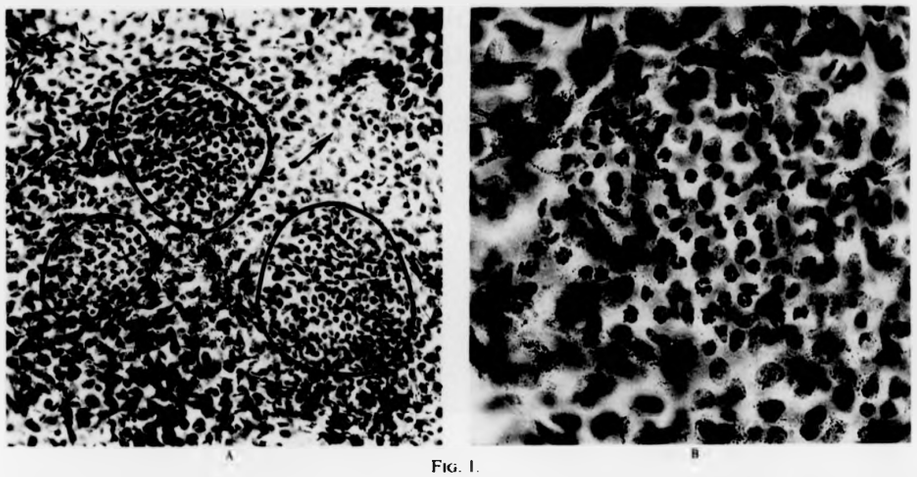

FIG. 1.

(A) Three neighbouring microcolonies at day 15 after 15 Gy. The arrow points to a hair follicle opening. ×60. (B) A single microcolony at day 15 after 17.5 Gy, showing a high proportion of mitotic cells. ×120.

colonies were observed between the squame mounds on the tail, *i.e.* associated with hair follicles. Colony size was estimated initially using cell number and later by colony area (measured using a Kontron-MOP Videoplan), which was proportional to cell number. Colonies were counted in the middle 2 cm of the irradiated 3 cm length of tail to avoid problems of cell migration and dose inhomogeneity at the edges of the irradiated field (Hendry, 1984). For the cell survival data, colonies containing 32 cells or more were counted. Tails were sampled between 10 and 21 days after irradiation, most being sampled between days 13 and 17.

Other mice receiving doses of 30 Gy or more to the tail were left for longer intervals of time until the skin reactions were more severe and when macrocolonies could be counted, as described by Hendry (1984). The macrocolonies were scored between 19 and 28 days after irradiation, and most scoring was performed between days 20 and 23.

A survival curve was fitted to the data points using a multitarget equation including a single-hit term, in the computer programme described by Gilbert (1969). A bending survival curve, described for example by a linear/quadratic equation, could have been used, but there was no clear curvature in the majority of the data in the high dose range, as judged by eye (see Figs 3 and 4). A similar lack of curvature was reported for epidermal colony forming units (CFU) over five decades of depopulation by Withers (1976b).

RESULTS

The assay time for microcolonies for 80% of the mice was between 13 and 17 days after irradiation (Table I).

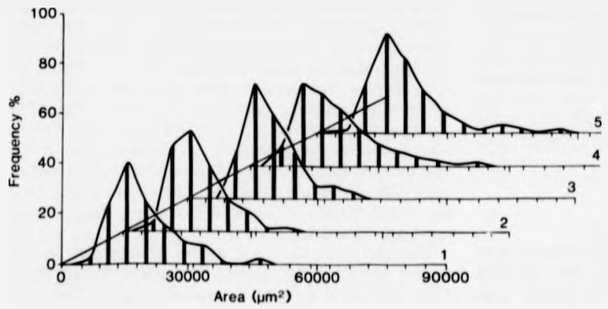

FIG. 2.

Clone-size distributions for five mice each assayed on a different day after 21.25 Gy, from day 14 (labelled 1) to day 18 (labelled 5). The skin reaction at the time of assay was erythema for all five mice.

TABLE I

PERCENTAGE OF MICE ASSAYED FOR MICROCOLONIES, AND COLONY SIZE

	Time after irradiation (days)											
	10	11	12	13	14	15	16	17	18	19	20	21
% of mice assayed	1	2	4.5	16.5	19	24.5	12	10	3.5	3.5	1.5	2
Colony No. – mean from all assay times	—	—	—	0.96 ±0.06	0.96 ±0.11	1.09 ±0.1	0.83 ±0.08	1.05 ±0.16	1.15 ±0.13	—	0.88 ±0.05	—
Mean No. of cells per colony	—	—	—	—	87 ± 3.3	78 ± 2.5	91 ± 2.6	84 ± 3.9	89 ± 3.9	—	—	—

± 1 Standard error.

To demonstrate the validity of using a specified skin reaction level as the criterion for choosing the assay time for scoring colonies, rather than using a fixed time interval, the mice used over all the doses generating Fig. 5 were grouped according to the day they were assayed. At each time, the colony count for a particular mouse was divided by the mean count in the group of mice used at a given dose. The average values of these ratios at each day after all doses are given in Table I. The values do not increase significantly with the time of

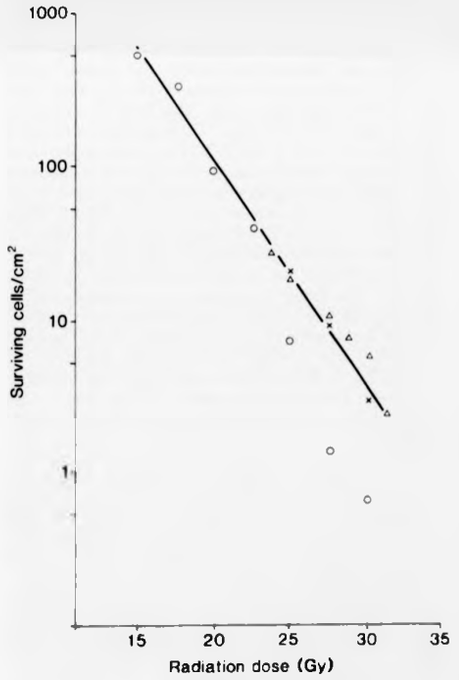

FIG. 3.

Surviving colony-forming cells per cm² versus radiation dose. Standard sampling errors are about 3% of the mean after the lower doses, increasing to about 30% after high doses. Tail skin assayed when showing erythema (○); with severe erythema (×) and with slight desquamation (△). Line fitted by eye through the circles up to 22.5 Gy, together with the crosses.

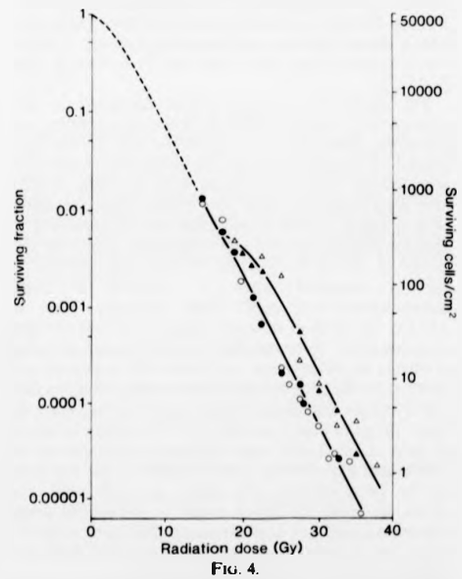

FIG. 4.

Survival after single doses (○, ●), and to a range of second doses given 24 h after a first dose of 17.5 Gy (△, ▲). The origin of the latter curve is the value of survival at 17.5 Gy on the curve fitted to the single-dose data. Open and closed symbols represent two separate experiments. Sampling errors (not shown), as stated for Fig. 3. The dashed curve represents the initial part of the single-dose curve, assuming it has the same shape as the initial part of the second-dose curve. For survival parameters see text.

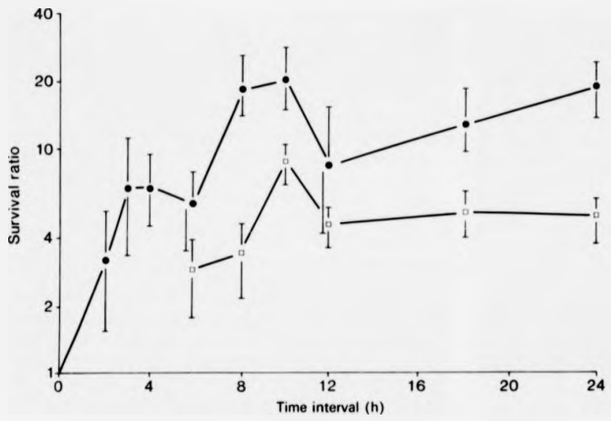

FIG. 5.
Survival ratio versus the time interval, using two equal doses of 17.5 Gy. Present data (□); data taken from Withers (1967a) (●). Error bars are 95% confidence limits.

assay. Also, in these same groups, the mean number of cells per colony is similar between assay times of 14 and 18 days (Table I). The frequency distribution of colony sizes is shown for five samples in Fig. 2, where a sample was assayed on each day from day 14 to day 18 after 21.25 Gy.

The single-dose survival curve measured in one experiment is shown in Fig. 3. When the criterion was erythema, there was a curvature in the survival data plotted on a log linear graph (open circles). However, it was noticed that the colonies were generally smaller after high doses than after low doses. When the degree of skin reaction used as the criterion was increased to severe erythema, survival after high doses increased by a factor of about 5 (crosses). No further increase in survival occurred when a criterion of slight desquamation was used (open triangles). Also, at 23.5 Gy the level of survival using a criterion of slight desquamation followed the curve generated using erythema as the criterion, and hence this dependence of survival on the criterion used to determine the assay time was a feature only of the higher range of doses. After the lower range of doses, virtually all the colonies consisted of more than 32 cells when erythema had appeared. In subsequent experiments it was decided to use erythema as the assay criterion for doses up to 22.5 Gy, and severe erythema for higher doses. A criterion of severe erythema could not easily be used after both ranges of dose, because for doses less than 22.5 Gy there was some confluence of adjacent large colonies.

The survival curve measured in two subsequent experiments is shown in Fig. 4 (left curve), using the above criterion. For the pooled single-dose data, $D_0 = 2.70 \pm 0.12$ Gy and n (right ordinate) $= 2.2 \times 10^5$ per cm^2. In order to produce a complete survival curve for these colony-forming cells, a split-dose experiment was performed where it was assumed that the response to a series of second doses, after a primary dose, reflected the response to solely the second doses (Hendry, 1979). First, two doses of 17.5 Gy were used, separated by intervals of time from 0 to 24 h (Fig. 5). On the basis of these results, it was decided to obtain a survival curve at 24 h after 17.5 Gy, which is shown in Fig. 4 (right curve) using data pooled from two experiments. The origin of the second-dose curve is the survival level at 17.5 Gy on the fitted curve for single doses ($D_0 = 3.13 \pm 0.29$ Gy; $n = 2.1 \pm 0.4$). The data are expressed as surviving colony-forming cells per cm^2 on the right ordinate, and as surviving fraction on the left ordinate. The latter procedure was accomplished by assuming that the second-dose curve reflected the initial shape of the single-dose curve (Hendry, 1979), shown as a dashed curve joining the single-dose curve to the origin in Fig. 4. In this case the surviving fraction after a first dose of 17.5 Gy was assumed to be the same as that measured from 17.5 Gy given as a second dose (right curve, Fig. 4). Hence the total surviving fraction after both first and second doses could be calculated, and these are shown on the left ordinate in Fig. 4. The origin corresponded to 59 000 cells per cm^2 (right ordinate). The "complete' single-dose curve was characterised by $D_0 = 2.71 \pm 0.16$ Gy, a ratio of initial to final slopes of 0.35 ± 0.10, and $n = 3.8 \pm 1.4$ (left ordinate) or $2.2 \times 10^$ cells per cm^2 (right ordinate).

The macrocolony data are shown in Fig. 6 (open circles) ($D_0 = 4.1 \pm 0.6$ Gy, and $n = 6.57 \times 10^3$ cells per cm^2). A discrepancy between the microcolony and macrocolony survival curves may be expected if there is coalescence of several microcolonies to form a macrocolony after the lower doses. This possibility was tested at several dose levels by counting the number of pairs (or groups) of microcolonies in a total of between 30 and 20 microcolonies (depending on the dose), where the distance between microcolonies was less than 3.05 mm (the average diameter of a macrocolony). When each pair was counted as one colony, predicted

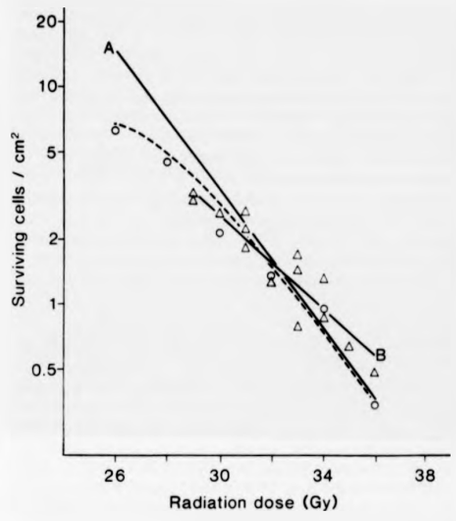

FIG. 6.
Macrocolony and microcolony survival data. (△) and line B, macrocolonies. Dashed curve, macrocolony survival predicted mathematically from the microcolony curve (see text). (○), macrocolony survival predicted from measurements of the distances between microcolonies (see text). Line A, taken from Fig. 4.

values of macrocolony survival could be calculated, and these are shown as open circles in Fig. 6. A mathematical way to demonstrate this is to assume a Poisson distribution of surviving cells. If there are m microcolonies per cm^2 at a given dose, and the average area of a macrocolony is A, there are mA microcolonies in the average area of a macrocolony. The probability of there being only one microcolony in this area is $mA.\exp(-mA)$, and the probability of there being one or more than one microcolony is $[1-\exp(-mA)]$. Hence the number of macrocolonies will be less than the number of microcolonies by a factor:

$$\left[\frac{1-\exp(-mA)}{mA.\exp(-mA)}\right]$$

The factor tends to unity for low values of m. The expected number of macrocolonies at each dose (dashed curve in Fig. 6) was very similar to the number calculated using the measured distribution of the microcolonies described above. The survival curve for macrocolonies (curve B, Fig. 6) was above the curve for microcolonies after high doses. This was due to the greater weighting of the low-dose points because of the greater number of colonies scored. A similar exercise, calculating the (Poisson) probability of microcolonies arising from more than one microcolony, showed only a 6% underestimate of colony numbers at 15 Gy, decreasing to 1% at 20 Gy. This was due to the much smaller size of microcolonies compared with macrocolonies.

DISCUSSION

In the present technique, samples are taken for scoring microcolonies when the skin reaction has reached a specified level. This procedure was shown to give similar colony-size distributions among different samples assayed at various times after a given dose. This indicates that there is a direct relationship between the development of gross skin reactions and the growth of microcolonies.

TABLE II

SURVIVAL PARAMETERS OF EPIDERMAL COLONY-FORMING UNITS TO SINGLE DOSES

Site	Dose to give one colony/cm² (Gy)	Maximum test area (cm²)	D_0 (Gy)	n/cm²	CFU/cm²	Reference
Macrocolonies						
Dorsum (plucked 1–12 h before irradiation)	20	1.13	1.37 (1.26–1.50)*	1.39×10^6	$\simeq 9 \times 10^4$	Withers, 1967 a, b
Dorsum (plucked 20–24 h before irradiation)	22	2.85	1.35 (1.20–1.55)*	1.35×10^7	$\simeq 2 \times 10^5$	Emery et al, 1970
Tail (unplucked)	34	1.7	4.10 ± 0.58†	6.57×10^3	—	Present data
Tail (unplucked)	34	1.4	3.45 ± 0.36†	2.03×10^4	$\simeq 3030$	Hendry, 1984
Microcolonies						
Dorsum (plucked)	22	Whole body	2.33 ± 0.11†	1.23×10^6	—	Al-Barwari & Potten, 1976
Dorsum (unplucked)	—	—	4.4	4.6×10^5	—	Keech, 1982
Tail (unplucked)	33	1.7	2.70 ± 0.12†	2.24×10^5	$\sim 6 \times 10^4$	Present data

* 95% confidence limits.
† Standard error.

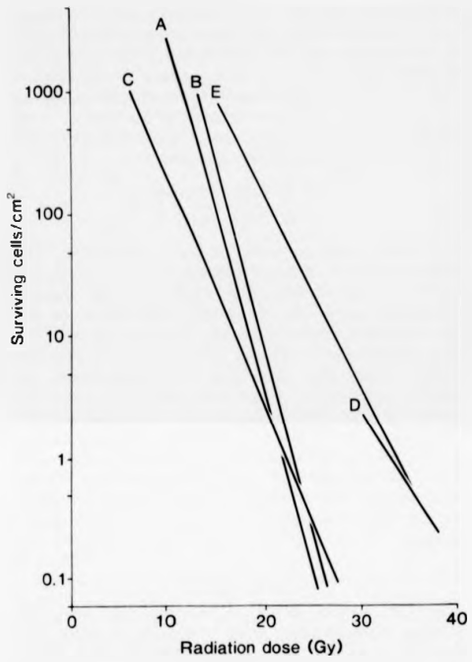

FIG. 7.

Comparison of survival curves. A, macrocolonies on dorsum (Withers, 1967b). B, macrocolonies on dorsum (Emery et al. 1970. C, microcolonies on dorsum (Al-Barwari & Potten, 1976). D, macrocolonies on tail (Hendry, 1984). E, microcolonies on tail (present data).

The comparison of microcolony and macrocolony survival curves demonstrates for the first time with epidermis that microcolonies containing more than 32 cells, *i.e.* where surviving colony-forming cells completed five divisions after irradiation, all develop into macrocolonies. Also, that in the low-dose range the macrocolony count is lower than expected because of the greater probability of several microcolonies surviving in the area covered by a single macrocolony. This accounts for the difference between the apparent sensitivity of macrocolony-forming cells, and the sensitivity of microcolony-forming cells, by a factor of $4.1/2.7 = 1.5$ (Table II).

A comparison of survival curves for colony-forming cells reported in the literature is shown in Fig. 7. Curves A, B and D refer to macrocolonies, and C and E to microcolonies. Also, curves A, B and C pertain to dorsal epidermis, and D and E to tail epidermis. The curves for tail epidermis are displaced to higher doses compared to dorsum by about 10 Gy in the high-dose range. At lower doses the curve for tail converges towards the macrocolony curves for dorsum, but not towards the microcolony curve. It should be noted that the macrocolony technique for dorsum does not involve a correction for colony coalescence as is required with the present technique, and hence the comparison of these curves remains valid. Also, very few colonies were associated with hair follicle openings in the present work, in contrast to microcolonies in dorsum (Al-Barwari & Potten, 1976) and possibly also with macrocolonies in dorsum (discussed by Potten, 1985).

Reported survival parameters for epidermal colony-forming units are shown in Table II. The reason for the higher D_0 value for colony-forming cells in tail epidermis (D_0 approx. 2.7 Gy) or microcolonies in dorsal epidermis (2.3–4.4 Gy) compared with macrocolony-forming cells in dorsal epidermis (D_0 approx. 1.35 Gy) remains unknown. The number of CFU per cm^2 is calculated by reducing the extrapolation number by the recovery factor measured in split-dose experiments (*e.g.* Potten & Hendry, 1973). This factor was 3.8 at 24 h in the present studies, being the extrapolation number on the second-dose survival curve (Fig. 4). (A factor of 4.8 was measured in Fig. 5, and if 6 h or 10 h had been chosen instead, the factor might have been respectively lower or higher by at most a factor of 2.) The factor of 3.8 is lower than the factors of 15 to 18 (Withers, 1967a) and about 70 (Emery et al. 1970) calculated using dorsum. However, because of the lesser sensitivity of tail epidermal CFU, the corresponding values of $(D_2 - D_1)$ are more similar, namely 3.6 Gy (present data), 3.75 Gy (Withers, 1967a) and 5.7 Gy (Emery et al, 1970). The present data provide a value of 6×10^4 CFU per cm^2, which is slightly lower than, but of the same order as, the values reported by Withers (1967b) and Emery et al (1970). Using a value of about 2×10^6 basal cells per cm^2 of tail epidermis (Potten et al, 1982), a fraction of about 0.0 would be clonogenic, *i.e.* CFU. This would represent a lower limit to the fraction if a radioresistant proportion of cells is being assayed. Also, a fraction of 0.03 would correspond to about five divisions of transit cells (*i.e.* $1/2^5 \sim 0.03$) in the basal layer (Potten, 1981), which would be an *upper* limit to the number of transit cell divisions if the proportion of CFU in the basal layer is truly higher.

Dose–response curves for macrocolonies are correlated with gross skin reactions (Hendry, 1984). The deduced sensitivity of target cells for epidermal healing was 2.8 ± 0.5 Gy, and this is now closer to the measured D_0 for colony-forming cells (2.7 ± 0.2 Gy) than the D_0 of 3.5 ± 0.4 Gy reported previously for macrocolony-forming units (Hendry, 1984). The close relationship described here between ~~macrocolonies~~ and microcolonies indicates that this microcolony technique can be used to study the cellular basis of epidermal responses in the tail in an accurate, quantitative way.

skin healing

The technique is now being used with epidermis in other sites.

ACKNOWLEDGMENTS

We are grateful to the Cancer Research Campaign (UK) for financial support, to the National Science Council (Taiwan) for the support of Fu-du Chen, and to Dr C. S. Potten for helpful suggestions and comments.

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Book reviews

The Ionising Radiations Regulations 1985. The Protection of Persons Against Ionising Radiation Arising from Any Work Activity. Health and Safety Commission, UK. pp. xii + 40. 1985 (HMSO, London). £5.65.
ISBN 0-11-883838-5

This EEC-stimulated document brings into operation a new regulatory system of protection in the UK, replacing the rather informal arrangements that have hitherto governed radiological safety. It considers the 35 Ionising Radiations Regulations (1985) and indicates how compliance is to be achieved by physical, engineering, procedural, administrative and educational means. Although the Code is not mandatory, failure to comply may be used as evidence in criminal proceedings.

A commendable feature is that the fundamental safety standards laid down by ICRP remain inviolate. The concept of "permissible" dose is now rightly superseded by that of dose limit, combined with a policy of keeping doses as low as reasonably achievable, or practicable. Readers should note that the Code explicitly defines those methods that should be considered "reasonably practicable". Their professional knowledge, skill and concern will, in future, need to be supplemented—not, one hopes, replaced—by the ability to digest complex technico-legal phraseology! The RPA will usually help as interpreter: the wise radiodiagnostician or radiotherapist will ensure that he is fully involved not just with safety but with the achievement of clinical goals.

Radiology is the largest man-made contributor to the collective radiation detriment—and probably to the collective benefit also. However, medicine is uniquely unamenable to control by regulation since it involves a subtle balancing of risks to the individual. The HSC acknowledges that special considerations apply to medical exposures and has made several welcome amendments following consultation. Some specific difficulties remain, for example, dose-rate is sometimes elevated to a status unjustified by radiobiological evidence. It is to be hoped that the HSC will encourage review of such problem areas.

This is an important document, which must be readily available to everyone in the UK with responsibility for the safe and beneficial use of radiation in medicine, industry, scientific research and education.

M. J. DAY

Assessment of Radioactive Contamination in Man 1984. International Atomic Energy Agency, pp. 565 + index, 1985 (IAEA, Vienna), 1140 Austrian Schillings.
ISBN 92-0-020085-0

This book contains the full texts of papers presented at an international symposium held in Paris in November 1984. The International Atomic Energy Agency is to be congratulated on the speed with which these proceedings have been published but the short time-scale has two consequences: the papers are published in the original language, only the abstracts of foreign-language papers being translated into English, and there is no record of the discussions that followed each paper.

This was the first symposium devoted to the assessment of radioactive contamination in man since one held in Stockholm as long ago as 1971. The meeting and the proceedings have, therefore, been eagerly awaited by those who are interested in new developments in techniques and in recent operational experience. They will find many papers of considerable interest. The major areas of progress are: the increasing use of high-resolution, solid-state detectors in preference to low-cost, but poor-resolution, scintillators; methods of calibration for low-energy photons in the body, including phantoms, *in-vivo* procedures and the use of cadavers; improved methods both of detecting plutonium isotopes in urine and of interpreting the results; and the impact of the recommendations of the International Commission on Radiological Protection concerning limits on intake of radionuclides on the design of monitoring programmes and the interpretation of results.

The major area of difficulty remains the evaluation of exposure to uranium and transuranic radionuclides at levels less than the accepted limits. Adequate surveillance of worker exposure can only be achieved by complementary techniques of assessing airborne levels, monitoring excreta and direct measurements of radionuclides in the body. Although many papers were addressed to these techniques in isolation (possibly because of editorial limits on length), an impressive collection of papers on the uranium industry illustrates the success that can be achieved by comprehensive investigations.

In summary, all those concerned with monitoring and assessment of internal radiation exposure will find papers of interest in this volume.

F. A. FRY

Br. J. Cancer (1986), 53, Suppl. VII, 73–74

Effects of field size on the incidence of skin healing and the survival of epidermal colony-forming cells after irradiation

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In the well-known effect of field-size in radiotherapy, it is generally considered that the level of target-cell survival is constant, but that the primary effect is modified by secondary responses e.g. of the vasculature. However, the validity of this assumption of constancy of cell survival has never been tested. The mouse-tail necrosis system demonstrates a field-size effect (Hendry, 1978), and colony techniques have been developed for tail epidermis (Hendry, 1984; Chen & Hendry, 1985). The present study was designed to measure cell survival and skin healing (lack of necrosis) after irradiation of different lengths of tail.

Materials and methods

Female B6D2F1 mice at an age of 3 months were used. Irradiations were given using 10 MeV electrons at a dose-rate of ~900 Gy min^{-1}; doses were measured by ferrous sulphate dosimetry (Hendry *et al.*, 1982). Beam flatness was confirmed using densitometry with irradiated PVC and X-ray films. The unanaethetised mice were confined in perforated perspex tubes and were positioned 5 min before irradiation to allow the tail to acclimatise to the 37°C air environment which was maintained during irradiation (Hendry *et al.*, 1982). Mice were examined every day from about day 14 to day 28. Both skin reactions and the number of macrocolonies were scored (Hendry, 1984) and most macrocolonies developed between days 20 and 23. In order to avoid possible cell migration from the unirradiated and penumbra regions, colonies only in the middle 3 cm of an irradiated 4 cm length of tail (or middle 1 cm for a 2 cm length) were counted. A recently developed microcolony technique was used (Chen & Hendry, 1985). The mice were killed and skin samples were taken between days 12 to 20. The samples were fixed and whole-mount epidermal sheets were prepared for colony counting. For experiments involving both healing and macrocolonies, 5 experiments were performed using 12 mice per point. Only one experiment was performed where microcolonies were counted. The fraction of tails healed (HF) was determined at the end of the 9th week after irradiation. The data for healing and for colonies were analysed using respectively the probit and Puck programmes described by Gilbert (1969).

Results

Data for the fraction of tails healed are shown in Figure 1. Probit analysis using a common slope for both curves gave HF (2 cm length) = 36.8 ± 0.7 Gy, and HF (4 cm) = 34.4 ± 0.7 Gy. Hence the HF doses were greater for the 2 cm length (area irradiated = 1.3 cm^2) than for the 4 cm length (area = 3.2 cm^2) by a factor of 1.07 ± 0.03.

Survival curves for macrocolony-forming cells are shown in Figure 2. The slopes of the fitted lines for each set of data were not significantly different from each other, and the common D_0 was 6.6 ± 0.4 Gy. More dose was required for the same number of colonies per unit area on the 2 cm length of tail than on the 4 cm length by a factor of ~1.10.

The microcolony data are shown in Figure 3. These data extended over a larger range of dose than the data for macrocolonies or tail healing, and in this case there was evidence for a change in sensitivity. The D_0 values were 5.3 ± 0.2 Gy (2 cm) and 4.0 ± 0.2 Gy (4 cm). The increase in dose for equivalent effect ranged between factors of about 1.01 to 1.10, with a mean of 1.06.

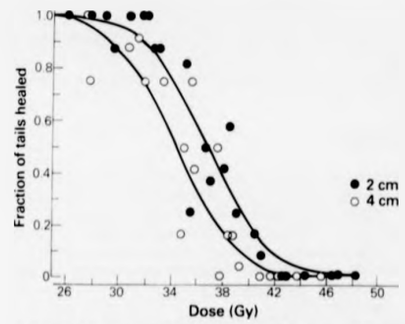

Figure 1 Fraction of tails healed (HF) *versus* dose.

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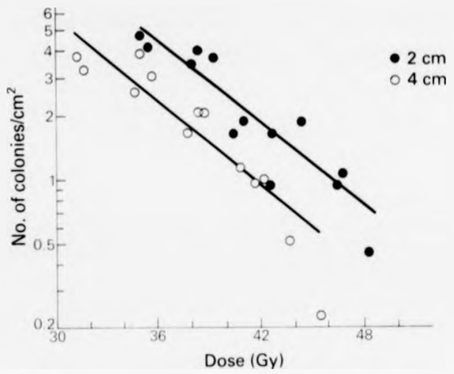

Figure 2 Number of macrocolonies cm^{-2} of epidermis *versus* dose.

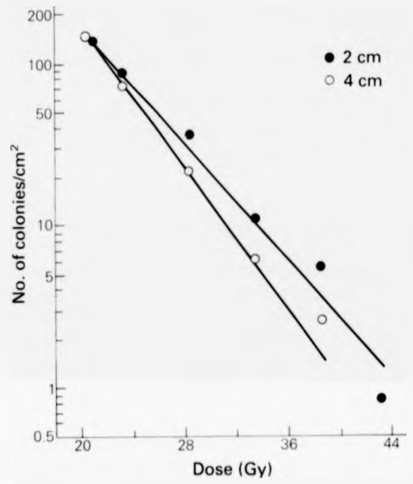

Figure 3 Number of microcolonies cm^{-2} of epidermis *versus* dose.

Discussion

The present data using all 3 assay systems demonstrate an effect of field size between areas of 1.3 and 3.2 cm^2. The change in dose for equivalent effect is between about 7 and 11% for the healing and macrocolony data and for the microcolony data in the high dose range. With the assay system capable of assessments over the largest range of dose (the microcolony technique) the sensitivity increased when the area was increased. This was not detected when the other assays were used, due to the more limited range of dose over which measurements were made, and the greater scatter in the data because of fewer colonies counted or smaller samples in the case of the healing data. D_0 values are 4.0–5.3 Gy with the microcolony technique and 6.6 Gy with macrocolonies. The higher latter value for macrocolonies is due partly to colony confluence after the lower doses (Chen & Hendry, 1985). Values of 4–5 Gy are higher than the value of 2.7 Gy reported previously for tail epidermis using the microcolony technique (Chen & Hendry, 1985), but the latter value pertains to 300 kVp X-rays and the present data pertain to 10 MeV electrons which are less efficient than X-rays.

For human skin, the field-size effect can be described by the equation:

$$\text{Dose} = k(\text{area})^{-0.16}$$ (Von Essen, 1968).

If this relationship is applied in the present work, a change in area from 1.3 to 3.2 cm^2 should change the isoeffect dose by 16%. This is greater than the observed change of 7–11%.

It is concluded that the field-size effect in this system, in terms of gross skin response, is reflected by similar changes in the survival of colony-forming cells. Also, that the effect may be caused by a difference in sensitivity of the cells.

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