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MECHANISMS OF RADIATION RESISTANCE IN CULTURED CELLS

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE FACULTY OF MEDICINE

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THIS THESIS IS DEDICATED TO MY PARENTS TO WHOM I OWE MORE THAN I COULD EVER SAY.

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CONTENTS

List of Figures

List of Tables

SUMMARY

To investigate cellular radioresistance, three approaches were adopted. These were: to examine claims in the literature that growing cells as spheroids enhances their resistance to ionising radiation; to follow reports that cells transfected with *ras* oncogenes became more radioresistant: and to isolate a radioresistant subline from a radiosensitive human neuroblastoma cell line.

It is known that for some cell lineages, culturing the cells as three-dimensional aggregates (spheroids), leads to an increase in their radioresistance, relative to monolayers, and it has been proposed that this "contact effect" is related to the presence of inter-cellular gap junctional communication in the spheroid. This claim was investigated by testing two cell lines which were shown to form gap junctions. No contact effect could be demonstrated in either case. The existence of gap junctional communication is not sufficient to confer a contact effect, though the possibility remains that it is a necessary component. The prevalence of the contact effect in human tumour cell lines was examined by means of a literature survey. Of 27 lines for which published data was available, approximately one third showed a contact effect, and these lines were, on average, relatively radiosensitive, when grown as monolayers. However, an attempt to identify more instances of the contact effect by examining human neuroblastoma cell lines failed. (Neuroblastoma lines are known to be, in general, more than usually radiosensitive.)

The effect of transfection with the oncogenes H-*ras* and c-*myc* was examined using a mink epithelial line, Mv1Lu. No alteration in radiosensitivity was observed in the transfected lines. These transfected lines were monoclonal in origin. There seemed to be little clonal variation in the parent line in terms of radiosensitivity. Five independent clones were established. Analysis of variance showed that in terms of radiosensitivity, they all belonged to the same population. This suggests that background variation in the radiosensitivity of the Mv1Lu cells is unlikely to have obscured an oncogene-mediated effect. However, analysis of several oncogene-transfected clones would be desirable to confirm this conclusion.

By subjecting a radiosensitive human neuroblastoma derived cell line, NB1-G, to a regime of fractionated X-irradiation, a radioresistant variant, XRNB1-G was obtained. Beginning with an original population of approximately 10^7 cells, selection was carried out by once or twice weekly irradiation with doses of 2 Gy. After 15 such treatments, an increase in the radioresistance of the population was noted. A monoclonal line was derived from the resistant population, (clone F) which remained resistant to ionising radiation over 40 passages, after the end of the fractionation protocol. The D_0 of the parent line was 0.7 Gy $\pm$ 0.1 (95% confidence limits); the resistant line had a D_0 of 1.1 Gy $\pm$ 0.3. Both the parent and resistant lines had almost exponential survival curves with no shoulder region and little overall curvature. Increased radioresistance was confirmed independently of colony formation by means of the spheroid growth delay

assay. Using flow cytometry, the cell cycle distributions of the lines were found to be almost identical. Studies with neutral filter elution showed no difference between the lines in the level of initial DNA double strand breaks. Double strand break repair did not differ between the cell lines.

The resistant and parent lines sustained equal amounts of chromosome damage, as judged by the micronucleus assay. However there seemed to be substantially more radiation-induced apoptosis in the parent cells. A reduced tendency to undergo apoptosis in response to radiation presumably underlies the resistant phenotype of the XRNB1-G/clone F cells. Because of the established link between p53 and radiation-induced apoptosis, the p53 status of the NB1-G and clone F lines was investigated. Most p53 mutations occur in exons 5 to 8. cDNA sequencing of that region did not identify any mutation in either line. Western analysis showed p53 protein was induced equally in both lines following radiation exposure. The difference between the resistant and parental lines seems to lie in the chain of events which leads from radiation damage to apoptotic death; however which step is altered in the resistant cells remains unknown.

1 THE RADIOBIOLOGY OF MAMMALIAN CELLS

Radiobiology is the study of how ionising radiation affects living things. At the cellular level, the most important effects of radiation are a consequence of its capacity to damage DNA. Depending on the damage induced, cells may be sterilised (i.e., rendered incapable of sustained division) or may suffer non-sterilising mutations. The ability of radiation to sterilise cells is used in the radiotherapy of cancer. Because of the importance of this form of therapy as a treatment for cancer, much work has been done on the radiobiology of cancer cells, and of critical normal tissues, with the ultimate aim of improving radiotherapy.

1.1 THE PHYSICS OF IONISING RADIATION

Ionising radiation acts by depositing energy in matter as it passes through. This energy is absorbed by atomic electrons causing them to undergo either excitation - to be raised to a more energetic quantum state - or liberating them from the atomic orbit thus leaving a free electron and a positive ion. This transfer of energy is usually quantified in units known as gray (Gy), where 1 Gy is equivalent to 1 joule absorbed per kilogram of absorber.

Ionising radiation may be either particulate - electrons, protons, neutrons and atomic nuclei such as Helium - or it may be electromagnetic radiation, i.e., X- and gamma-rays. These radiations vary in the intensity with which they produce ionisations, heavy charged particles being much more densely ionising than electromagnetic radiation. The gradient of transfer of energy to the surrounding matter by a radiation track is expressed as the Linear Energy Transfer (LET) factor, which gives the energy lost from a unit length of radiation track in keV/μm. The efficiency of cell sterilisation depends on the LET of the radiation: as the LET rises, the sterilising cytotoxicity of the radiation increases to reach a maximum at an LET value of about 200 keV/μm. Beyond this point any increase in LET leads to a reduction in the efficiency of cell sterilisation. This phenomenon is usually known as "overkill". It is explained on the basis that cell sterilisation requires several ionisations to occur within the nucleus. Tracks of sparsely ionising radiation may, on average, produce only one ionisation per nucleus. More densely ionising radiations are more efficient at cell killing, since there is a higher probability that a single track will deliver all the "hits" necessary for cell death. However when LET increases beyond a certain point, radiations may "waste" energy, if individual particle tracks generate more ionisations in the target than are required for cell death.

X- and gamma-rays belong to that portion of the spectrum of electromagnetic radiations that are sufficiently energetic to produce ionisations

in matter. X- and gamma-rays differ from each other in their origins. X-rays are produced when electrons lose energy: in radiotherapy this is achieved by accelerating a beam of electrons onto a metal target. Gamma-rays are the products of the radioactive decay of certain isotopes, for example, ^{60}Co and originate from the nucleus.

X-rays deposit energy in a number of different processes, depending on the energy of the photon. Most radiotherapy treatments work with photon or electron beams with energies in the range 1 to 10 MeV, though lower energy X-rays are also used, particularly for superficial tumours. There are three important mechanisms of energy absorption. At low energies, absorption is by the photoelectric effect. The incoming photon transfers all its energy to an electron bound in one of the inner atomic orbits. The electron is ejected, while the photon is completely absorbed. The vacancy created in the inner orbital is filled by an electron from an outer orbital that loses energy in the transition, emitting an X-ray. The freed electron is called a photoelectron. It will collide with bound electrons in its path, losing kinetic energy and causing further ionisations. In tissue the photoelectric effect is negligible for photons of energy greater than 100 keV and consequently is only relevant to radiations produced by the lowest energy treatment machines.

At higher photon energies, energy is lost through Compton scattering. Photons collide with outer orbital electrons, transferring some of their kinetic energy to the liberated electrons. The photons are deflected by the collision.

Compton scattering falls off with increasing photon energy, but only slowly. For those energies used in radiotherapy, Compton scattering represents the most significant energy transfer process.

The third process is known as pair production. At photon energies greater than 1.02 MeV, a photon can interact with the nuclear field to form an electron and a positron. The photon disappears in this process, which is an example of the interchangeability of energy and mass, as predicted by Einstein's special theory of relativity. Pair production is the dominant process at very high energies, greater than those used in clinical radiotherapy.

In all these processes the photon's energy is transferred to an electron. It is the liberated, or secondary, electrons created in matter by X-rays that are responsible for most of the ionisation produced. For this reason X- and gamma-rays are said to be indirectly ionising radiations.

These secondary electrons do not deposit their energy evenly. Much of their energy is spent producing dense clusters of ionisation in small volumes around the end of the electron track. The lower the energy of the original photon beam, the greater the proportion of the total energy that will be deposited in these track end events. It is often argued that these track ends are responsible for much if not all of the cytotoxicity of X-rays (Ward 1985). This point will be returned to later.

1.2 CELL KILLING AND CELL STERILISATION

Ionising radiation sterilises proliferative cells so that they lose their capacity for indefinite cell division. Sterilised cells may remain physically intact and may even undergo a few divisions before all mitotic activity ceases. Alternatively, they may enter mitosis, fail to complete it successfully and lyse. This is known as mitotic death. In cell culture experiments, any irradiated cell that can give rise to a colony of 50 cells or more, that is., complete between five and six divisions, is deemed to be viable. Such viability is known as clonogenic survival. In radiobiological discussion, the terms cell killing or cell lethality are often used synonymously with cell sterilisation. Though this is common practice, it should be remembered that cell sterilisation will usually precede cell death - sometimes by substantial periods of time.

However, this description of cell death is not complete, for it is now known that at least in some cell types, cell death can be an active process, initiated by the cell itself. This mode of cell death is known as apoptosis and was first described by Kerr *et al.* (1972). For general reviews see Wyllie (1992 and 1993). Apoptosis is characterised by a number of morphological and biochemical changes. Cells shrink in volume, and separate from their neighbours. The cell membrane folds in on itself to form apoptotic bodies that break free from the cell. In the nucleus there is marked chromatin condensation. An endonuclease, DNAse I, is activated that digests the DNA

of the cell. Apoptosis is important in controlling cell numbers in developing tissue. It can be induced by a variety of stimuli, for example, transforming growth factor ß1, and loss of anchorage (reviewed in Hoffman and Lieberman, 1994; Ruoshlati and Reed, 1994). In some cell types it is known to occur in response to a variety of DNA damaging agents, (Hickman, 1992) including ionising radiation. It has been suggested that apoptosis may selectively suppress mutant cells and so prevent the emergence of transformed cells (Lane, 1992). Radiation induced apoptosis has been demonstrated in normal lymphocytes (Sellins and Cohen, 1987) and gastro-intestinal crypt cells (Potten, 1977, 1992); it has also been found in human leukaemic and lymphoma cell lines (Foster *et al.*, 1992; Shinohara and Nakano, 1993; Gorczyca *et al.*, 1993) and in several murine carcinomas (Stephens *et al.*, 1993; Meyn *et al.*, 1993). The latter authors also found that those tumours that did not display radiation-induced apoptosis were the most radioresistant. However, it is only recently that apoptosis has received much attention, and the contribution it makes to the radiosensitivity of different cell types requires further research.

1.3 THE MOLECULAR MECHANISM OF MITOTIC CELL KILLING

Much work has gone into investigating how radiation insult is translated into cell kill, though most of the studies have concerned cell lines that do not undergo radiation-induced apoptosis, at least to any appreciable extent. Hence most of the evidence is related to mitotic death as opposed to apoptosis. The unique importance of DNA as a target for mitotic death was demonstrated by Munro (1970). Using ^{210}Po - a short range α emitter - in the form of a fine needle, he selectively irradiated different parts of Chinese hamster ovary (CHO) cells. Irradiation was only cytotoxic if it was delivered to the cell nucleus. Warters *et al.* (1977) selectively irradiated cell membranes and nuclei by feeding cells ^{125}I labelled concavalin A and tritiated thymidine respectively. (Both isotopes have very short range emissions, so that most of their energy is deposited at the site of isotope accumulation.) These authors found that the dose to the nucleus was critical in determining cell survival.

Ionising radiation inflicts several types of damage on DNA. It causes the formation of DNA-protein crosslinks, the modification of nitrogenous bases, the production of base free sites and single and double strand breaks (ssb and dsb). Because such a variety of lesions are produced simultaneously, it is difficult to identify those which are responsible for cell lethality. Most cells can to some extent repair each of these classes of damage. There are

mutant cell lines deficient in single strand break repair (Thompson *et al.*, 1982), double strand break repair (Kemp *et al.*, 1984), and excision repair (Fuller and Painter 1988). Excision repair corrects some types of base damage and DNA-protein crosslinks. These mutants have all been found to be more radiosensitive than their parent lines. This suggests that most forms of DNA damage may be lethal if they are unrepaired. However, it is generally agreed that for cells with normal repair capacity, dsbs are the most significant cause of cell lethality. Further evidence that dsbs are an important cause of cell lethality comes from experiments where restriction endonucleases have been artificially introduced into cells. These enzymes cut DNA at specific base sequences, producing double strand breaks. When this is done, a dose-dependent increase in cell killing is observed (Bryant, 1985). By contrast, high levels of single strand breaks can be produced by hydrogen peroxide, (equivalent to 15 Gy) without any cell killing (Ward *et al.*, 1985; Prise *et al.*, 1989a). Hydrogen peroxide is toxic to cells, but only at concentrations that produce measurable levels of double strand breaks (Prise *et al.*, 1989b). Prise *et al.* (1987) showed that compared to 250 kVp X rays, ultra-soft X rays were more cytotoxic. Ultrasoft X-rays were also more efficient in the generation of double strand breaks, but less effective in the production of single strand breaks.

There is much evidence to suggest that whatever the molecular nature of the lethal lesion, at a microscopic level mitotic cell death is usually

associated with gross chromosomal damage. Irradiated cells often show a range of aberrant chromosomes. Using Chinese hamster cells, Dewey *et al.* (1971) found the mean lethal dose for cell killing gave an average of one chromosome aberration per cell. Similar results were obtained by Prosser *et al.* (1990). By microscopically following the fate of irradiated cells as they grew in culture, Joshi *et al.* (1982) found that non-viable colonies of Syrian hamster cells almost invariably displayed micronuclei. These are chromosome fragments present in the cytoplasm of post-mitotic cells. However, they also found that the presence of micronuclei was not necessarily a lethal event, particularly after low doses. A tetraploid clone was better able to tolerate the presence of micronuclei than its diploid parent line (Revell 1983), suggesting that loss of genetic information is critical in determining a cell's fate. However a hypotetraploid sub-line was more tolerant of micronuclei than the tetraploid line (Revell, 1983), so DNA content cannot be the only factor.

Double strand breaks can be produced in cells by permeabilizing their membranes so that they take up restriction enzymes. Such treatment results in chromosome aberrations (Subrahmanyan *et al.*, 1976; Bryant, 1984; Natarajan and Obe, 1984; Obe *et al.*, 1985; Zhang and Dong, 1987). Although enzymatically produced dsbs may differ chemically from radiation produced dsbs, it is reasonable to suppose that dsbs, regardless of the mechanism of their creation, will also lead to chromosome aberrations.

Because of this work, it now seems likely that mitotic death is due to

unrepaired or incorrectly repaired double strand breaks that lead to large scale chromosome damage. However, one cannot rule out the possibility that other lesions contribute to cell killing.

The technique of introducing restriction enzymes into cells has been used by Nelson and Kastan (1994), to demonstrate that DNA double strand breaks can initiate the apoptotic pathway. However there is also some interest in the possibility that reactive oxygen intermediates may stimulate apoptosis, particularly since a major suppressor of apoptosis *bcl-2*, also regulates an anti-oxidant pathway (Korsmeyer *et al.*, 1993).

1.4 THE CELL SURVIVAL CURVE

Over the last 40 years, several theories have been developed to explain radiation survival curves in terms of lesion production and repair. Since the molecular changes that lead to cell death are even now only partly understood, these models are all to some extent speculative. However, it is well known that ionising radiation deposits energy in a random fashion: that ionisations occur through the chance collisions of photons and secondary electrons with bound electrons. In consequence, radiation-induced lesions are not produced evenly in a population of irradiated cells but conform to a Poisson distribution. From this, it follows that if a dose of radiation produces an average of n lesions per

cell in a cell population, the fraction of cells with no lesions, F_0, is:

$$F_0 = e^{-n}$$

If the number of lesions is proportional to the dose, D, and if only lesion-free cells survive, then the surviving fraction, SF, is:

$$SF = e^{-kD}$$

where k is a constant. Graphically, this is represented by plotting the log of the surviving fraction against dose. This relationship holds good for bacterial cells. For eukaryotic cells, more complex formulae are needed to describe cell survival, though these too are based on Poisson statistics.

Most mammalian cells are relatively insensitive to low doses of radiation (up to 2 Gy), but higher doses are more effective at cell killing. This leads to a survival curve that steepens with increasing dose. It is possible to represent this curve using several different equations. Of the two most commonly used, one is derived from target theory and the other is the linear-quadratic model. Both of these are discussed below. The simplest version of target theory leads to the expression:

$$SF = 1-(1-e^{-D/Do})^n$$

(Puck and Marcus, 1956) where D_o and n are constants. The linear-quadratic equation is written:

$$SF = e^{-(\alpha D + \beta D^2)}$$

where α and β are constants. Although both equations provide good fits to most cell survival data, it now seems that the linear-quadratic model is usually the more accurate (Peacock *et al.*, 1988; Holmes *et al.*, 1990; McMillan *et al.*, 1992).

Recently, it has become possible to analyze the survival curve at very low doses, where survival is only slightly less than unity. This requires an assay that can resolve very small differences in survival. The conventional clonogenic assay cannot define this region of the curve because it measures the number of colonies obtained from a starting population whose size is estimated rather than exactly known. This problem was overcome by Spadinger and Palic (1993) who devised a method of following the fate of individual cells. Using this technique, it was found that at doses below 0.5 Gy the linear-quadratic equation does not apply (Marples and Joiner, 1993; Lambin *et al.*, 1993). This region of the survival curve seems to represent high radiosensitivity. This may be due to the presence of a small radiosensitive sub-population, since radiosensitivity varies through the cell cycle (e.g., Sinclair and Morton 1966; Vos *et al.*, 1966). Alternatively, it may reflect an induced protective response, which low doses fail to activate. For a number of reasons, Marples and Joiner (1993) feel the latter explanation to be more likely, though there is no conclusive proof.

1.5 MECHANISTIC MODELS OF CELL KILLING

1.5.1 Target theory

Target theory assumes that eukaryotic cells contain several molecular targets, all of which must be hit to inactivate the cell. The identity of the targets was not initially specified, though later they were considered to be genes, of which both copies had to be hit to inactivate diploid cells.

The probability that a particular target is unaffected by a radiation dose (D) is taken to be an exponentially decreasing function of dose, viz:

$$e^{-D/Do}$$

where D_o is a constant, with the dimensions of radiation dose. The probability of the target being inactivated is:

$$1 - e^{-D/Do}.$$

The probability of inactivating all n targets in a cell is:

$$(1 - e^{-D/Do})^n$$

and this is equal to the probability of killing the cell. Therefore cell survival can be related to dose by the equation:

$$S = 1-(1-e^{-D/Do})^n$$

At high doses, this approximates to:

$$\ln(S) = -D/D_o + \ln (n)$$

so that the slope of the survival curve reaches a final value, equal to the reciprocal of D_o. The intercept of this line with the ordinate equals ln (n), and the point where it intercepts the abscissa is known as the quasi-threshold dose, or D_q. These three parameters are commonly used to describe cell survival curves. At low doses, target theory predicts that the initial slope of the curve should equal zero. Because this was soon shown to be false, the formula was modified to:

$$S = e^{-D/D1}(1-(1-e^{-D/Do})^n)$$

(Bender and Gooch, 1962). The added term implies a second process, where only one hit to some critical target, distinct from the n targets is sufficient for cell killing to occur. Target theory predicts that the extrapolation number, n, obtained from survival curves should be an integer, as it corresponds to the number of cellular targets, and that the survival curve should become linear at doses beyond the shoulder region. Integral extrapolation numbers are rare in experimental practice, though this could be explained by cell asynchrony.

An important concept in radiobiology is that of sub-lethal damage. That some lesions are not cytocidal alone, but can contribute to cell killing, either through accumulation or interaction with other lesions. Evidence for this came from the work of Elkind and Sutton (1959) who exposed cells to radiation, delivered either in single doses or as two fractions, separated by several hours. They found that splitting the treatment into two fractions significantly reduced its cytotoxicity. Those cells that survived the first dose responded to the second

as though they had not been irradiated: that is they experienced two low doses rather than one high dose. The sparing effect of dose fractionation was maximal when the doses were separated by a few hours and progressively reduced if the treatments were moved closer together in time. Target theory explains this by proposing that immediately after irradiation, some of the surviving cells will have n-1 targets inactivated, and that recovery is due to repair of these targets. The dependence of the sparing effect on time between the radiation treatments is considered to provide a measure of repair of the presumptive sub-lethal lesions.

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1.5.2 Linear-quadratic models

The linear-quadratic equation has already been mentioned. It is generally accepted as providing the best available empirical description of survival curves. However it is not definitely known why the dose-response relation for cell survival should take this form. Chadwick and Leenhouts (1973) proposed a linear-quadratic survival expression, based on the idea that lethal lesions could arise either through the passage of one track of ionising radiation, or through the interaction of two sublesions produced by two independent tracks. The one track events would lead to the linear component of the dose-response relationship, the two track events to the dose-squared component. These authors suggested that the lethal lesion is a double strand break, which can be

produced either by a single track breaking both strands of DNA or by two separate tracks producing opposing single strand breaks in a small length of DNA. However, it has subsequently been shown that at relevant doses, single strand breaks are too far apart for this mechanism to operate (Blazek *et al.*, 1989). Therefore, the Chadwick-Leenhouts interpretation is probably wrong.

Blocher and Pohlit (1982) found that while dsbs were initially produced linearly with respect to dose, the relationship between residual dsbs and dose is linear-quadratic. They explained these findings by postulating that dsb repair occurs through recombination of homologous DNA subunits. Repair would only fail when both subunits were damaged. Such a mechanism would explain why repair is not 100% successful and introduce a dependence on dose-squared into the survival equation. The linear component of the dose-response curve is assumed to be due to either irreparable damage or to events in which a single track deposits a dsb in both homologous sub-units. Recombinational repair is known to occur in yeast (Resnick and Martin, 1976; Game *et al.*, 1980; Prakash *et al.*, 1980). Rejoining of cut plasmid by HeLa cell nuclear extract has been shown to depend on the presence of an intact homologue (Lopez and Coppey, 1987) and direct evidence of recombinational repair has been found in Xenopus laevis (Sweigert and Carroll, 1990). However, Resnick and Moore (1979) found only limited recombination in CHO cells after radiation. Also using CHO cells, Godwin *et al.* (1994) found no evidence of homologous recombination in response to restriction enzyme generated breaks. Severe

combined immune deficiency (*scid*) mice are deficient in genetic recombination. They are also hypersensitive to radiation (Fulop and Phillips, 1990) due to an inability to rejoin dsbs (Biederman *et al.*, 1991). This could mean that radiation-induced dsbs are rejoined in cells of mice by genetic recombination. Alternatively, dsb repair and recombination may be different processes; the product of the *scid* locus may participate in both of them.

1.5.3 Repair-misrepair and lethal-potentially-lethal damage models

As discussed above, radiation damage leads to a range of chromosomal aberrations, some of which - translocations, dicentric chromosomes - seem to arise through incorrect rejoining of broken DNA. There are two theories that formally relate the misrepair of radiation damage to cell survival: these are the repair-misrepair (RMR) model of Tobias (1980) and the lethal-potentially-lethal damage (LPL) model of Curtis (1986). The models are very similar, the main difference being that the RMR model assumes all lesions can be repaired, while the LPL model assumes that some damage is irreparable. These models are not linear-quadratic in form, though they do approximate to the linear-quadratic formula over the relevant dose range. The simplest forms of these models are outlined below.

Both models assume that the number of lesions initially produced is

proportional to dose. These lesions can either be repaired correctly or misrepaired. Cells only have a finite time in which to complete repair: lesions that are unrepaired at the end of this time are assumed to be lethal. Correct repair is understood to mean rejoining of a dsb, so that the original chromosome is restored. The term misrepair means the joining together of fragments of two different chromosomes. The more double strand breaks a cell contains, the more likely it becomes that the cell will ligate fragments of separate chromosomes. Since the number of lesions increases with dose, the probability of misrepair also increases with dose. At some point, true repair is all but abolished. Consequently, the predicted shape of the survival curve is one that becomes progressively steeper with increasing dose, while tending towards a final gradient. This final gradient is equivalent to the rate of lesion induction - its presence implies a situation where all lesions are being misrepaired.

The rate of dsb induction has been estimated at 40-100 dsb per cell per Gy (Blocher and Pohlit, 1982; Radford and Hodgson, 1985). If dsbs are taken as the lethal lesion, then these models predict cell survival curves tending towards a final slope of -40 per Gy on a natural log-linear plot. This is clearly unrealistic. To agree with most mammalian cell survival curves, both models must assume that lesions are being induced at a rate of about one per cell per Gy. These models therefore imply that only a small proportion of dsbs are capable of causing cell death.

There is evidence to support such a suggestion. Dsbs as measured by physical assays of DNA breakage may be a heterogeneous group of lesions. If some forms of strand break are always correctly repaired, they would not be biologically relevant lesions. As mentioned earlier, because of electron track structure, ionisations are distributed unevenly through irradiated matter: highly concentrated in some places, less densely in others. Applying this to DNA, it is easy to conceive of strand breaks of different severity. Some dsbs could occur surrounded by other forms of damage that might make the whole lesion difficult or impossible to repair. The concept of these sites was proposed by Ward (1985) who named them locally multiply damaged sites (LMDS). Prise *et al.* (1989b) have shown that double strand breaks produced by hydrogen peroxide are less likely to be lethal than those produced by 250 kVp X-rays.

A different argument has been advanced by Bryant (1984), which also suggests that some dsbs may not be cytotoxic. By introducing restriction endonucleases into cells permeabilized by Sendai virus, he showed that enzymes which cut the DNA strands leaving a cohesive-ended break are much less cytotoxic than enzymes that produce blunt ended breaks. (A cohesive ended break is one where the breaks on the two strands of the DNA are not directly opposite each other. This means that between the two breaks there are a few bases complementary to those on the opposite strand.) However other workers have found that both cohesive and blunt cutting enzymes are effective in producing chromosome aberrations (Chung *et al.*, 1991). It is not possible

to say what fraction of dsbs measured in physical assays of DNA fragmentation are lethal or potentially lethal lesions, though the LPL and RMR models predict that the fraction must be very small.

1.5.4 Saturable response models

Saturable response models were first proposed by Powers (1962) and later developed by Orr *et al.* (1966), and Laurie *et al.* (1972). Like the multitarget model, these theories treat the survival curve as shouldered, rather than continuously bending. However, they can be made to approximate to a linear-quadratic form, at least over the dose range used in clonogenic assays. Cells are assumed to have limited supplies of repair enzymes, or other radioprotective molecules. With increasing dose, these compounds become depleted, and the cells become more sensitive to ionising radiation. When cells have lost all their repair capacity, the survival curve reaches a terminal value. Two of the most recent saturable-response proposals have been made by Goodhead (1986) and Radford *et al.* (1988).

Goodhead's model is concerned with DNA repair enzymes. These are assumed to bind irreversibly to damaged DNA - for every lesion repaired, one

enzyme molecule is lost. Goodhead described this as a suicide enzyme. With increasing dose, cellular repair capacity is progressively lost, till eventually no repair enzymes remain. Consequently, the rate of cell inactivation becomes equal to the rate of lesion induction and the survival curve displays a terminal slope. Against this, it must be said that there are no known examples of suicide enzymes. Repair saturation can also be considered in terms of conventional Michaelis-Menten kinetics, as proposed by Calkins (1971). Some workers have reported that the half-time for repair of radiation damage increases with increasing dose, implying a saturable repair system (Wheeler and Nelson, 1987; Reddy *et al.*, 1990), although this is not a universal finding (Blocher and Pohlit, 1982; Dikomey and Lorenzen, 1993).

Radford *et al.* (1988) proposed that the shape of the survival curve may be due to dose-dependent depletion of endogenous thiols. Such compounds reduce free radicals and so protect against indirect radiation damage. If, during irradiation, such compounds were depleted faster than they could be regenerated, this could lead to a shouldered survival curve. Although the idea seems similar to Goodhead's, there are a number of important differences. Radford's model requires that the dose response curves for survival and lesion induction should have the same shape, i.e., curvilinear. Most models have assumed that lesion numbers are linearly related to dose. Radford's own experimental work supports his hypothesis (Radford, 1985a, 1985b). Measuring dsbs by neutral filter elution, he found that the relatively insensitive low-dose

region on the survival curve was matched on the elution profile, and for both plots this region was approximately the same size. These results are impressive because Radford correlated survival curves and elution profiles for both V79 and mouse L cells irradiated with a variety of radio-modifying treatments. Using neutral filter elution, Prise (1989b) also found a curvilinear relationship between eluted DNA and dose. Similar results have been reported by Blazek *et al.* (1989). However, the shape of the elution profile depends on the assay conditions (Okayasu and Iliakis, 1989). Dsbs can also be measured by sucrose gradient centrifugation and pulsed field gel electrophoresis. Results from these experiments suggest that dsb induction is linear with respect to dose (Blocher and Pohlit, 1982; Giaccia *et al.*, 1992). Levels of single strand breaks are directly proportional to dose (Hanson and Grdina, 1987; Ono and Okada, 1974; Rydberg 1980). If radioprotectors were being depleted as Radford suggests, one would expect a curvilinear relationship for these as well as dsbs - and indeed all - forms of low LET radiation damage. Although the balance of the evidence seems to be against Radford, his theory has not been conclusively disproved.

1.5.5. Conclusions regarding survival curve models.

Although there are many competing theories, some basic ideas found in the most recent of them seem to have gained widespread acceptance. These ideas can be summarised as follows. The survival curve for eukaryotic cells is

usually linear-quadratic, at least over the dose range used in clonogenic assays. Lesions themselves are probably induced linearly with respect to dose. The curvature of the dose-response plot is probably due to interaction of lesions in a misrepair process. There is still uncertainty over several questions: for example whether all dsbs should be considered as relevant lesions or not. There is no way at present of settling these issues. However, current theories such as the LPL model clearly represent a great advance over earlier ones such as target theory.

1.6 TUMOUR RADIOBIOLOGY

The object of radiotherapy is selectively to kill malignant cells without exceeding tolerable levels of damage to critical normal tissue. The first step in achieving this is to try to ensure that the tumour receives a higher dose than adjacent normal tissue. The simplest way to do this in external beam radiotherapy is to irradiate the tumour from different angles. However even when this is done, the need to spare normal tissue still limits the dose that can be given.

External beam radiotherapy is usually given as a series of doses, most commonly 2 Gy, separated by at least twenty four hours. Dose fractionation has been employed ever since Regaud (1922) found that ram testes could not

be sterilised by single doses of radiation without causing severe skin reactions. However he found that fractionation preferentially spared the skin relative to the spermatogonia. In this way, sterilisation could be achieved with lower levels of skin damage than for single doses. This shows that the relative radiosensitivity of two tissues can be modified by radiation. The experiment stimulated clinical interest in fractionation of treatment schedules. Eventually the practice of fractionating treatments was widely adopted by clinicians and found to be superior to single doses.

In a famous paper, Withers (1975) proposed that four factors would govern the response of clonogenic cells in tumours to fractionated treatment. Known as the four Rs of radiotherapy, these were: repair, reoxygenation, repopulation and redistribution. However, these topics though important in radiotherapy, are not the subject of this thesis. For relatively recent reviews in this area see Denekamp (1986), Kallman (1988) and Fowler (1992).

One factor excluded by Withers was the radiosensitivity of tumour cells. This was because early studies on cells in culture suggested that most mammalian cell lines did not differ greatly in intrinsic radiosensitivity. This conclusion was reversed when Fertil and Malaise (1981) reviewed all the then published *in vitro* survival curve data for human tumour cell lines. They found that the radiosensitivity of cell lines was related to the type of tumour from which the line was derived. Those lines that came from clinically radioresistant types of tumour, such as melanoma, were usually more radioresistant than lines

from clinically radiosensitive tumour types - e.g., neuroblastoma. This difference in radiosensitivity could only be observed in the low-dose region of the survival curve. The original study has been updated twice (Fertil and Malaise, 1985; Malaise *et al.*, 1987). Similar reviews have been carried out by Deacon (1984) and Steel (1988). These later reviews, based on more data, largely confirmed the initial findings. Fertil and Malaise used three categories of radiocurability, while Deacon and Steel used five. Tumours which respond poorly to radiotherapy include melanomas and glioblastomas; lymphomas and neuroblastomas are typically radioresponsive. The most common tumours - squamous cell carcinomas and adenocarcinomas - fall between these two extremes. It is worth noting that Steel did not find any marked difference in the radiosensitivity of cell lines derived from the three least curable categories (Steel, 1988), the correlation being conferred by the existence of more sensitive groups. This suggests, not surprisingly, that radiosensitivity is not the only factor influencing clinical radiocurability.

The parameters that describe the low dose region are α, in the linear-quadratic model, D_q or n in the multi target model, and the mean inactivation dose, a model-free parameter. A particularly important finding was that survival at 2 Gy (SF_2) also discriminated well between resistant and sensitive lines. This is relevant since 2 Gy is the most commonly employed daily dose in radiotherapy. Ignoring hypoxia and repopulation, one would expect cell survival in tumours treated with 30 fractions to be

$$SF_{2x30} = SF_2^{30}.$$

The mean SF_2 for the most sensitive group reported by Steel (1988) was 0.19; for the most resistant it was 0.52. Applying these values to the equation above leads to final survival levels of approximately 2×10^{-22} and 3×10^{-9}, respectively. There may be 10^9 - 10^{12} clonogenic cells in a macroscopic tumour. If the same range of SF_2 values is found in human tumours, as is found *in vitro*, then clearly SF_2 will be the major determinant of radiocurability. This has been shown mathematically by Tucker and Thames (1989), using more realistic models which include repopulation and reoxygenation. Intrinsic radiosensitivity is now considered the "fifth R" of radiotherapy (Steel *et al.*, 1989a).

However, it does not follow from this that *in vitro* data suffice to describe the absolute level of cell kill produced in human tumours. Other factors such as hypoxia, redistribution and repopulation will modify tumour response. The cell cycle distribution of cells growing exponentially in vitro will very likely be different from that found in a tumour. In addition, the process of taking cells from a tumour and establishing them in long term culture may select unrepresentative clones. Nevertheless, the fact that a good agreement between *in vitro* survival data and tumour response has been found implies that the radiosensitivity of cells in culture is related to the radiosensitivity of cells in tumours.

The existence of differences in the radiosensitivity of tumour cell lines is now well established. What is less clear is the biological basis for these differences. Steel *et al.* (1989b) argued that differences in induction of radiation damage (as opposed to repair) would account for all the differences in cell survival at 2 Gy. This theory was based on two observations. Firstly, variations in radiosensitivity appear as variations in the linear-quadratic parameter, α. This parameter is often assumed to represent the rate of induction of irreparable lesions. Secondly, Peacock *et al.* (1988) had shown that radiosensitive neuroblastoma cells had a capacity for inter-fraction recovery equal to that of more resistant cells. However, studies using neutral elution to measure DNA damage in nine human tumour lines, showed that differences in lesion induction alone could not explain the differences in α found between the cell lines (McMillan *et al.*, 1990). Eguchi-Kasai *et al.* (1991) found a good correlation between residual (rather than initial) damage and survival in five mammalian cell lines. Similar results were reported by Giacca *et al.* (1992). However, de Almodovar *et al.* (1994) who studied five human tumour lines, found that initial damage correlated well with radiosensitivity. The relative importance of DNA damage and repair is still a matter of controversy and further data will be needed to resolve the issue.

As discussed above, conventional survival assays underestimate the sensitivity of some cells to low doses of radiation. This has been interpreted as evidence for cellular radioresistance being induced by doses beyond a

certain threshold. Lambin *et al.* (1994) have shown that this phenomenon is absent in a radiosensitive human colorectal carcinoma line, though present in two more resistant lines. The authors speculate that lack of an inducible response may be a general difference between sensitive and resistant cells.

Currently, there is much interest in a possible link between radiation-induced apoptosis and radiosensitivity. In radiosensitive murine tumours, irradiation produces high numbers of apoptotic cells, compared to more resistant cells (Stephens *et al.*, 1993; Meyn *et al.*, 1993). Similar results have been reported by Radford *et al.* (1994) using murine lymphoid lines. The apoptotic response is often absent in tumour cells. The connection between apoptosis, malignancy and radiosensitivity will be discussed in the next chapter.

2 MOLECULAR MECHANISMS OF RADIORESISTANCE

It is well known that different tumour types respond very differently to radiation, and it is now generally believed that a major explanation for this must lie in differences in the radiosensitivity of tumour cells themselves. However, it is not clear why such a spectrum of radiosensitivity should exist. One approach to this problem is to look for differences in DNA damage induction and repair between sensitive and resistant lines, as discussed in chapter 1. At a more fundamental level, one can argue that systematic differences in radiosensitivity between, e.g., neuroblastoma and glioma must be due either to the tumours arising through different oncogenic events, which somehow affect radiosensitivity, or to differences in the original normal tissue, which are preserved in the malignant cells. Concerning the second alternative, not enough is known about the clonogenic survival of cells from normal human tissues, to reach any firm conclusions, though Miller *et al.* (1987) have shown that thyroid epithelial cells and carcinoma cells are both similar in terms of radiosensitivity.

Studying the molecular biology of radioresistance requires cell systems where radiation response can be modified in some way and the changes compared to alterations in gene expression or protein activity. There are three

approaches to this problem that have been widely used.

1 It is sometimes possible to alter the radiation response of cells by changing their culture conditions. Growing cells as three dimensional aggregates sometimes promotes a more resistant phenotype (Durand and Sutherland, 1972).

2 Direct transfection of genes, usually oncogenes can lead to enhanced radioresistance in some cell types. This effect was first noted by Fitzgerald (1985).

3 It is also possible to select mutants of altered radiosensitivity (e.g. Jeggo *et al.*, 1982). These mutants are generally hypersensitive to ionising radiation. This approach has recently led to the isolation of a gene Ku80, (Taccioli *et al.*, 1994) whose product is directly involved in double strand break repair.

These routes to the study of cellular radiosensitivity are considered in detail below.

2.1 THE CONTACT EFFECT

The contact effect was discovered by Durand and Sutherland (1972). They found that Chinese hamster cells were more resistant to radiation when grown as small three dimensional aggregates (spheroids), than when they were grown as monolayers. This effect was not due to hypoxia in the spheroids nor to an altered cell cycle distribution. It therefore seemed that some change in the

biology of the cells occurred when they were grown as spheroids, which altered their response to ionising radiation. A contact effect has been found in various cell lines, both animal and human in origin. In some cases, the terminal portion of the multi-target survival curve is affected; in others it is the shoulder region. It is therefore possible that these are two separate effects. Dertinger and Hulser (1981), and Dertinger *et al.* (1983) found that a contact-associated increase in the D_0 was only present in cells that were electrically coupled. The effect could be abolished by inhibitors of gap-junctional communication. However, monolayer cells showed stronger coupling than cells in spheroids (Dertinger *et al.*, 1982). These workers also found an inverse correlation between strength of coupling and adenylate cyclase activity, and postulated that this might explain the contact effect. However, they did not attempt to correlate adenylate cyclase activity with the magnitude of the contact effect, which would have been a better test of their hypothesis. Using rat 9L cells, Rodriguez *et al.* (1988) have shown that what appeared as contact enhancement of D_0, was in fact due to increased repair of PLD. After trypsinisation, cells plated from spheroids took longer to re-attach to plastic than cells from monolayers, allowing more time for PLD repair. This phenomenon may explain other cases where cells from spheroids showed a greater D_0 than cells from monolayers. However, some cells, such as V79, have a contact effect that only alters the shoulder region of the survival curve, suggesting that PLD repair might not be the explanation.

In the case of V79 cells, Olive and Durand (1985) observed differences in the alkaline unwinding behaviour of monolayer and spheroid-derived DNA. They suggested that some differences in chromatin conformation, which presumably caused the altered unwinding pattern, might underlie the contact effect. The authors found that cells in V79 spheroids had lower levels of topoisomerase II activity, and speculated that this might underlie the contact effect (Olive *et al.*, 1991). These findings are inconsistent with the results of Cunningham *et al.* (1991), who found that high levels of topoisomerase II activity coincided with increased values of Dq. Similarly, the topoisomerase II inhibitor, novobiocin, has been found to display a slight radiosensitising effect on V79 cells (Utsumi *et al.*, 1990), while Warters *et al.* (1989) found that the drug decreased the radiosensitivity of HeLa cells. These contradictory results suggest that topoisomerase II may not have much influence on radiosensitivity and that the molecular basis for the contact effect is to be sought elsewhere.

2.1.1 Prevalence of the contact effect among human tumour cell lines.

Whatever its molecular basis, an interesting question regarding the contact effect is its prevalence in human tumour lines. If a contact effect commonly occurs in tumours, it could be a significant determinant of the clinical outcome of radiotherapy and one that could not be evaluated in any study of

monolayer-derived data.

To investigate this, a literature survey was carried out by the author. It was possible to find data regarding the presence or absence of a contact effect for a total of 27 human tumour cell lines. The results are summarised in the table below.

Table 2.1 The fraction of human tumour cell lines which display a contact effect.

Tumour type	Fraction of lines displaying a contact effect	References
Melanoma	3/13	West *et al.*, 1984; Rofstad, 1986: Rofstad *et al.*, 1986; Kuwashima *et al.*, 1988
Small cell lung carcinoma	2/3	West *et al.*, 1984; Duchesne *et al.*, 1986
Squamous cell carcinoma	1/2	Kwok and Sutherland, 1991
Rectocolonic adenocarcinoma	1/2	Barone *et al.*, 1980; West and Sutherland, 1987
Ovarian carcinoma	1/6	Rofstad and Sutherland, 1988
Neuroblastoma	1/1	Deacon *et al.*, 1985
Total	9/27	

In all these results, hypoxia could be excluded as a likely determinant of cell survival, either because of spheroid size (diameter < 200μm), or because the

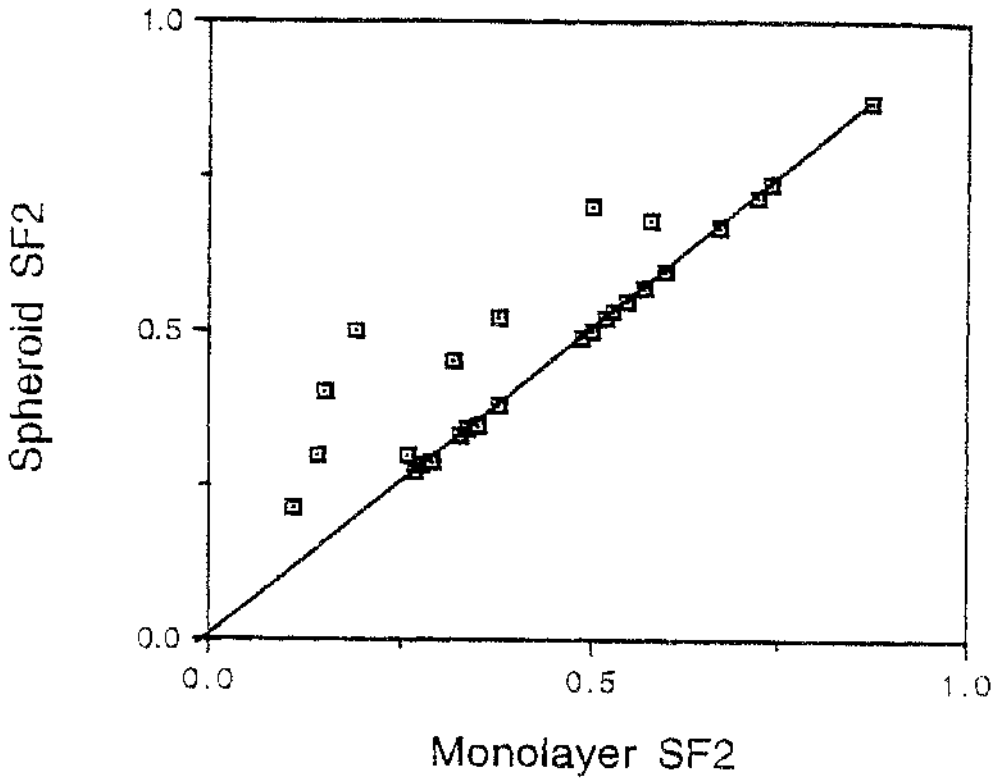
authors directly demonstrated that the spheroids were not hypoxic. The contact effect is found in only a minority of human tumour lines (9/27), and even this may be an overestimate, since there may be a tendency for workers not to publish negative data.

The contact effect may be quantified in terms of the ratio of survival at 2 Gy of cells from spheroids to cells from monolayer (or cells from spheroids disaggregated pre-irradiation). SF_2 values were obtained from the lines fitted by the authors of the publications, as the original data could not always be obtained. In cell lines displaying a contact effect, the ratio of SF_2 values ranges from 1.4 to 2.5, with a median value of 1.8. Figure 3.1 shows the relationship between monolayer and spheroid radiosensitivity expressed as SF_2. Those cell lines that display no contact effect lie on a straight line with a slope equal to 1. (The fact that they all lie exactly on the line is a consequence of the SF_2 values being obtained from published work where both monolayer and spheroid data had been fitted to one line.) Cell lines that show a contact effect lie above the line. Although the contact effect is not confined to radiosensitive cells, a striking feature of figure 2.1 is that the four most radiosensitive lines all display a contact effect. The mean monolayer SF_2 of the contact effect positive lines was 0.31, against 0.5 for the others.

A lot of importance is now attached to the *in vitro* radiosensitivity of tumour cells, as measured by monolayer survival. However, the existence of a contact effect implies that spheroid culture may be a better model for the *in*

vivo response of solid tumours to radiation. This is particularly so if the contact effect is concentrated in radiosensitive cell lines. In this case, an analysis based on monolayer survival will tend to overestimate the variation in cellular radiosensitivity.

Figure 2.1 The contact effect in human tumour cell lines.



2.2 ONCOGENES AND RADIORESISTANCE

Over the last ten years, there has been much interest in the effects of activated oncogenes on radiosensitivity. For recent reviews see Kasid *et al.* (1993) and Cassoni (1994). Several groups have studied oncogene-transfected cells, the object being to see whether oncogene transfection alters the radiobiology of the cell. Much of the work has been carried out using NIH 3T3 and rat embryo (RE) cells. However, the use of NIH 3T3 cells has been criticised by Kasid *et al.* (1989a) who found significant clonal heterogeneity in the radiobiology of the line, with 2 out of 6 clones being significantly more sensitive than the parent population. RE cells are non-immortalised primary cultures and so should constitute a better model of cell behaviour *in vivo* than NIH 3T3 cells.

2.2.1 *Ras, myc* and *raf* oncogenes

The first group to investigate the effect of oncogenes on radiosensitivity were Fitzgerald *et al.* (1985), who transfected NIH 3T3 cells with activated human N-*ras* and found a significant increase in D_o. These transfectants only showed enhanced resistance to high dose-rate radiation. A subsequent transfection generated a line that was more resistant only to low dose-rate radiation

(Fitzgerald *et al.*, 1990). Using eleven separate transfectants, Sklar (1988) also found that *ras* transfection increased the radioresistance of NIH 3T3 cells. Harris *et al.* found that *ras* transfection increased the radiosensitivity of NIH 3T3 cells (1990).

Ling and Endlich (1989) showed that rat embryo (RE) cells displayed an increased D_o when transfected with H-*ras*; this effect was slightly increased when the cells were transfected with v-*myc* as well as H-*ras*. Using the same system, McKenna *et al.* (1990) found that both oncogenes had to be transfected to produce a major effect on radiosensitivity. Compared to the parent cells, the transfected lines show an increased post-irradiation G_2 delay (McKenna *et al.*, 1991) and DNA synthesis following radiation is also reduced compared to untransfected cells (Wang and Iliakis, 1992, 1993). Studies on synchronised cell populations have shown that the normal and transfected cells are equally radiosensitive in G_1, but differ in S-phase sensitivity (Cheong *et al.*, 1993). The transfected cells are also more likely to undergo apoptosis after irradiation (Chen *et al.*, 1994), an effect that depends on *c-myc* rather than H-*ras*. Aldridge *et al.* (1995) have also found c-myc transfection to increase radiation induced apoptosis without affecting clonogenic survival. The involvement of *c-myc* in apoptosis is well established (Evan *et al.*, 1992). However, it is surprising that apoptosis can increase substantially without a parallel rise in clonogenic death. This suggests that for RE cells at least, apoptosis may only represent an alternative pathway for cells that are in any case doomed to

clonogenic death.

Using a variety of cell lines, other workers have produced conflicting results. Rat rhabdomyosarcoma cells have been shown to become more radioresistant following *ras*-transfection (Hermens and Bentvelzen, 1992). Taking micronuclei formation as their endpoint, Miller *et al.* (1993) found that *ras* transfection rendered human osteosarcoma cells more radioresistant. Alapetite *et al.* (1991) found that H-*ras* transfection into human mammary epithelial cells did not alter their radiosensitivity. H-*ras* transfection was shown by Mendonca *et al.* (1991) not to alter the radiosensitivity of a human keratinocyte line. Harris *et al.* (1990) found a slight increase in the radiosensitivity of normal rat kidney cells after transfection with a viral K-*ras* oncogene. Suzuki *et al.* (1992) found that the radiosensitivity of Syrian Hamster cells transfected with H- and Ki-*ras* was unaltered, though a clone transfected with N-*ras* was slightly more resistant than the parent line.

There are some clues as to how the *ras* product could affect radiosensitivity. *Ras* protein is located in the cell membrane, where it participates in mitogenic signal transduction pathways. (Reviewed in Barbacid, 1987; Chao. 1922; Egan and Weinberg, 1993). However, it is known that part of the mammalian cellular response to UV irradiation is to activate the transcription factor AP-1, through a *ras*-dependent pathway (Devary *et al.*, 1992). AP-1 is also activated following ionising radiation (Hallahan *et al.*, 1993; Brach *et al.*, 1993; Atherton *et al.*, 1993). This raises the question of

which genes are being transcribed via this pathway and how they affect radiosensitivity.

Other oncogenes have been found to modulate radioresistance in transfection experiments. Pirollo *et al.* (1989) found that NIH 3T3 cells transformed by c-*raf* displayed an increased radioresistance. This is consistent with the effects of *ras* on NIH 3T3 cells, since the normal *raf* protein is activated by *ras*. However, also using NIH 3T3 cells, Kasid *et al.* (1989a) found that transfection with c-*raf* failed to enhance radioresistance. It has also been shown that blocking c-*raf* expression in human squamous carcinoma cells led to a reduction in radioresistance as measured by D_o (Kasid *et al.*, 1989b). These experiments used pooled as opposed to monoclonal populations and so the results are more reliable. Against this, Warenius *et al.* (1994) studied 19 human tumour cell lines and found that the most radiosensitive cells had the highest levels of c-*raf*-1 proto-oncogene expression.

Aberrations in c-*raf* have also been linked by Pirollo *et al.* (1989) to increased radiation resistance in fibroblasts obtained from individuals suffering from Li-Fraumeni syndrome, which is a genetic predisposition to the development of certain cancers (Li and Fraumeni, 1969). Cunningham *et al.* (1991) have produced evidence that the c-*raf* product acts on the enzyme topoisomerase II and differences in topoisomerase II activity in Li-Fraumeni fibroblast lines correlate positively with differences in D_q. However, members of this family also carry a germline mutation for the tumour suppressor gene,

p53 (Srivastava *et al.*, 1990) and it is now clear that p53 is intimately involved in the cellular response to ionising radiation. This involvement is further considered below.

2.2.2 The p53 tumour suppressor gene

p53 is a tumour suppressor gene that co-ordinates the cellular response to genetic damage (Lane, 1992). Transgenic mice lacking p53 develop normally but are prone to spontaneous tumours (Donehower *et al.*, 1992). p53 has been shown to suppress gene amplification (Livingstone *et al.*, 1992; Yin *et al.*, 1992), and to be toxic to tumour cells carrying a variety of genetic lesions (Takahashi *et al.*, 1992). Tumour cells transfected with wild-type p53 undergo apoptosis (Yonish-Rouach *et al.*, 1991). Loss of p53 function is frequently observed in tumour cells. In carcinomas, mutations in the p53 gene are common (Bartek *et al.*, 1991); sarcomas often show amplification of the mdm2 gene whose product inactivates p53 protein (Oliner *et al.*, 1992).

p53 protein is normally present in cells at low levels, but expression is significantly increased following irradiation. p53 product is a transcription factor, which leads to the activation of several genes involved in G_1 arrest (Kastan *et al.*, 1991). It also suppresses transcription of protein(s) involved in inhibiting apoptosis (Shen and Shenk, 1994; Caelles *et al.*, 1994). Thymocytes enter apoptosis in response to glucocorticoid treatment (Wyllie, 1980); this

behaviour does not require p53 (Lotem and Sachs, 1993). Radiation can also invoke apoptosis in the absence of p53 (Strasser *et al.*, 1994), suggesting that p53 is not the only element controlling programmed cell death.

Since activation of p53 both enhances cellular repair, and initiates a cell death pathway, the gene seems to exert conflicting influences on clonogenic survival, though increased radiation-induced apoptosis might not always affect clonogenic survival (Chen *et al.*, 1994). Ataxia telangiectasia cells, which are unusually radiosensitive, fail to induce p53 following ionising radiation and lack a G_1 delay (Kastan *et al.*, 1992). However, the AT gene may have other targets besides p53, since several cell cycle checkpoints seem to be lost in AT (Beamish and Lavin, 1994). In contrast, most other work suggests that loss of p53 function increases radioresistance. Su and Little (1992) have shown that inactivation of p53 in human diploid fibroblasts results in an increased D_o. Bristow *et al.*, (1994) showed that transfection with a dominantly-acting p53 mutant conferred radioresistance on a RE fibroblast line. However, these cells do not normally show much radiation-induced apoptosis (Chen *et al.*, 1994), so abolition of apoptosis is unlikely to account for their findings. Slichenmyer *et al.* (1993) found that p53 status did not affect fibroblast radiosensitivity. Other normal cells, including those from the crypt of the gastro-intestinal tract and the haematopoietic system, are more prone to radiation-induced apoptosis. In p53 null or mutant cells, this response is lost (Lee and Bernstein, 1993; Lotem and Sachs, 1993; Merritt *et al.*, 1994) or modified (Strasser *et al.*,

1994). Transfecting tumour cells with wild-type p53 is known to trigger apoptosis (Yonish-Rouach *et al.*, 1991, Liu *et al.*, 1994). Cell suicide seems to arise from a conflict between the p53 mediated growth arrest signals and the transformed cells' constitutively active mitogenic pathways (Morgenbesser *et al.*, 1994; Hermeking and Eick, 1994).

Although p53 function is lost in many of the most common human cancers, there are several malignancies - such as leukaemias, Wilms tumour, and neuroblastoma (Imamura *et al.*, 1993; Malkin *et al.*, 1994; Vogan *et al.*, 1994) - in which p53 aberrations are uncommon. These tumours tend to be highly responsive to cytotoxic therapy. If these tumours have retained normal p53 function, this may account for their radiosensitivity.

2.3 ISOLATION OF MUTANTS WITH ALTERED RADIOSENSITIVITY

The most direct way to investigate cellular response to ionising radiation is to isolate mutant cell lines that differ from their parent lines in their radiosensitivity. If the techniques of molecular biology can be applied to such lines, this should provide a direct and powerful method of identifying which gene products are important in determining radiosensitivity.

There are now several such sublines in existence, the most studied of

which are the xrs lines, derived by Jeggo *et al.* from CHO cells (1982). These cells are more radiosensitive than the parent line, due to decreased ability to repair dsbs (Kemp *et al.* 1984). Other radiosensitive mutant lines have been identified that are defective in single strand break repair (Thomson *et al.*, 1982). For these lines, the defective genes have been identified and cloned (Thompson *et al.*, 1992; Taccioli *et al.*, 1994). Thacker and Ganesh (1990) isolated an AT-like mutant that was unable to halt temporarily DNA synthesis after irradiation. These lines are all rodent in origin. Powell *et al.* (1992) have derived a mutant from a human bladder carcinoma line that is prone to inaccurate rejoining of strand breaks.

Apart from artificially induced mutants, a number of tumour cell populations have been shown to contain clones of differing radiosensitivity (Alexander, 1961; Leith *et al.*, 1982; Jenkins *et al.*, 1986; Spang-Thomsen *et al.*, 1986; Yang 1992). Where such radioresistant variants are present, it should be possible to select them by irradiating the total population. Alexander (1961) showed that the radiosensitivity of murine lymphoma L5178Y cells changed in culture, with faster-dividing radiosensitive cells overgrowing the more resistant clone that had initially dominated the population. Courtenay (1969) showed that it was possible to recover these radioresistant cells by exposing the population to continuous low dose-rate ß radiation. The differences in radiosensitivity between the two sub-lines are believed to be due to differences in dsb repair (Wlodek and Hittelman, 1988).

Comparing radioresistant and sensitive cells (e.g., gliomas and lymphomas) suggests that radioresistance is a relative phenomenon. That is, there are no known examples of cells that are totally immune to radiation killing. This is in contrast to some forms of drug resistance, where drugs can be completely exported from the cell without any DNA damage occurring (Riordan *et al.*, 1985). Using ionising radiation as a single-step selective agent requires doses large enough to sterilise all the radiosensitive cells in the culture while leaving some of the more resistant population viable. However, if a resistant clone makes up a small fraction of the total population, the possibility exists that any dose large enough to remove the radiosensitive majority might also eradicate the resistant clone. (This is because the probability of a given dose sterilising a population depends not only on the radiosensitivity of the constituent cells but also on the number present.) To minimise this problem it is expedient to repeatedly expose a cell population to a relatively low dose of radiation, the irradiations being separated by sufficient time to allow the surviving cells to repopulate the culture. In this way the resistant fraction - if such cells are present - will increase after each irradiation. This is essentially the principle that Courtenay used in selecting the radioresistant lymphoma cell line, as described above. In the present project, this procedure was applied to a culture of human neuroblastoma cells that were subjected to repeated fractions of 2 Gy X-irradiation.

2.4 OBJECTS OF THIS THESIS

1 To test the hypothesis that the contact effect is due to gap junctional communication.

2 To look for human tumour cell lines displaying a contact effect. In the light of the possible link between radiosensitive lines and the contact effect, it was decided to concentrate on neuroblastoma lines, which are generally radiosensitive.

3 To test the claim that transfection with activated *ras* leads to increased radioresistance, and to examine the effect of c-*myc* transfection on radiosensitivity.

4 To attempt to select for increased radioresistance in a human neuroblastoma cell line by repeated irradiation.

5 Assuming that it was possible to modify cellular radiation response either through spheroid culture, oncogene transfection or Darwinian selection, to investigate the underlying mechanisms, particularly DNA damage and repair.

3 MATERIALS AND METHODS

3.1 CELL LINES

The cell lines used in this study were: a Chinese hamster lung line DON (Hsu and Zenzes, 1964); a mink lung epithelial line Mv1Lu (American tissue culture collection, certified cell line 64; Henderson *et al.*, 1974) and two sublines, MLMC and HO6T1, which had been transfected with the c-*myc* and activated H-*ras* oncogenes respectively, (Khan *et al.*, 1991); the human leukaemic line HL60 (Collins *et al.*, 1977), the human squamous cell carcinoma line SCC4 (Burns *et al.*, 1993) and the human neuroblastoma lines NB1-G, (Carachi *et al.*, 1987), IMR 32 (Tumilowicz *et al.*, 1970), and NB 100 (Yuhas *et al.*, 1984). The DON line was a gift from Dr J D Pitts (Beatson Institute). Mv1Lu and its transfected sublines were supplied by Professor D J Kerr (Birmingham University). SCC4 was a gift from Dr E.K. Parkinson (Beatson Institute) All cell lines, except for IMR 32 and HL60 were maintained in Eagle's Minimum Essential Medium (MEM), with 10% foetal calf serum, L-glutamine (2 mM), penicillin/streptomycin (100 I.U./ml) and amphotericin B (2.5 µg/ml). IMR 32 was cultured in a 50:50 mix of MEM and Ham's F-12, and HL60 in RPMI 1640, both with supplements as described above.

Medium with serum and supplements will be referred to as growth medium. All media ingredients were supplied by GIBCO, (Paisley, Scotland). Cells were grown in Nunc tissue culture flasks (Paisley, Scotland) in humidified incubators at 5% CO_2 and 37°C. Except for the HL60 line, cells were sub-cultured once a week using 0.5% trypsin (w/v), 0.2% ethylene diamine tetra-acetic acid (EDTA) (w/v) in Earle's balanced salt solution (GIBCO). HL60 cells grow in suspension and the culture density was kept in the range 5 x 10^5 - 1.5 x 10^6 cells/ml. For spheroid growth, cells were seeded into spinner flasks (all neuroblastoma lines) or tissue culture flasks which had been base-coated with 1% (w/v) agar (DON cells). The laboratory carries out routine screening of all cell cultures for mycoplasma. None of the data presented here comes from mycoplasma contaminated cells. Unless stated, cells were never kept in continuous culture for more than 3 months, after which time fresh cells were established in culture from frozen stock. Cells were frozen down in cryogenic ampoules (Nunc, Paisley) at a concentration of 10^6 cells/ml in medium containing 10% (v/v) dimethylsulphoxide (DMSO, BDH, Poole) and 20% (v/v) FCS. Ampoules, each containing one ml of cell suspension, were first frozen at -85°C for 4 hours before being transferred to a liquid nitrogen store. To defrost cells, ampoules were removed from liquid nitrogen and immersed in warm water. The cells were removed from DMSO as quickly as possible. In the case of the neuroblastoma lines, this was achieved by adding the cells to 20 ml of growth medium and centrifuging at

200g for 5 minutes. The supernatant was removed, the cell pellet resuspended and the cells transferred to 75 cm^2 flasks. For the mink and chinese hamster lines, which attached quite quickly to the plastic flasks, the ampoule contents were transferred directly into medium-containing flasks. After 2 hours, when the cells had attached, the medium was changed.

3.2 IRRADIATION

Most irradiations were performed using a ^{60}Co source (Thomson, Versailles, France), at a dose rate of approximately 1 Gy/min. However, as neutral filter elution required relatively high doses cells, were irradiated at a dose rate of approximately 2.7 Gy/min. The data shown in figures 4.1, 4.4, 4.11, and 4.15 to 4.18 were obtained by irradiating cells on a Siemens stabiliplan X-ray unit, (300 kV, 1 mm Cu filtration). The dose rate was approximately 1 Gy / minute.

3.3 CLONOGENIC ASSAY

Clonogenic assays on cells growing in monolayer were performed as follows: cells were seeded 3 to 4 days in advance, to ensure they were growing exponentially at the time of irradiation. They were then transferred to 25 cm^2

flasks and irradiated in suspension. Cell numbers were chosen to give approximately 100 colonies in each flask. All of the lines required a homologous feeder layer (irradiated to 50 Gy) to produce optimal plating efficiencies. For DON cells, feeder cells were added at 5×10^4 per flask; for the Mv1Lu line and its transfected derivatives, the concentration was 5×10^5 cells per flask. For NB1-G the concentration was 10^6 feeder cells per flask and for IMR 32 and NB100, 2×10^5 feeder cells per flask. DON cells required 7 - 10 days to form colonies; the other lines needed 10 - 14 days. Plates were prepared in duplicate for each dose point except for the controls which were prepared in triplicate. The cells were stained by carbol fuschin (Lamb, London) after removing the medium. Colonies were counted microscopically, using the standard definition that a colony consists of 50 or more cells.

For clonogenic assays of cells grown as spheroids, spheroids were irradiated prior to disaggregation. For these studies, it was important to ensure minimal variation in spheroid size. The neuroblastoma lines all produced spheroid cultures with little variation in spheroid size. The DON cells required filtering through nylon mesh (John Stannier, Manchester), to select spheroids with diameters in the range 100 - 200 μm. Before irradiation, spheroid size was measured using 24 randomly selected spheroids and an image analysis system (see section 3.4). All survival curves are described both in terms of the linear-quadratic and the single hit multi-target models.

Data were fitted to both linear-quadratic and multi-target models using

a programme provided by Professor Gordon Steel, Institute for Cancer Research, Royal Marsden Hospital, Sutton, Surrey, which calculates non-linear least squares fits to experimental data. Analysis of variance tests were performed using SSPS software (SSPS Inc.Chicago, IL).

3.4 SPHEROID GROWTH DELAY

After irradiation, individual spheroids were selected using a microscope and micropipette and placed into single wells of 24 well plates. The wells contained 0.5 ml growth medium and were base-coated with 1% (w/v) agar (Difco, Detroit) which was made up in growth medium. This treatment prevented the attachment of spheroids to the bottom of the wells. The cross-sectional area of each spheroid was measured using a microscope linked to an image analyzer (Analytical Instruments, Cambridge). Spheroids were measured two or three times per week; 0.5 ml of fresh medium was added every 7 days. One 24 well plate was used for each dose-point. Using a computer programme, (Dr G.M. Ford, Department of Clinical Physics and Bio-engineering, Glasgow) each measured area was converted into volume on the assumption that the spheroids were perfect spheres. Spheroid growth delay is presented in terms of specific growth delay, (Steel 1984). This expresses growth delay in terms of the number of additional doublings which treated

spheroids must undergo to reach the same volume as untreated controls. It can be defined as:

$$\text{Specific Growth Delay} = (T_{10,\text{treated}} - T_{10,\text{control}})/ T_{3,\text{control}}$$

where $T_{10,\text{treated}}$ and $T_{10,\text{control}}$ are the times taken by treated and untreated spheroids to reach 10 times their original volume and $T_{2,\text{control}}$ is the volume doubling time of the controls. Median values for these three parameters were obtained from the above-mentioned computer programme. Spheroids which failed to regrow were included in the analysis, provided they constituted less than 50% of the sample. These cured spheroids were assigned a T_{10} of 1000 days, an arbitrary value which did not influence the position of the median. Dose groups showing more than 50% cure cannot be assigned a growth delay. Growth delay data are presented for doses below this level.

3.5 SELECTION OF A RADIORESISTANT CELL LINE

Two subconfluent 75 cm^2 flasks of NB1-G cells were exposed to repeated fractions of 2 Gy X-irradiation. The total number of cells present in the flasks at the time of the first irradiation was approximately 10^7. A time interval of between 5 and 7 days was allowed between each fraction to enable the surviving cells to regenerate. In this way the cell number present at each

irradiation was kept roughly constant. Fresh medium was added at the time of irradiation. However the normal practice of subculturing was suspended and irradiation was the only check on cell growth. After every five treatments, the contents of the two flasks were pooled and a small fraction was transferred to new flasks for clonogenic assays and to establish frozen stocks.

3.6 NEUTRAL FILTER ELUTION

DNA strand breaks were measured by neutral filter elution (Bradley and Kohn, 1979). 75 cm^2 flasks containing cells in exponential growth were labelled by adding 37 kBq tritiated thymidine (specific activity, 2.6-3.1 TBq/mmol, Amersham International, Aylesbury, Bucks) in 10 ml fresh growth medium. After 24 hours, labelled cells were washed four times in 10 ml phosphate buffered saline (PBS) and resuspended in growth medium to a final concentration of 5 x 10^5 cells/ml. After irradiation, 1 ml of cell suspension was loaded on to each polycarbonate filter. Filters were supplied by Nucleopore (High Wycombe, Bucks) and had a pore diameter of 2 μm and an external diameter of 25 mm. The filters were held in Swinnex filter holders (Millipore, Molsheim, France) and cells were loaded via 10 ml syringe barrels. The cells were washed twice with 10 ml PBS by allowing the wash to run through the

filters under gravity. The flow of liquid through the filter holders was controlled by clamped tubing, fitted to the filter holders via hypodermic needles with their sharp end sawn off. Cell irradiation was carried out on ice; the loading and washing was done in a cold room (4°C) with pre-chilled PBS. In DNA repair studies, cells were held at 37°C between irradiation and loading.

Cells were lysed by incubating the filters in an oven at 60°C for 1 hour as recommended by Okayasu and Iliakis (1989). Lysis buffer consisted of tris(hydroxymethyl) methylamine, 20 mM, pH 7.2; glycine, 50 mM; EDTA (disodium salt), 25mM; 2 % (v/v) Sarkosyl and 0.5 mg/ml proteinase K. (All chemicals were supplied by BDH, Poole, Dorset, except for proteinase K which was obtained from Sigma.)

After lysis, 40 ml of eluting buffer was pumped through each filter using a multichannel peristaltic pump (Watson and Marlow, Falmouth, Cornwall) at a flow rate of 2 ml/hr. Eluting buffer was identical to lysis solution but for the absence of proteinase K. When elution was completed, the filters were placed in scintillation vials with 0.4 ml HCl (1M) and heated for 1 hour at 60°C. 2.4 ml NaOH (0.4M) was then added, the vials were mixed vigorously before the addition of 15 ml Ecoscint scintillation fluid (National Diagnostics, New Jersey). The radioactivity in the eluate was measured in 2 aliquots of 2.8 ml from each sample and adding to 15 ml Ecoscint. In each experiment, each data point was determined in duplicate. Beta-radiation counting was performed using a Packard Tri-carb 1600 scintillation counter.

3.7 FLOW CYTOMETRY

Cells were seeded as for the clonogenic assay (section 3.3), and grown for 2 to 3 days growth. They were then trypsinised, rinsed well in PBS and fixed in 70% ethanol. Before analysis, the cells were removed from ethanol by centrifugation at 200g for 5 minutes and resuspended in a solution containing 1.6 ml PBS, 0.2 ml propidium iodide, 400 μg/ml, (Sigma) and 0.2 ml RNAse, 1mg/ml, (Sigma). After incubation for 30 minutes at 37°C, the cells were again pelleted by centrifugation and resuspended in PBS. The cells were analyzed using a Coulter Epics Profile II flow cytometer (Coulter Electronics, Hialeah, FA), which has a 15 mW argon laser emitting at 488 nm. 10^4 cells were analyzed using a peak versus area fluorescence histogram to gate out debris and cell clumps. Cell cycle distribution was analyzed offline using Multicycle 2.5 software (Phoenix Flowsystems, San Diego. CA). This work was carried out in collaboration with Dr Peter Stanton, (Department of Surgery, Glasgow Royal Infirmary).

3.8 MICRO-INJECTION

The technique of iontophoretic microinjection has been described by Pitts and Kam (1985). Cells were injected with an aqueous solution of 4% w/v lucifer yellow (Sigma), a fluorescent stain capable of passing from cell to cell if gap junctional communication is present. Injections were carried out using spheroid and monolayer cultures. For monolayer injections, cells were grown on glass coverslips for 2 days prior to injection. Glass capillary tubes (outside diameter 1.0 mm, Clark Electromedical Instruments, Reading U.K.) were used to form micropipettes which were back filled with lucifer yellow. The micropipette was mounted on a micromanipulator with manual and electronic control. A platinum electrode was inserted into the micropipette, and a second electrode was dipped into the culture medium. Microinjection was monitored through a Leitz inverted microscope with UV epi-illumination. When the needle tip had entered the cell, the dye was injected by passing hyperpolarising current pulses through the electrode. Pulses were of 500ms duration, frequency 1 Hz and amplitude 10 nA. Each injection lasted 1 minute.

3.9 CHROMOSOME SPREADS

Chromosome spreads were prepared according to Freshney (1987). Cells were seeded into 175 cm^2 flasks. When they reached semi-confluence they were treated for 1 hour with demecolcine (Sigma) at a final concentration of 100 nM. The cells were then centrifuged at 200g for 5 minutes and resuspended in a hypotonic salt solution (KCl, 0.04M; Na citrate, 0.025M). Cells were incubated for 25 minutes at 37°C, after which an equivalent volume of fresh ice-cold acetic acid: methanol (1:3) was added. The suspension was vortexed while the fixative was added. The cells were spun down as before, and resuspended in fresh acetic acid: methanol. Cells were stored on ice for 10 minutes, pelleted for the final time and resuspended in 0.2 ml acetic methanol. The cell suspension was then dropped onto pre-chilled glass slides, which were then dried on a hotplate and either stained with Giemsa or set aside for trypsin-Giemsa banding.

Banding was performed as follows. Slides were rehydrated by standing overnight in SSC (NaCl, 0.15M; Na citrate 0.015M) and transferred to saline for 10 minutes. They were then sequentially immersed in 50%, 95% and 100% ethanol for 1 minute, after which they were allowed to air dry. The slides were treated with trypsin (0.1% w/v in saline) for 10 minutes, washed in saline and stained with Giemsa (BDH) diluted to a 20% v/v solution in 0.025M phosphate buffer, pH 6.8. Slides were washed in tap water, allowed to dry, and immersed

in xylene. The cells were mounted in DPX (BDH) and photographed at the Duncan Guthrie Institute of Medical Genetics, Royal Hospital for Sick Children, Glasgow. Cytogenetic analysis was carried out by Mr William Baird of the Institute.

3.10 FLUORESCENCE IN SITU HYBRIDISATION (FISH)

Chromosome spreads were prepared as described above, and fixed for one hour in acetic acid: methanol. Slides were rinsed twice in 2 x SSC and incubated in 2 x SSC with 100μg/ml RNAse A (Sigma) for one hour at 37°C. Slides were rinsed as before, and incubated with pepsin (Sigma) 0.01% w/v in 0.01M HCl, at 37°C for 10 minutes.Again, the slides were rinsed in PBS and fixed in 1% (v/v) paraformaldehyde in PBS for 10 minutes. Finally, the slides were rinsed in PBS, dehydrated in 70% (v/v) and 100% ethanol, and allowed to air dry.

The probes used were biotinylated pNb101 which hybridises to N-myc (generously supplied by Dr Manfred Schwab, German Cancer Research Centre, Heidelberg, and prepared by Anne Livingstone, CRC Beatson Laboratories) and dioxigenin (DIG) labelled α satellite 1, which hybridises to the chromosome 1 centromere (Oncor, Boltoph Claydon, Bucks). Probes were applied in a hybridisation mixture of: formamide 60% (v/v), dextran sulphate 7.5% (w/v), salmon sperm DNA (Sigma) 0.5 mg/ml, human Cot 1 DNA (0.8

mg/ml) and 2 x SSC. Probes were heated to 70°C for five minutes, and either incubated at 37°C for twenty minutes (N-myc) or held on ice for 2 minutes (chromosome 1) before application. Prior to probing, the chromosomes were denatured by immersing slides in 70% (v/v) formamide, 2 x SSC, at 70°C for two minutes, followed by rinsing in ice-cold ethanol (70%, v/v) and dehydration as before. The probe was applied followed by a coverslip, sealed with cowgum. Hybridisation to probes occurred overnight at 37°C in a humidified chamber.

Coverslips were removed using 2 x SSC, and the slides were washed twice in 50% formamide (v/v), 1 x SSC at 42°C for ten minutes. The slides were rinsed in 4 x SSC, 0.05% (v/v) Tween 20 and blocked in 4 x SSC, 0.05% Tween 20, 0.5% (v/v) block solution (Boeringher Mannheim, Lewes, Sussex) for ten minutes at room temperature. The slides were co-exposed to (a) fluorescein isothiocyanate (FITC) labelled sheep anti-DIG and FITC-avidin (b) rabbit anti-sheep and biotinylated anti-avidin and (c) FITC labelled goat anti-rabbit and FITC-avidin. Each exposure was for 45 minutes at room temperature, with intervening ten minute washes in 0.1M phosphate buffer (pH 8.0). DIG detection reagents were supplied by Oncor and used as recommended by the manufacturer. Biotin detection reagents were supplied by Vector Laboratories (Peterborough) and were used at final concentrations of 5μg/ml. After probing, the slides were counterstained in propidium iodide (400 μg/ml) for 20 minutes, dehydrated as before, mounted in fluorescent mounting

medium (Vector Laboratories) and examined on a confocal microscope (BioRad, Hemel Hempstead).

3.11 ASSAYS OF MICRONUCLEUS FORMATION AND APOPTOSIS

The micronucleus assay was performed using cytochalasin B, as described by Fenech and Morley (1985). Cells were seeded into 25 cm^2 square flasks at 10^5 cells per flask and allowed two days growth, after which they were irradiated and exposed to a 3μg/ml solution of cytochalasin B (Sigma) in growth medium. After 24 hours the cells were suspended in the medium by shaking the flasks. Cells were centrifuged (200g, 5 minutes) and resuspended in PBS. They were then fixed in acetic acid: methanol (1:3 v/v), spread on slides and stained in acridine orange (6μg/ml in PBS, Sigma) for five minutes. Slides were washed in tap water, dried and mounted in distilled water. Micronuclei were counted on a UV microscope at 100 x magnification. Following the conventions of Bush and McMillan (1993), micronuclei were defined as possessing each of three essential features:

(1) distinct from the main nuclei

(2) clearly smaller than the nuclei

(3) same staining density as the main nucleus.

At the time of irradiation the flasks were coded using randomly generated six

digit numbers, and this coding was only broken after counting was complete. For each dose point, at least 500 cells were counted over three independent experiments.

To count apoptotic cells after irradiation, cells were fixed and stained as described above. Apoptotic cells were recognised on the basis of chromatin condensation and fragmentation of the nucleus into separate regions. These features have been demonstrated in published photographs of apoptosis (Barry *et al.*, 1993). Additionally, HL60 cells treated with camptothecin were used as positive controls (Solary *et al.*, 1993). Again, a six digit coding system was used. Data points represent at least 800 cells counted over 3 independent experiments. To compare the apoptotic fractions at each time point, statistical analysis of the data was performed using Pearson chi-squared tests (BMDP software Los Angeles, CA),.

3.12 IMMUNOCYTOCHEMISTRY

Immunostaining was performed using the anti-p53 antibody, PAb1801 (Ab-2, Oncogene Science, Cambridge) and the Vectastain ABC kit (Vector Laboratories) in accordance with the manufacturer's instructions. Cells were seeded into 8 well chamber slides (Nunc, Paisley) at 10^4 cells per well and grown to near-confluence. The medium was removed and the cells were fixed

in ice-cold methanol with 0.3% (v/v) hydrogen peroxide for 30 minutes. The slides were air-dried and treated with 0.1 ml blocking serum (horse) per well for 25 minutes. This was replaced with an equal volume of primary antibody (1μg/ml) in bovine serum albumin/PBS (0.1 % w/v) for 1 hour. The slides were placed in washing buffer (NaCl 0.9% (w/v) / Tween 20 0.05% (v/v) / PBS) for 30 minutes, with three changes of buffer. Biotinylated secondary antibody (horse anti-mouse IgG) was used at 7μg/ml in BSA/PBS. Wells were exposed to 0.1 ml secondary antibody for 1 hour, and washed as above. The slides were exposed to an avidin-biotinylated-horseradish peroxidase complex (ABC reagent, Vector Laboratories) for 1 hour, washed as before, and treated with hydrogen peroxide/diaminobenzidine/ NiCl (Vector Laboratories) for 7.5 minutes. Slides were washed in tap water, counterstained in methyl green (2% (w/v) in 0.1M Na acetate/acetic acid buffer, pH 4.2) for two minutes, washed again, dehydrated and mounted in DPX. All incubations were performed at room temperature. Control cells which were not exposed to primary antibody were included in all experiments.

3.13 SDS POLYACRLYAMIDE GEL ELECTROPHORESIS

SDS polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970). Exponentially growing cells were suspended in PBS, centrifuged and the pellet washed carefully with PBS. The cells were suspended in 100µl PBS, from which 2 to 10µl was removed for protein estimation using Biorad reagents (Biorad, Hemel Hempstead, Herts.). Protein concentration was obtained by measuring absorbance at 590nm on a spectrophotometer and comparing the results to a standard curve produced with known quantities of bovine serum albumin (Sigma). For each sample, the aliquot volume was adjusted to give an absorbance in the range 0.6 to 0.8, bracketing the midpoint of the standard curve. The cell suspensions were added to equal volumes of 2 x sample buffer (0.125 M Tris base, pH 6.8, 4% (w/v) sodium dodedecylsulphate, (SDS) 10% (v/v) glycerol, 4% (v/v) ß-mercaptoethanol, 0.02% (w/v) bromophenol blue), boiled for 5 minutes and loaded on to polyacrylamide gels. Size markers (Amersham International, Bucks.) were also prepared by boiling in loading buffer.

Polyacrylamide gels were prepared from stock 30% (w/v) acrylamide: bisacrylamide (29:1) solution (Severn Biochemicals, U.K.), which was diluted to 10% in gel buffer. The final concentration of buffer was: Tris base, 0.375 M, pH 8.8, SDS 0.1% (w/v), with ammonium persulphate and TEMED (final concentrations 0.017% (w/v), 0.05% (v/v)). The gel was cast vertically

between two glass plates. Gel dimensions were 16 x 16 cm. A "stacking gel" was cast over the main gel. This consisted of 3% (w/v) polyacrylamide, Tris base 0.125 M, pH 6.8, SDS, ammonium persulphate and TEMED as above. Electrophoresis was continued until the bromophenol blue left the gel. The tank buffer consisted of Tris base 0.025 M; glycine 0.2 M, SDS 0.1% (w/v), pH 8.3.

3.14 WESTERN BLOTTING

Western blotting was performed on proteins transferred from SDS polyacrylamide gels to PVDF membranes (Millipore). Membranes were soaked in methanol followed by transfer buffer (0.02 M Tris base, 0.15 M glycine, 20% (v/v) methanol pH 8.3). Gel and membrane were sandwiched between 12 sheets of 3M filter paper which had been soaked in transfer buffer, and electroblotted for 1 hour to transfer protein from gel to membrane.

Membranes were blocked overnight at 4° C in 10% (w/v) Marvel, 10% (v/v) serum in Tris-buffered saline, with gentle rocking. (Serum was from the species in which the secondary antibody was raised.) Primary antibody was applied at 0.1µg/ml in blocking buffer and incubated for 24 hours at 4° C, as above. The antibodies used were: anti-p53, DO1; anti-pan-ras, Y13259; and

anti-c-myc, 9E10 (Ab6, Ab1, Ab1, Oncogene Science). The filters were subjected to four ten minute washes in Tris-buffered saline, 0.1% (v/v) Tween 20 and treated with biotinylated secondary antibody (Vector Laboratories: 7μg/ml in blocking buffer) for 1 hour at room temperature. Filters were washed as before, exposed to an avidin-biotinylated-horseradish peroxidase complex (ABC reagent; Vector Laboratories) for 1 hour at room temperature, washed again and immersed in Enhanced Chemi-luminescence solution (Amersham International) for one minute. The filters were exposed to X-ray film and processed. Where desired, signal strength was quantified densitometrically.

3.15 CALCIUM PHOSPHATE MEDIATED TRANSFECTION

Transfection was carried out according to the method of Graham and van der Ebb (1973). The plasmid used, Homer 6 (Spandidos and Wilkie, 1984), was generously provided by Professor Spandidos, National Hellenic Research Foundation, Greece. It contains the bacterial *aph* gene which confers resistance to geneticin. Plasmid was mixed with $CaCl_2$ solution (final concentrations 40μg/ml DNA, 250mM $CaCl_2$). To this was added an equal volume of 2 x Hepes buffered saline (NaCl, 270 mM; KCl, 10 mM: Na_2 HPO_4, 1.5 mM; HEPES, 42 mM; dextrose, 0.2% w/v). After 30 minutes at room temperature a precipitate had formed. The mixture was added to a Petri dish, containing

approximately 10^6 cells in exponential growth. The cells were incubated for 30 minutes at room temperature, followed by five hours at 37°C. The medium was removed and the cells were exposed to 15 % glycerol in Hepes buffered saline for 3 minutes at 37°C after which fresh medium was added. 24 hours later geneticin (GIBCO), was added at a final concentration of 1 mg/ml. The cells were maintained in these conditions for 14 days prior to cloning.

3.16 ISOLATION OF MONOCLONAL CELL LINES

Monoclonal cultures were derived from the resistant variant of NB1-G and from Homer 6 transfected mink epithelial cells, Mv1Lu. In both cases a similar procedure was followed. A very dilute cell suspension was prepared and cells were seeded into wells of 24 well plates in volumes that would be expected to result in an average seeding density of one cell per well. Heavily irradiated feeder cells were added to each well (7×10^4 NB1-G feeders per well, 3.5×10^4 Mv1Lu feeders per well). Mv1Lu cells were cultured in geneticin (1mg/ml). After ten days the plates were examined microscopically and those wells containing one colony only were selected for amplification. The selected wells were trypsinised, and the contents transferred to small petri dishes (9.6 cm^2). When the cells had grown to confluence they were seeded out in 75 cm^2 flasks and cultured as described in 3.1.

3.17 RNA ISOLATION AND cDNA SYNTHESIS

RNA was isolated using populations of approximately 5 x 10^7 cells. The cells were washed four times in PBS, and suspended into PBS. They were centrifuged at 200g for 5 minutes, and resuspended in RNAzol B (Cinna/ Biotecx Labs, Tx.). Autoclaved microcentrifuge tubes were used throughout. 0.1 volume of chloroform was added to the lysate, the mixture was vortexed, and held on ice for 5 minutes before centrifugation at 12000 g for 15 minutes at 4° C. The upper aqueous phase was collected, mixed with an equal volume of isopropanol and stored at 4°C for 15 minutes. RNA was precipitated by centrifugation (15000g for 15 minutes) at 4°C and resuspended in 70% (v/v) ethanol. This was centrifuged as above and the pellet resuspended in diethylpyrocarbonate (DEPC) treated water. RNA concentration was obtained spectrophotometrically, from the absorbance at 260 nm.

cDNA was prepared using the Gibco superscript preamplification system. 5μg of RNA was incubated with 0.5μg oligo (dT) in 13 μl DEPC treated water. The mixture was held at 70° C for 10 minutes and cooled on ice. 10 x synthesis buffer was added with dithiothreitol (DTT), an equimolar mixture of deoxynucleotide triphosphates (dNTP) and reverse transcriptase. The final concentrations of the components were: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$ 0.1μg/μl BSA, 10 mM DTT, 0.5 mM each dATP, dCTP, dTTP, dGTP, reverse transcriptase 20 U/μl). The reaction was held at

room temperature for 10 minutes, and at 42°C for 50 minutes. The reaction was stopped by incubating at 70°C for 15 minutes, followed by chilling on ice. RNase H was added (0.1 U/μl), and the reaction was incubated for 20 minutes at 37°C.

3.18 PCR AMPLIFICATION AND SEQUENCING

p53 cDNA was amplified over a region covering exons 5 to 8. The primer sequences 5'-TTTCCGTCTGGGCTTCTTGC-3' and 5'-TCTTCTTTGGCT GGGGAGAGG-3', were used in the amplification of the base pair region 458 to 1096. Primers were synthesised by Oswel DNA service, University of Edinburgh. Conditions for the polymerase chain reaction (PCR) were as follows: Tris-HCl (pH 8.4), 20 mM; KCl, 50mM; $MgCl_2$, 1.5 mM; dNTPs, 0.2 mM each; primers, 0.2 μM each; Taq DNA polymerase, 0.025 units/μl; W-1 detergent (GIBCO), 0.05% v/v. 10 μl of cDNA solution prepared as above, was used as template. The final volume was 100 μl. The mixture was overlaid with paraffin oil, and heated to 94° C for 3 minutes. 35 cycles of PCR were performed - 94° C for 45 seconds, 55° C for 30 seconds and 72° C for 90 seconds.

PCR products were run on 2% (w/v) low-melting point agarose gels in 0.5 x TBE (Tris base, 0.025M; boric acid, 0.025 M; EDTA 0.5mM; pH 8.0).

The gels contained ethidium bromide at 0.5µg/ml. The products were visualised using long-wavelength UV light, excised and melted in 100µl TE buffer (Tris base, 10mM; EDTA, 0.1mM, pH7.4) at 65°C for 10 minutes. The samples were extracted three times with equal volumes of salt-saturated phenol, and twice with chloroform, after which 0.2 volumes of ammonium acetate (10M) and 2.5 volumes of ethanol were added. The samples were held at -70°C for 15 minutes and the DNA precipitated at 15000g for 15 minutes at 4°C. The pellet was resuspended in 70% (v/v) ethanol and centrifuged as before. Finally, the DNA was resuspended in 20µl sterile distilled water.

Sequencing was performed using the in-house sequencing facility. The PCR primers were used with additional internal primers 5'-TCATCCAAATACTCCACACGC-3' and 5'-ATTTGCGTGTGGAGTATTTGG-3' to generate four sense and anti-sense sequences. The internal primers gave rise to 300 and 360 base pairs sequence respectively. Primers were added at a final concentration of 0.2 µM with 9.5 µl terminator mix (Applied Biosystems, Warrington, Lancs.) and cycled at 96°C, 30 seconds; 50°C, 15 seconds; 60°C, four minutes for 25 cycles. After cycling, the reaction mixture was extracted twice with equal volumes of phenol chloroform water (68:18:14). DNA was precipitated with 0.15 volumes of 2M sodium acetate (pH 4.5) and 2.5 volumes ethanol at -70°C for 15 minutes, followed by centrifugation (15,000g, 15minutes, 4° C). The pellet was washed in 70% ethanol and lyophilised (15 minutes, 55° C). Samples were electrophoresed through polyacrylamide gels by Mr Robert MacFarlane (Beatson Institute).

4 RESULTS

4.1 THE CONTACT EFFECT

Figure 4.1 shows the radiation survival curves of DON cells, irradiated as spheroids or monolayers. Additional experiments were also performed using spheroids which were disaggregated before irradiation, since differences in cell cycle distribution between monolayer and spheroid cultures could complicate the experiment. In addition, unlike monolayer cultures, cells in suspension would lack all intercellular communication at the time of irradiation. There is no significant difference between the two curves, and therefore no evidence for a contact effect. The parameters are given in table 4.1.

Table 4.1. The radiation survival parameters of DON cells grown as monolayers and spheroids (Indicated errors are 95% confidence limits).

Culture conditions	D_o (Gy)	n	α (Gy^{-1})	ß (Gy^{-2})
monolayer	2.60 ± 0.4	1.38	0.257 ± 0.085	0.010 ± 0.010
spheroids disaggregated before irradiation	2.38 ± 0.1	1.47	0.336 ± 0.006	0.004 ± 0.001
spheroids disaggregated after irradiation	2.66 ± 0.2	1.40	0.261 ± 0.036	0.036 ± 0.004

Figure 4.1 Radiation survival of DON cells grown as spheroids or monolayers. Points were derived from four independent experiments. The surviving fraction is represented as $\log_{10}$, error bars represent standard errors of the mean.

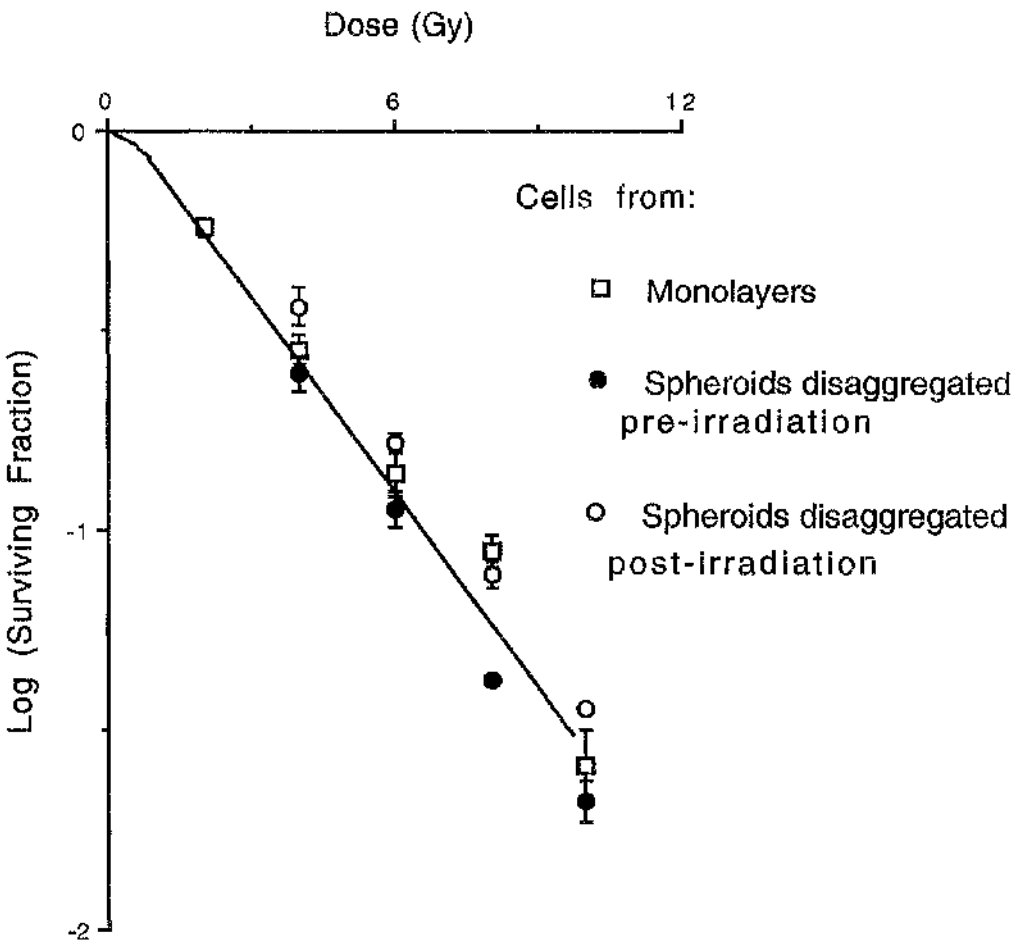
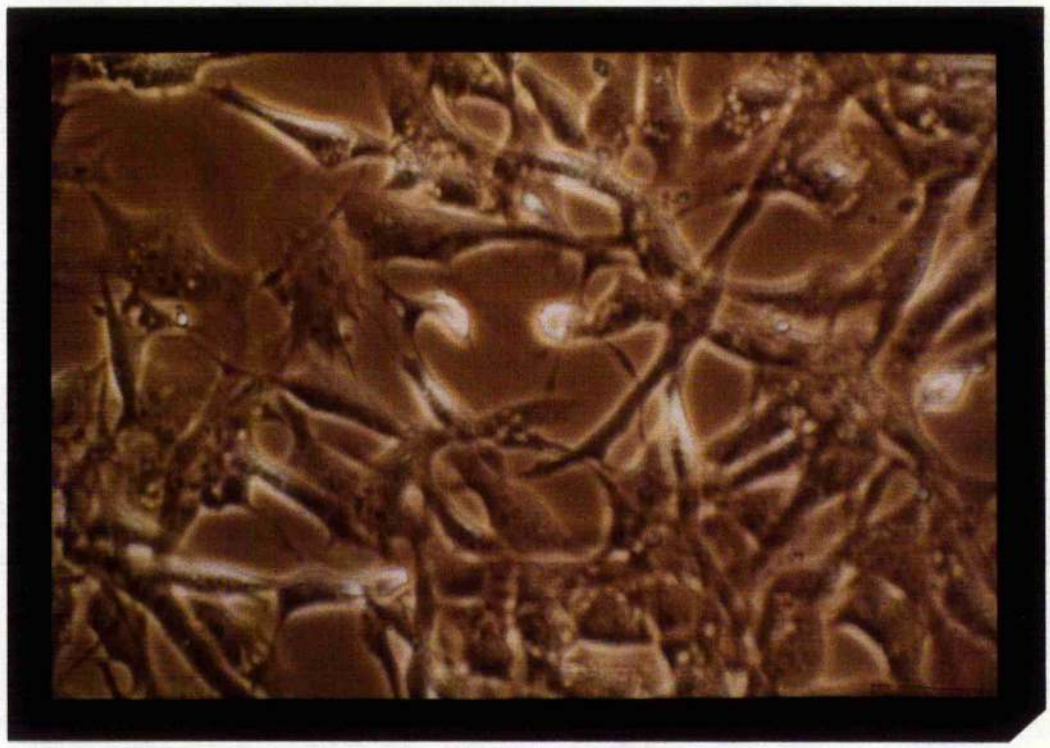


Figure 4.2 shows the results of microinjecting a single DON cell in a monolayer with the fluorescent dye, lucifer yellow. The neighbouring cells are fluorescing, indicating that dye has spread from the injected cell. Because the dye will not diffuse across cell membranes, this spread indicates the presence of gap junctional coupling. Figure 4.3 shows the results of microinjecting dye into a DON cell on the surface of a spheroid. It is not possible to obtain good resolution of cells on the spheroid surface, though individual cells can just be identified in the bright field image (figure 4.3a). The large surface area fluorescing (figure 4.3b) indicates that several cells have taken up the dye. Dertinger's original hypothesis was that cell lines that showed such coupling (in monolayer and in spheroid culture) also would display a contact effect. The results presented here seem to refute that theory.

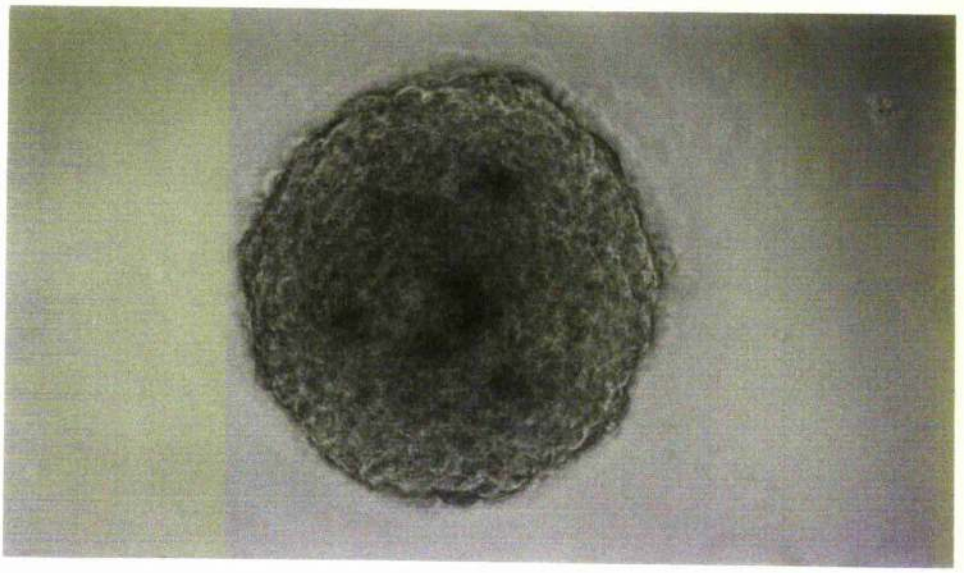
Figure 4.2 Micro-injection of DON cells grown as monolayers with lucifer yellow.



4.2a phase contrast illumination

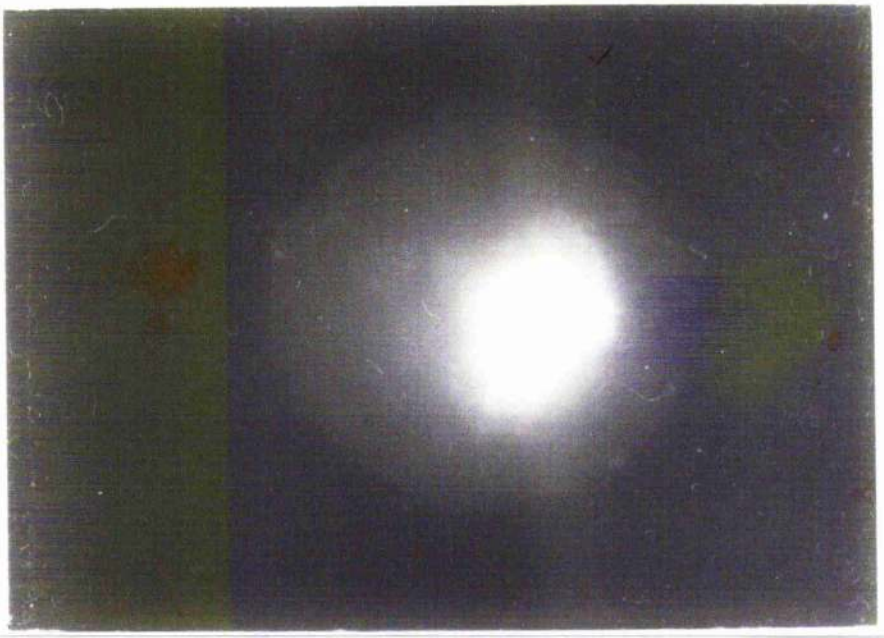
4.2b fluorescence photomicrograph.

Figure 4.3 Microinjection of DON cells grown as spheroids with lucifer yellow.



4.3a brightfield

4.3b fluorescence photomicrograph



Rofstad and Sutherland (1988) suggested that the contact effect might be more prevalent in radiosensitive cell lines.Accordingly, three human neuroblastoma lines were also investigated, to see if they displayed a contact effect. Neuroblastoma lines are generally radiosensitive (Fertil and Malaise, 1985). Figures 4.4 - 4.6 show the survival curves of NB1-G, IMR 32, and NB100 cells grown as monolayers and spheroids. The survival curve parameters are given in table 4.2. None of the three lines shows a contact effect.

Table 4.2 Radiation survival parameters of three human neuroblastoma lines, grown as monolayers and spheroids.

Cell line	Culture conditions	D_o (Gy) ± 95% c.l.	n	SF_{2*}	α (Gy^{-1})	ß (Gy^{-2})
NB1-G	monolayer	0.8 ± 0.1	1.4 ± 0.4	0.12	0.965 ± 0.187	0.051 ± 0.048
	spheroid	0.72 ± 0.29	1.97 ± 0.82	0.13	0.856 ± 0.339	0.0865 ± 0.08
IMR32	monolayer	0.66 ± 0.12	1.65 ± 0.81	0.085	0.812 ± 0.408	0.204 ± 0.179
	spheroid	0.61 ± 0.27	3.1 ± 3.0	0.15	0.354 ± 0.713	0.301 ±0.267
NB100	monolayer	1.15 ± 0.13	2.6 ± 1.04	0.42	0.284 ± 0.118	0.078 ± 0.026
	spheroid	1.26 ± 0.26	2.29 ± 1.35	0.40	0.355 ± 0.212	0.051 ± 0.041

* SF_2 values are derived from fitting the data to the linear quadratic equation.

Figure 4.4 Radiation survival of NB1-G cells grown as monolayers or spheroids.
Points were derived from three independent experiments, error bars represent standard errors of the mean.

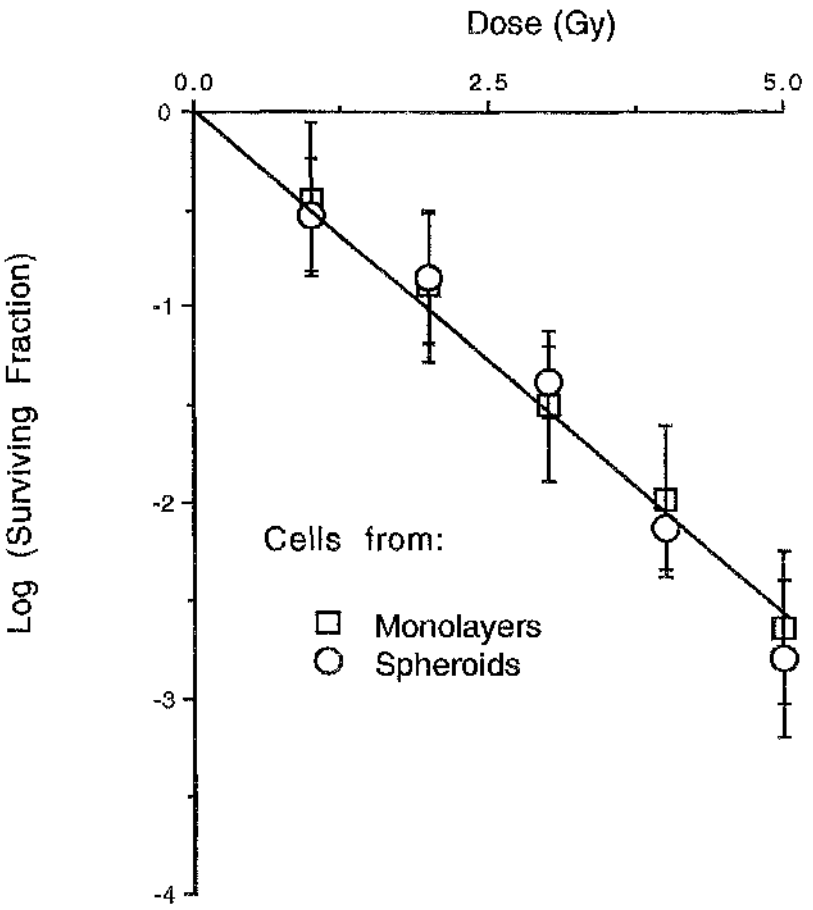


Figure 4.5 Radiation survival of IMR 32 cells grown as monolayers or spheroids. Points were derived from three independent experiments, error bars represent standard errors of the mean.

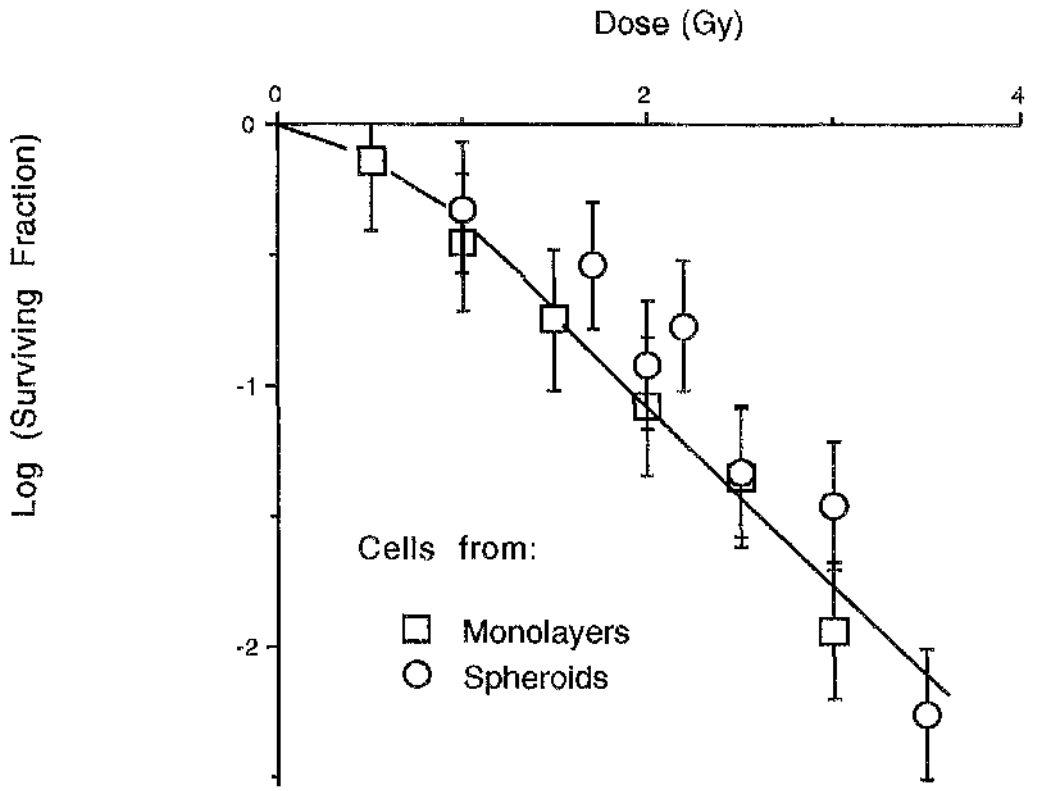
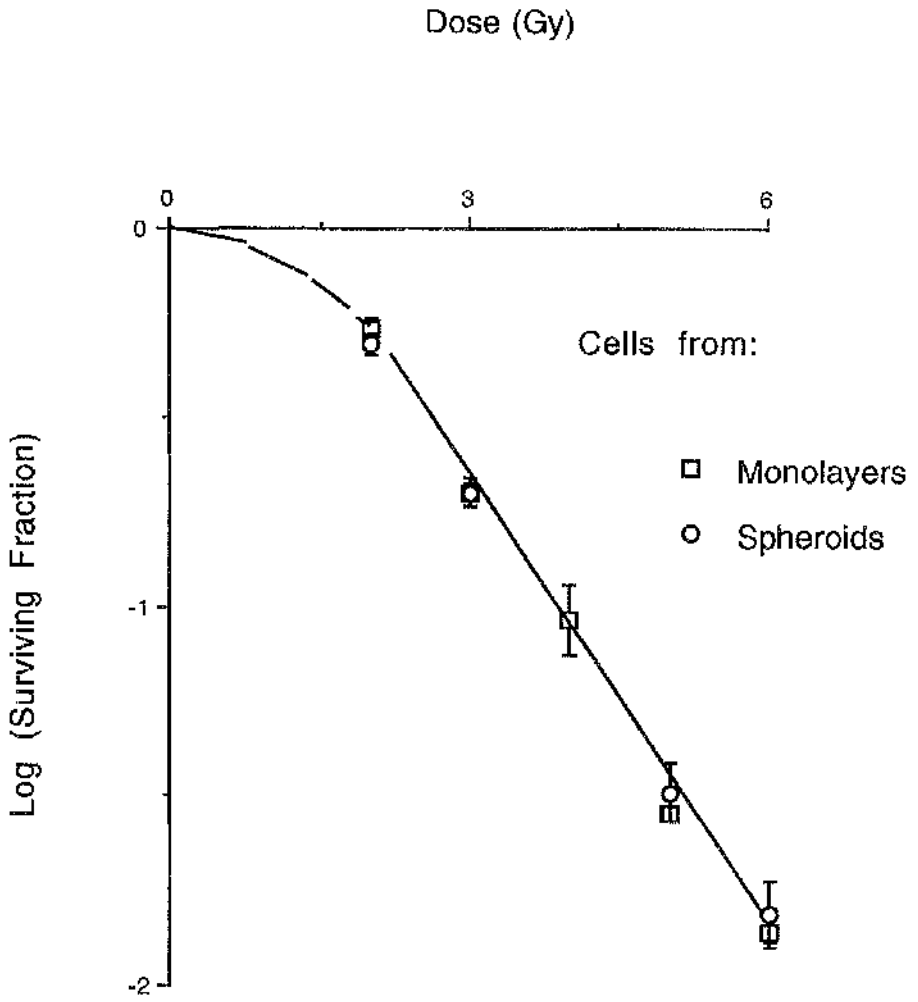


Figure 4.6. Radiation survival of NB100 cells grown as monolayers or spheroids. Points were derived from three independent experiments, error bars represent standard errors of the mean.



NB100 cells were also examined for gap-junctional communication (figure 4.7). Again dye transfer was observed between adjacent cells, confirming that the presence of gap junctions cannot by itself confer a contact effect.

Figure 4.7 NB100 cells microinjected with lucifer yellow

4.7a fluorescent photomicrograph

4.7b brightfield photomicrograph

4.2 THE EFFECT OF TRANSFECTION WITH THE ONCOGENES H-*ras* AND c-*myc* ON THE RADIOSENSITIVITY OF A MINK EPITHELIAL CELL LINE

Khan *et al.* (1991) described the transfection and characterisation of a mink cell line with the oncogenes c-*myc* and H-*ras*. The radiosensitivity of the transfectants was examined here. Controls for the H-*ras* transfectants were generated by transfecting Mv1Lu cells with the Homer 6 vector which had been used to transfect the H-*ras* oncogene. The vector used for the c-*myc* transfection was pMCGM1 (Spandidos, 1985). Like Homer 6 it carries genes for ampicillin and G418 resistance. However, vector controls for this transfection were not generated, since pMCGM1 had been constructed by inserting the aph gene, which codes for G418 resistance, into a plasmid already containing c-*myc*.

Figure 4.8 shows elevated expression of c-*myc* and H-*ras* proteins in the transfectants compared to two vector controls. Table 4.3 shows that *ras* expression led to a significantly increased growth rate. It also allowed cells to proliferate in low serum concentrations. This is consistent with previous growth data on these cells (Khan *et al.*, 1991). Activated *ras* generates constitutive activity in the mitogenic pathway and would be expected to reduce the need for growth factor stimulation. However, the vector controls and c-*myc* transfectants both continued to divide in low serum, albeit at a reduced rate. Growth factor withdrawal has been linked to apoptosis in fibroblasts, an effect enhanced by over-expression of c-*myc* (Evan *et al.*, 1992). Tolerance of low serum concentrations may be characteristic of epithelial cells. Alternatively it

Figure 4.8 c-*myc* and H-*ras* protein expression in mink epithelial cells. Panel a) probed with anti c-*myc*. Lane 1 cells transfected with Homer 6; lane 2, H-*ras*; lane 3 c-*myc*. Panel b) probed with anti H-*ras*, lanes as before.

may indicate that Mv1Lu cells have changed in culture.

Table 4.3 The effect of serum concentration on the growth of the transfected cell lines.

Cell line	Doubling time (hours) ± s.e.m.	
	10% FCS	0.1% FCS
Vector control(clone 1)	30 ± 5	60 ± 13
c-*myc* transfected	27 ± 6	38 ± 4
H-*ras* transfected	16 ± 2	17 ± 1

The data are derived from 2 independent experiments.

Figure 4.9 shows the survival curves of the transfected lines. The survival curve parameters are given in table 4.4. To assess whether significant differences existed between the vector controls, an analysis of variance test was carried out, using the statistical package SPSS (SPSS Inc, Chicago IL.). The lines were analysed according to survival at each dose-point. The significance (p value) of the F ratio for the test was 0.173, indicating that in terms of radiobiology, the vector controls all belong to the same population. By the same test, the significance of the difference between the c-*myc* transfected cells and the pooled data for the vector controls was 0.09 and between the *ras* transfectants and the vector controls, 0.07. The same comparison between the vector controls and the parental line gave a p value of 0.12. If the criterion of $p < 0.05$ is employed, then none of these lines differ significantly

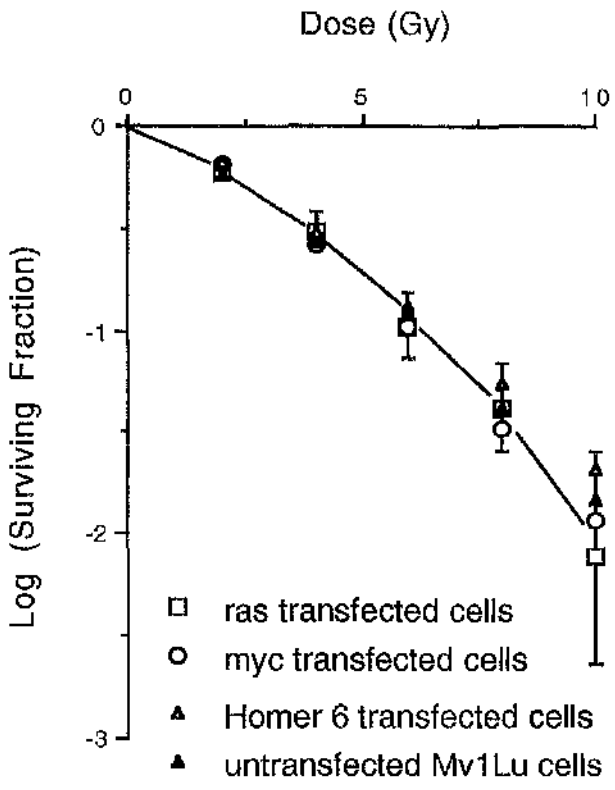
in terms of radiosensitivity.

Table 4.4. Survival parameters of the mink epithelial line, Mv1Lu, and the oncogene transfected derivative lines (Errors indicate 95% confidence intervals).

Cell line	SF_2*	$\alpha(Gy^{-1})$	$ß(Gy^{-2})$	$D_0(Gy)$	n
Vector controls					
1	0.48	0.358 ±0.032	0.004 ±0.005	2.49 ±0.35	1.12 ±0.72
2	0.57	0.251 ±0.02	0.014 ±0.002	2.27 ±0.35	1.85 ±0.92
3	0.64	0.187 ±0.032	0.018 ±0.004	2.15 ±0.44	2.69 ±1.91
4	0.61	0.213 ±0.021	0.017 ±0.002	2.13 ±0.25	2.42 ±0.99
5	0.62	0.201 ±0.039	0.021 ±0.005	1.98 ±0.39	2.74 ±2.04
Vector controls, pooled results.	0.60	0.242 ±0.015	0.006 ±0.001	2.20 ±0.1	2.03 ±0.27
Untransfected Mv1Lu cells	0.56	0.253 ±0.093	0.017 ±0.011	1.95 ±0.35	2.0 ±1.0
HO6T1 (*ras*)	0.62	0.179 ±0.076	0.03 ±0.009	1.68 ±0.78	3.65 ±3.65
MCMG1 (*myc*)	0.56	0.253 ±0.025	0.02 ±0.002	1.87 ±0.55	2.54 ±1.29

* SF_2 values are derived from fitting the data to the linear quadratic equation. The data for the untransfected cells represent four independent experiments, and for each of the other lines, two independent experiments.

Figure 4. 9 Radiation survival of mink lung epithelial cells. Cells were transfected with H-*ras* or c-*myc* and compared to the parent line and to clones transfected with only the vector Homer 6. The data for the *ras* and *myc* transfectants were derived from two independent experiments and for the untransfected cells, points represent four independent experiments. The Homer 6 data represent the results of 5 separate monoclonal lines, each assayed twice. The error bars represent the standard error of the mean for the *ras* transfectant. The errors for the other lines were omitted for the sake of clarity.



4.3 SELECTION OF A RADIORESISTANT VARIANT BY EXPOSING A HUMAN NEUROBLASTOMA LINE TO REPEATED FRACTIONS OF X-RADIATION.

4.3.1 Clonogenic and spheroid growth delay studies

NB1-G is a radiosensitive cell line with a D_o of 0.68 Gy. Two flasks of this cell line were subjected to repeated fractions of 2 Gy, separated by 5-7 days. After 15 fractions of 2 Gy, a clear change became apparent in the survival curve of the treated cells. This is illustrated in figure 4.10. The increased radioresistance of the treated cells was hypothesized to be due to a selection process which favoured the emergence of resistant cells. The resistant subline has been designated XRNB1-G.

The XRNB1-G data relate to cells that were exposed to between 15 and 30 fractions of 2 Gy irradiation. Ongoing irradiation of the XRNB1-G cells was required due to the instability of the cultures. It was found that the XRNB1-G cultures maintained a radioresistant phenotype for at least 3 passages after the cessation of fractionated radiation. With further passages, the cells reverted to wild-type radiosensitivity (figure 4.11). It is possible that the instability of the resistant population was due to surviving wild-type NB1-G cells overgrowing the XRNB1-G cells. The wild-type cells had a faster doubling time (22 hours) than the variants (33 hours) (figure 4.12).

Figure 4.10 Radiation survival of NB1-G and XRNB1-G cells. The data represent three and four independent experiments respectively, error bars represent 95 % confidence limits.

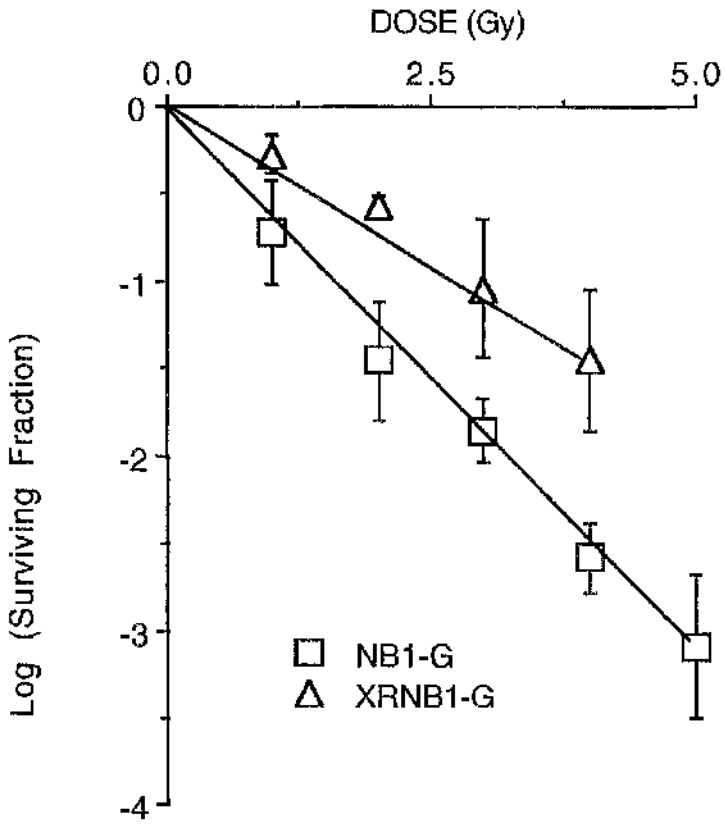


Figure 4.11 Loss of radioresistance in the XRNB1-G population, following the cessation of fractionated irradiation. Each point is derived from one clonogenic assay, with survival measured at five different doses.

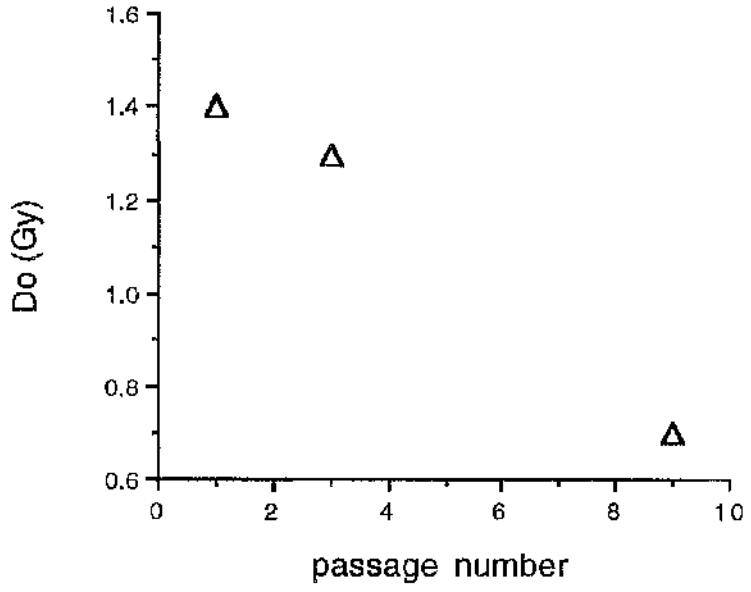
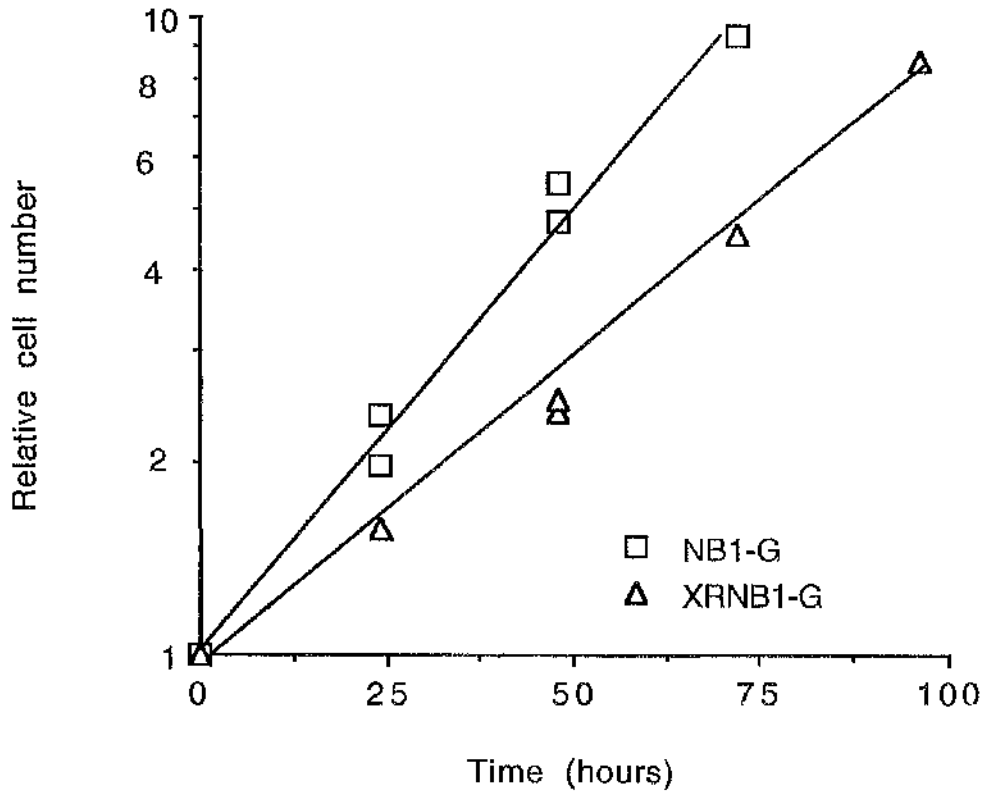


Figure 4. 12 Growth rates of NB1-G and XRNB1- G cells in culture. Data are from two independent experiments. The data was normalised to correct for variation in the number of cells obtained at the initial measurement.



After two rounds of cloning, a single clone, designated clone F, was isolated from the XRNB1-G population. This clone maintained the radioresistant phenotype in the absence of ionising radiation over 37 passages, as shown in figures 4.13 and 4.14. (Cells from the first round of cloning were not phenotypically stable: possibly these cultures were contaminated with wild-type cells.) The survival curve parameters for all three lines are shown in table 4.5. The plating efficiencies of the two lines did not differ greatly: 14% for NB1-G as opposed to 12% for XRNB1-G.

Two sets of clonogenic assays were performed to compare the NB1-G and XRNB1-G cells. The first employed a 300 kV X-ray machine, the second used a ^{60}Co source. The experiments were separated by approximately 18 months. Both sets of experiments yielded significant differences between the lines. The parameters are shown in tables 4.5a and b. The ^{60}Co data seem to show greater radiosensitivity. This is surprising since X-rays generated by orthovoltage machines have a higher RBE than ^{60}Co γ rays. The question is whether this unexplained inter-experimental variation compromises the differences between the NB1-G and XRNB1-G/clone F cells. To allow comparison between NB1-G and resistant cells, clonogenic assays were performed so that parent and resistant cells were tested together. In this way, environmental influences, such as serum batch or small absolute differences in dosimetry, ought to act equally on all the lines. Also, both sets of experiments demonstrated enhanced radioresistance in the XRNB1-G line, indicating that there is a real difference in radiosensitivity between the parental and XRNB1-G/clone F lines.

Table 4.5a Clonogenic survival parameters of NB1-G, clone F and XRNB1-G cells following exposure to ^{60} Co γ-rays. (Indicated errors are 95% confidence limits.)

Cell line	SF2 **	D_o (Gy)	n	α (Gy^{-1})	ß (Gy^{-2})
NB1-G *	0.053	0.68 ± 0.04	1.0	1.47 ± 0.1	0.0
clone F	0.19 p < 0.001	1.1 ± 0.14	1.1 ± 0.46	0.824 ± 0.039	0.012 ± 0.01
XRNB1-G	0.23 p = 0.002	1.02 ± 0.4	1.85 ± 2.03	0.548 ± 0.102	0.073 ± 0.028

Table 4.5b Clonogenic survival parameters of NB1-G and XRNB1-G cells following exposure to 300 kV X-rays. (Indicated errors are 95% confidence limits.)

Cell line	SF2 **	D_o (Gy)	n	α (Gy^{-1})	ß (Gy^{-2})
NB1-G	0.12	0.8 ± 0.1	1.4 ± 0.4	0.965 ± 0.187	0.051 ± 0.048
XRNB1-G	0.24 p=0.041	1.4 ± 0.3	1.0 ± 0.5	0.720 ± 0.051	0.0 ***

* Fitting the data to multitarget and linear-quadratic models gave a value of n below unity and a negative value of β; the parameters α and D_o were obtained by regression forced through the origin.

** p values obtained by comparing clone F and XRNB1-G against NB1-G, using Students t-test.

*** l.q. fit to the data gave a negative value of β; α was calculated by linear regression.

Figure 4.13 Radiation survival of NB1-G cells compared to a monoclonal line, clone F, established from the XRNB1-G population. The NB1-G data are from figure 4.10. Clone F data were obtained from five independent experiments. Error bars represent 95% confidence limits.

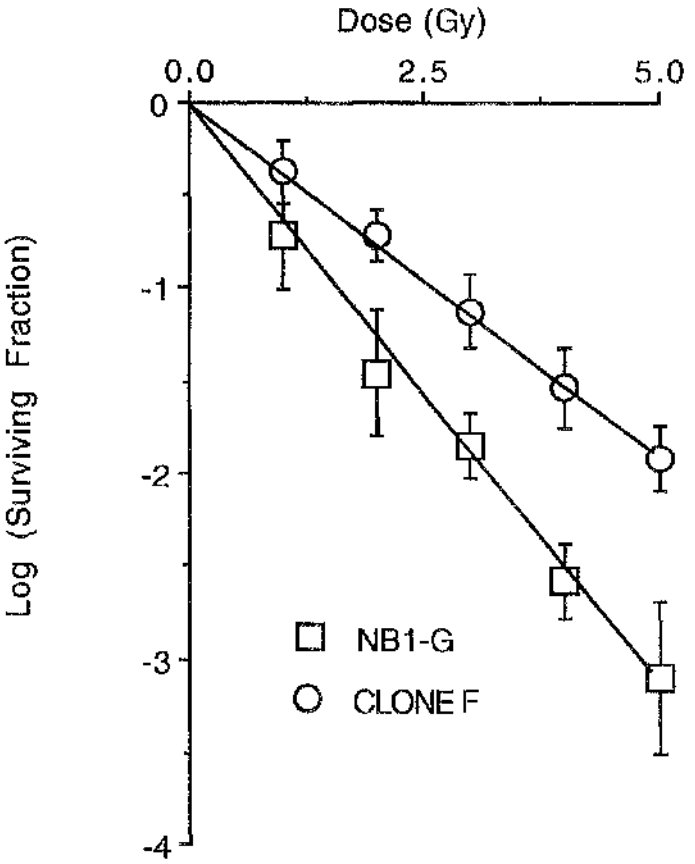


Figure 4.14 Relationship between Do and passage number for clone F cells, cultured in the absence of further irradiation. (Same data as in Figure 4.13.)

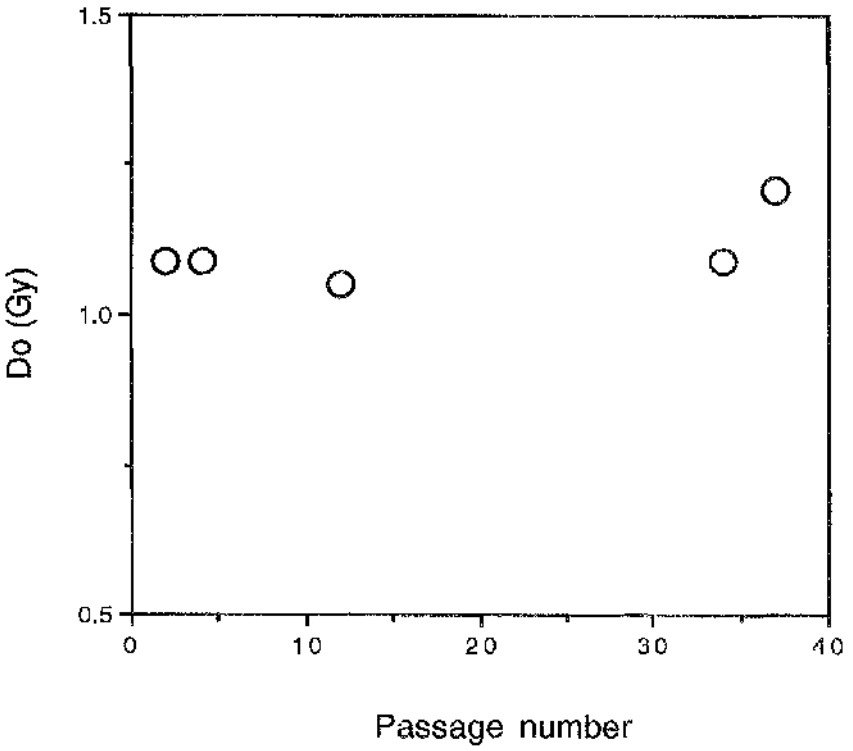


Figure 4.15 shows the regrowth curves of treated NB1-G and XRNB1-G spheroids. Figure 4.16 shows the growth delay for these lines as a function of dose. The fact that increased radioresistance was observed in the XRNB1-G line using the spheroid growth delay assay reduces the possibility that the clonogenic assay results are due to an experimental artefact. Spheroid growth delay is expressed as specific growth delay, defined as:

$$SGD_D = (T_{10,D} - T_{10,C})/T_{2,C}$$

where SGD_D is the specific growth delay of spheroids given a dose D; $T_{10,D}$ is the time taken by such spheroids to reach ten times their pre-treatment volume; and $T_{10,C}$ and $T_{2,C}$ are the times taken by untreated spheroids to reach ten times and twice their pre-treatment volume respectively. Because specific growth delay is equivalent to the number of doublings that surviving cells must undergo to repopulate the treated spheroid to its original level, it is possible to infer surviving fractions using the formula:

$$SF = 2^{-SGD}$$

The validity of this manipulation depends on spheroids being in exponential growth and treated spheroids regrowing at the same rate as the controls. Figure 4.15 shows that both NB1-G and XRNB1-G spheroids satisfied this condition. Spheroid growth delay was not performed on clone F cells as they grew poorly as spheroids. Figure 4.17 shows the inferred survival curves obtained in this way. There is a good but not perfect agreement between the inferred surviving fractions and those obtained from the monolayer clonogenic assay. Whatever the reasons for the spheroid-monolayer difference, it does not seem to be due to a contact effect (figures 4.4 and 4.18).

Figure 4.15 Regrowth curves of spheroids following irradiation , a) NB1-G b) XRNB1-G. Each point represents the median volume of 18 to 24 spheroids.

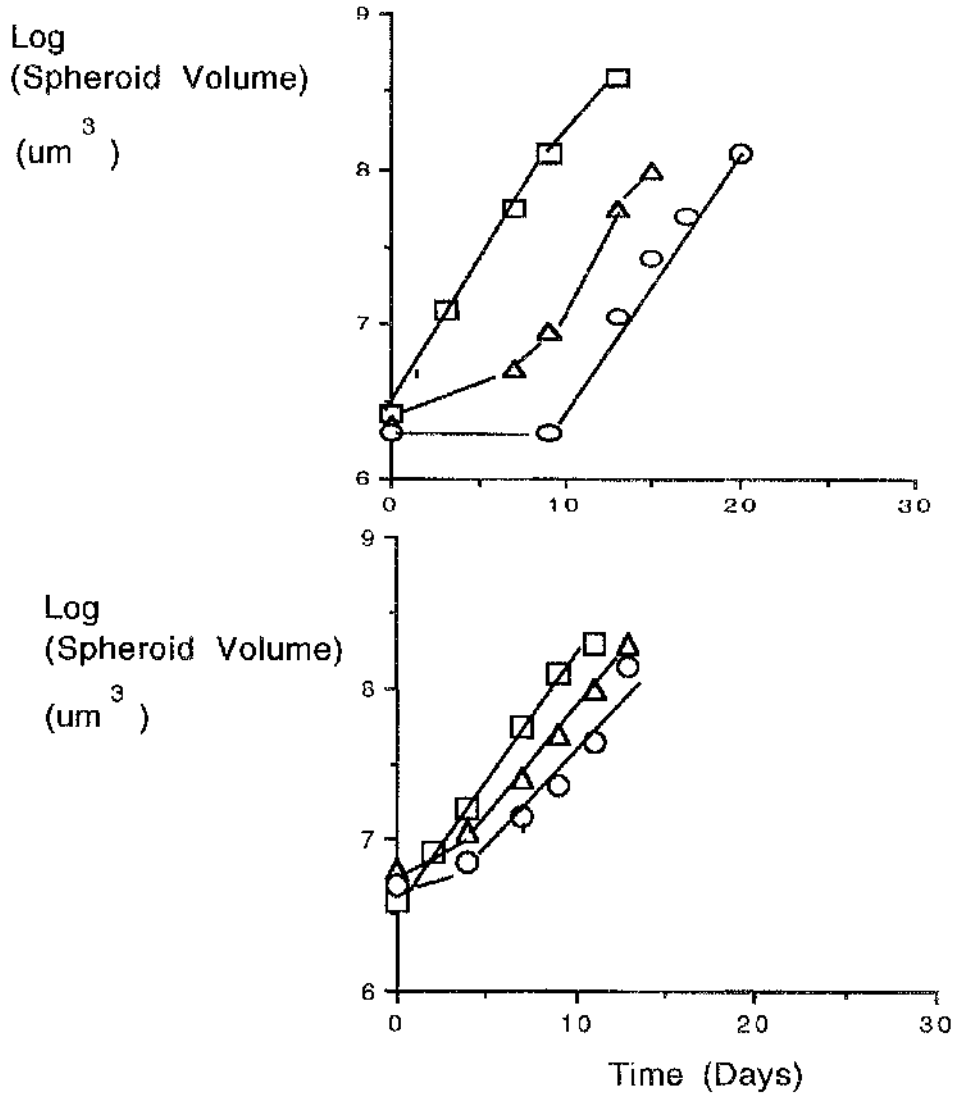


Figure 4.16. Specific growth delay of NB1-G and XRNB1-G spheroids. Each point was calculated from the median growth delay of 18 to 24 spheroids.

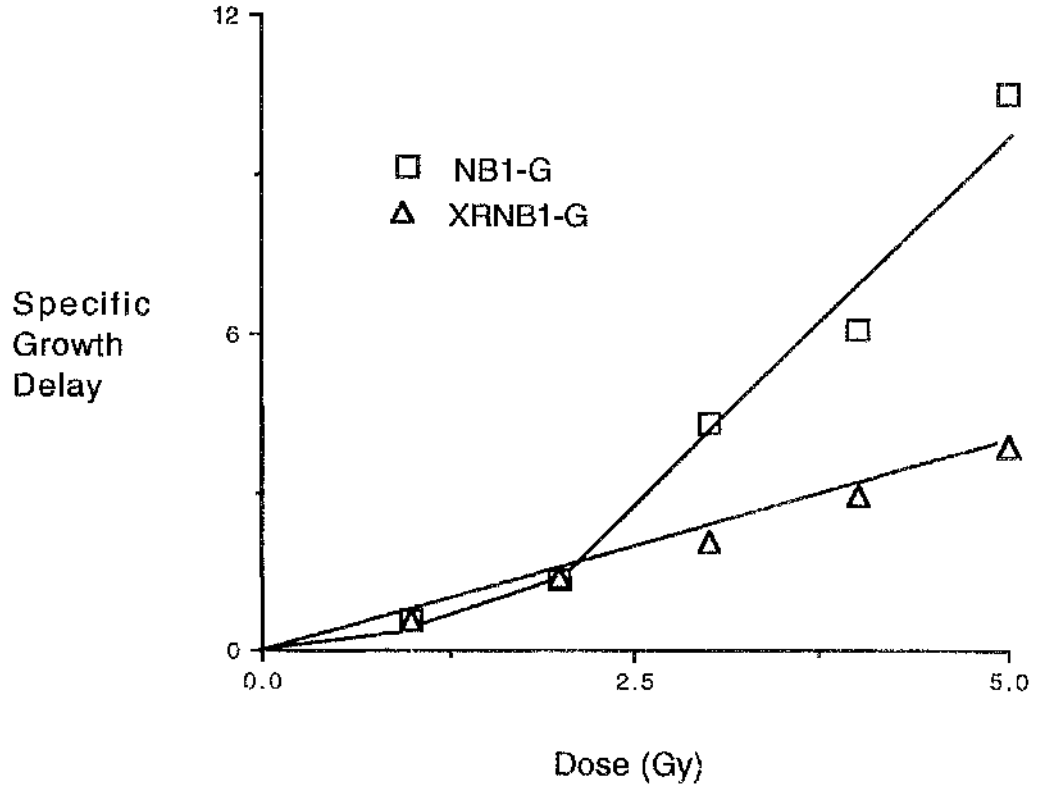


Figure 4.17 Clonogenic survival inferred from specific growth delay. Monolayer survival curves for cells irradiated with 300kV X-rays are shown for comparison. Each monolayer survival curve is derived from four independent experiments. Error bars represent 95% confidence intervals.

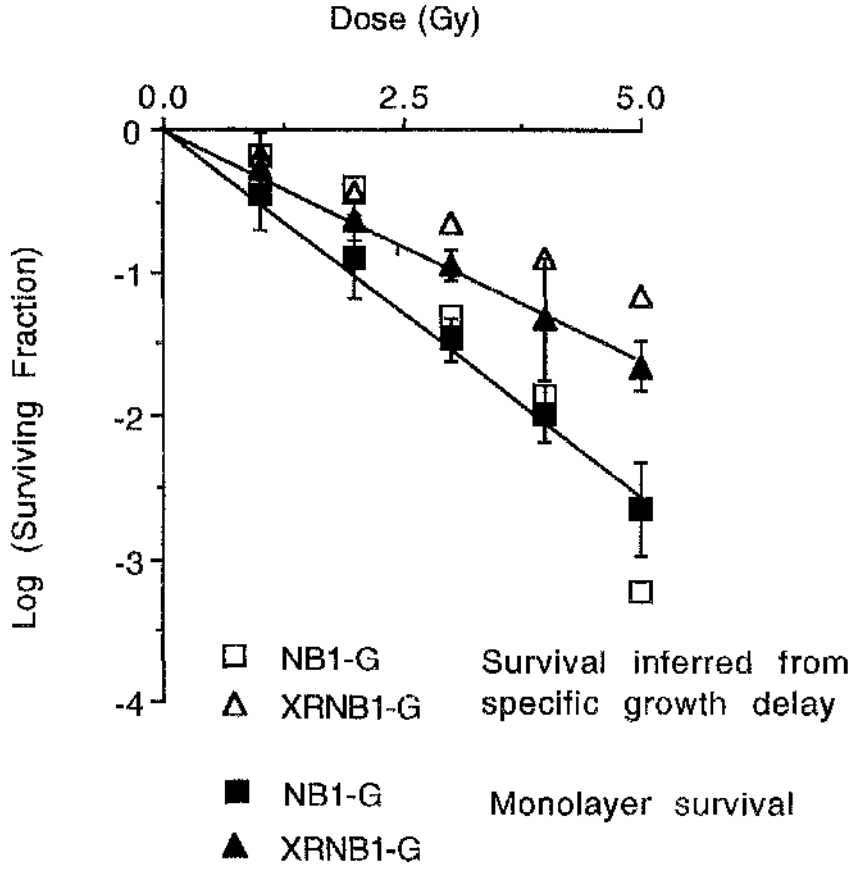
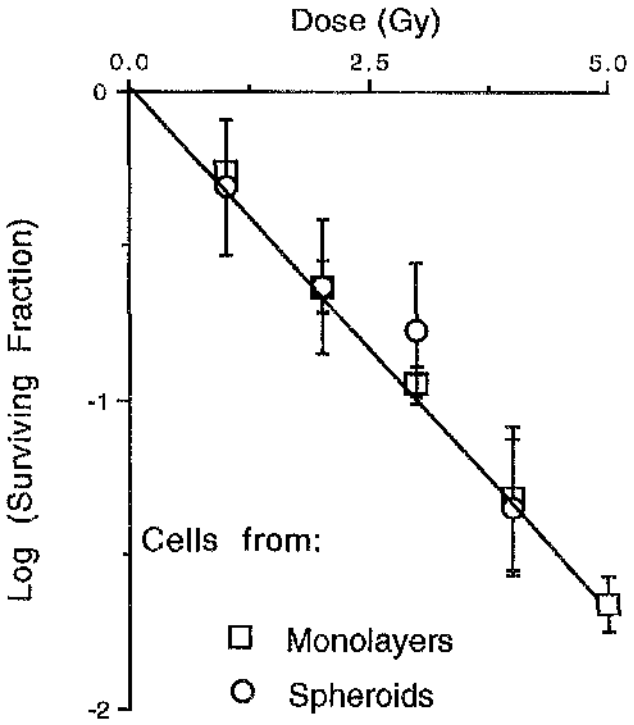


Figure 4.18 Radiation survival curves of XRNB1-G cells grown as monolayers or spheroids and irradiated with 300kV X-rays. Data represent four and two independent experiments respectively. Error bars represent standard errors of the mean.



4.3.2 Cell cycle distribution of the parental and resistant lines

Because of the well known relationship between position in the cell cycle and radioresistance, we examined the cell cycle distribution of the lines using flow cytometry. The results in table 4.6 show that all the lines display identical cell cycle distributions, and that this cannot be the explanation for the altered radiosensitivity of XRNB1-G / clone F cells.

Table 4.6 Cell cycle distributions of NB1-G, XRNB1-G and clone F cells

Cell line	Experiment number	% of cells in		
		G_1	S	G_2/M
NB1-G	1	51	35	14
	2	47	37	15
XRNB1-G	1	54	33	13
	2	52	37	11
Clone F	1	51	34	15
	2	55	33	12

4.3.3 Karyotypes of NB1-G and XRNB1-G

Both lines were karyotyped using trypsin-Giemsa banding, to see if there were any gross differences at the chromosomal level which might be related to the altered phenotype of the XRNB1-G cells. Gene amplification can be detected in this way. The amplified genes may form double minute chromosomes or be present as homogenously staining regions (hsr). Gene amplification is one mechanism for the acquisition of methotrexate resistance (Schimke *et al.*, 1978) and the multi-drug resistant phenotype (Riordan *et al.*, 1985). Typical spreads are shown in figures 4.19 and 4.20. Both lines have several aberrations in common. These are: trisomy of chromosome 1, with additional material on 1p; a deletion on chromosome 9p; chromosomes 15 and 16 are both abnormal, carrying extra material on the p and q arms respectively, and loss of the Y chromosome (NB1-G was derived from a male (Carachi *et al.*, 1987)). The XRNB1-G spreads show other abnormalities randomly distributed throughout the population. Chromosome 2 is altered in four out of six spreads. However, the specific aberrations differ - they are: three additions, two on the q arm and one on p; and one deletion on the q arm. Chromosome 17 is also affected in four of the spreads. Again a range of aberrations was evident. These additional aberrations are presented in table 4.7. They presumably arose because of the repeated irradiations used in generating the line.

Table 4.7 Chromosome aberrations unique to the XRNB1-G line.

Spread Number	Altered Chromosome
1	6 17 21 22 X
2	2 4 7 8 15 17 X
3	2 14 17 20
4	2 4 5 11 20
5	2 9 11
6	4 5 10 13 17 20 X

However, analysis of six XRNB1-G spreads suggested that four had lost an hsr that is present in NB1-G cells on one copy of chromosome 1. The hsr is clearly visible in figure 4.19, resulting in the normally short p arm being approximately 2.5 times the length of the q arm. Loss of the hsr from the resistant cells would be surprising, since the hsr is known to contain amplified N-*myc* gene (Carachi *et al.*, 1987) and both NB1-G and XRNB1-G cells display twentyfold N-*myc* amplification (Livingstone *et al.*, 1994). To clarify the situation, the N-*myc* amplicon was investigated using FISH, with probes raised against N-*myc* and the centromere of chromosome 1. Figure 4.21 shows clone F and NB1-G cells probed in this way. Both lines show N-*myc* amplification on one copy of chromosome 1. In both lines, the amplicon seems to be present on an abnormally large chromosome, suggesting that loss of material from this region is not involved in the radioresistant phenotype.

Figure 4.19 Karyotype of NB1-G cells. The arrows indicate abnormal chromosomes.

Figure 4.20 Karyotype of XRNB1-G cells. The arrows indicate abnormal chromosomes.

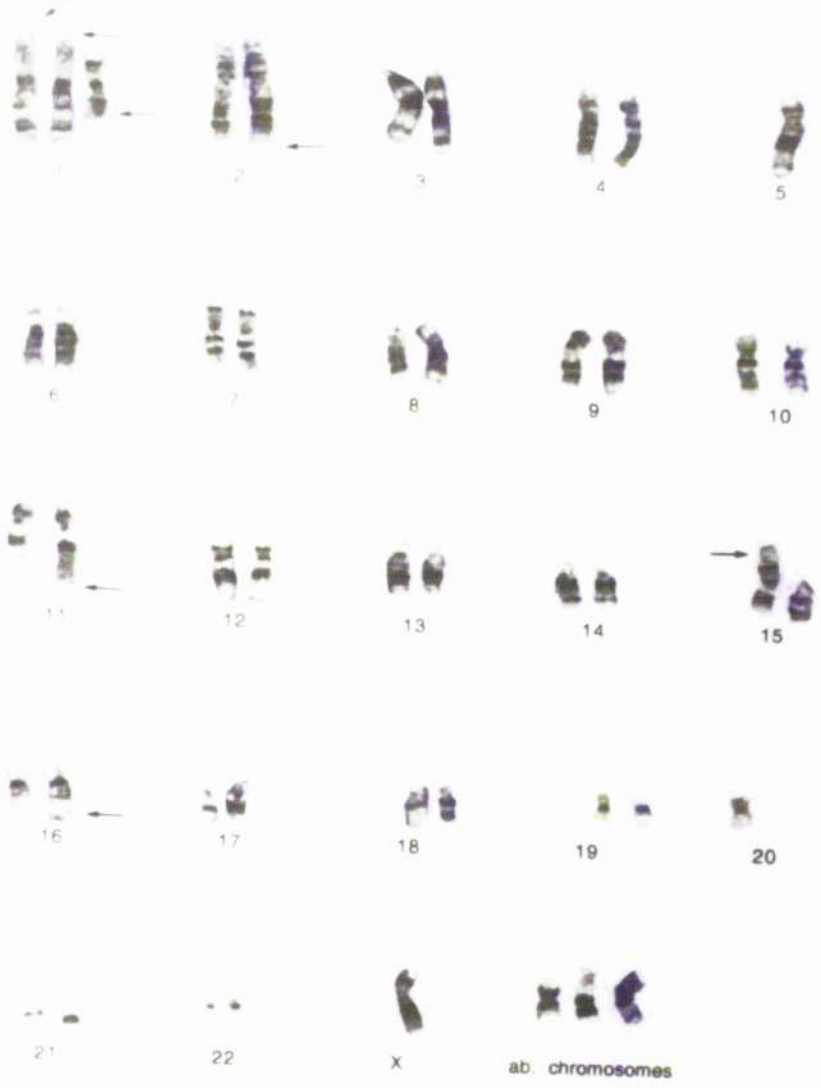
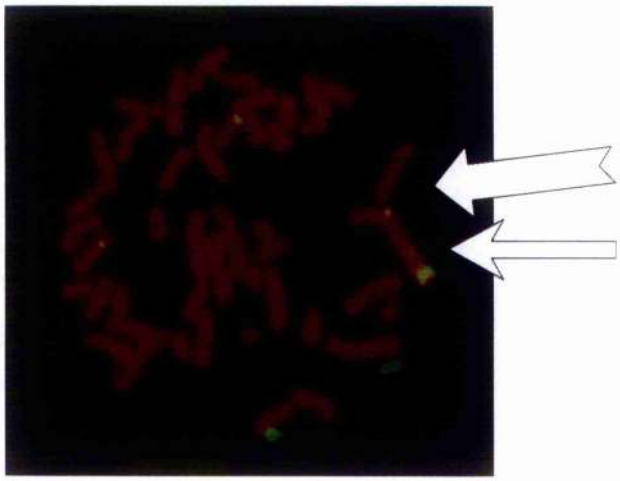


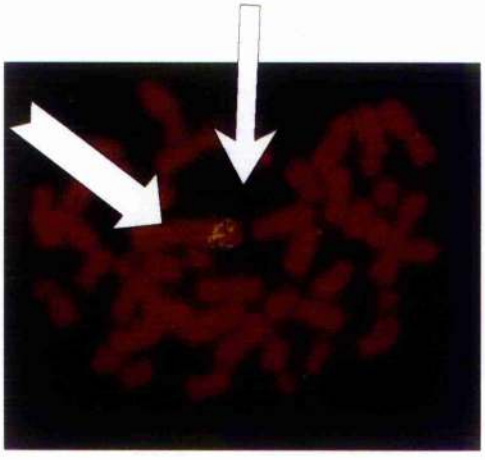
Figure 4.21 FISH analysis of clone F and NB1-G cells.



Clone F cells

Centromere 1

N-myc



NB1-G cells

4.3.4 Levels of initial DNA damage and repair

DNA double strand breaks were studied using neutral elution. Figure 4.22 shows the dose-response plots for dsb induction in the two lines. The data can be fitted by linear regression. The slopes and intercepts are shown in table 4.8.

Table 4.8 Neutral elution parameters of NB1-G and XRNB1-G

Cell line	Repair Time (Hours)	Slope x 10^{-3} (Gy^{-1}) (±95% c.l.)	Intercept
NB1-G	0	16 ± 5	0.023 ± 0.18
	2	5.27 ±1.4	-0.032 ± 0.089
XRNB1-G	0	18 ± 6	-0.048 ± 0.1
	2	3.9 ± 1.2	-0.013 ± 0.075

There is no evidence for any difference between the two lines in terms of dsb induction. The cell lines also show the same capacity for DNA double strand break repair. The levels of residual damage found after the two cell lines had been allowed two hours repair time at 37°C are shown in Figure 4.23. By comparing the slopes of the elution profiles in the presence and absence of repair, it appears that after two hours both lines repair approximately 70% of the initial damage.

Figure 4.22 Fraction of DNA eluted as a function of dose. Data represent three independent experiments, error bars represent standard errors of the mean.

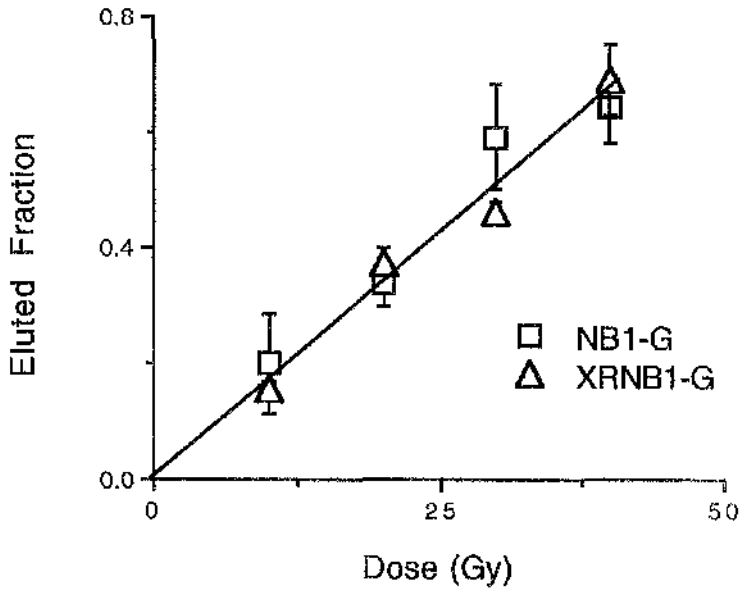
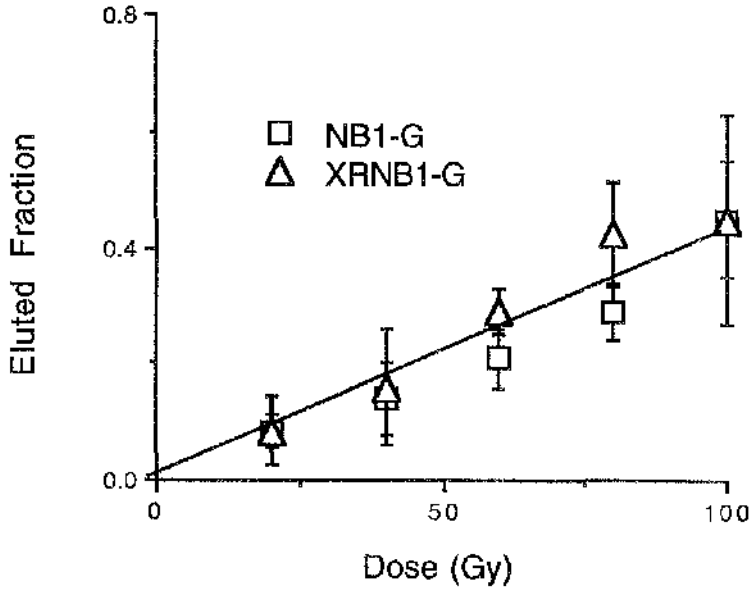


Figure 4.23 Fraction of DNA eluted after 2 hours repair. Data represent three independent experiments, error bars represent standard errors of the mean.



4.3.5 The consequences of radiation damage: micronucleus formation and apoptotic death.

Chromosomal damage to NB1-G, XRNB1-G and clone F cells following radiation was compared using the micronucleus assay. Micronuclei are acentromeric chromosomal fragments extruded from the nucleus after mitosis. Using cytochalasin B to prevent cells separating after mitosis, micronuclei can be observed in binucleate cells, as shown in figure 4.24a. Figure 4.25 shows that the dose-response relationship for micronucleus formation is essentially the same for all three lines.

The assay depends on scoring binucleate cells, and this requires cells to pass through mitosis. Inter-line differences in cell cycle kinetics, particularly in G_2 delay, could affect the proportion of binucleate cells and influence the result. However, after 24 hours in cytochalasin B the irradiated parent and resistant cells had a binucleate fraction of approximately 0.5. For unirradiated cells the proportion of binucleate cells was higher (0.66).

The contribution that micronuclei are making to cell kill can be assessed if micronucleus formation is plotted as a function of the mean number of lethal lesions per cell. According to Poisson statistics, cell survival can be related to lethal lesion frequency by the expression:

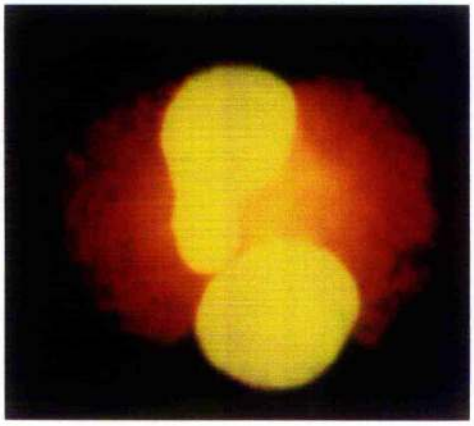
$$SF = e^{-N}$$

where N is the average number of lethal events per cell. Using the linear-quadratic equation,

$$N = \alpha D + \beta D^2$$

(continued p114)

Figure 4.24 Micronucleus formation and apoptosis



A Micronucleus formation in a binucleate NB1-G cell

B Apoptotic HL 60 cell treated with camptothecin

C Apoptotic NB1-G cell 12 hours after 5 Gy

Figure 4.25 Micronucleus formation as a function of dose. Points are derived from counts of at least 500 cells . Error bars represent standard errors of the mean.

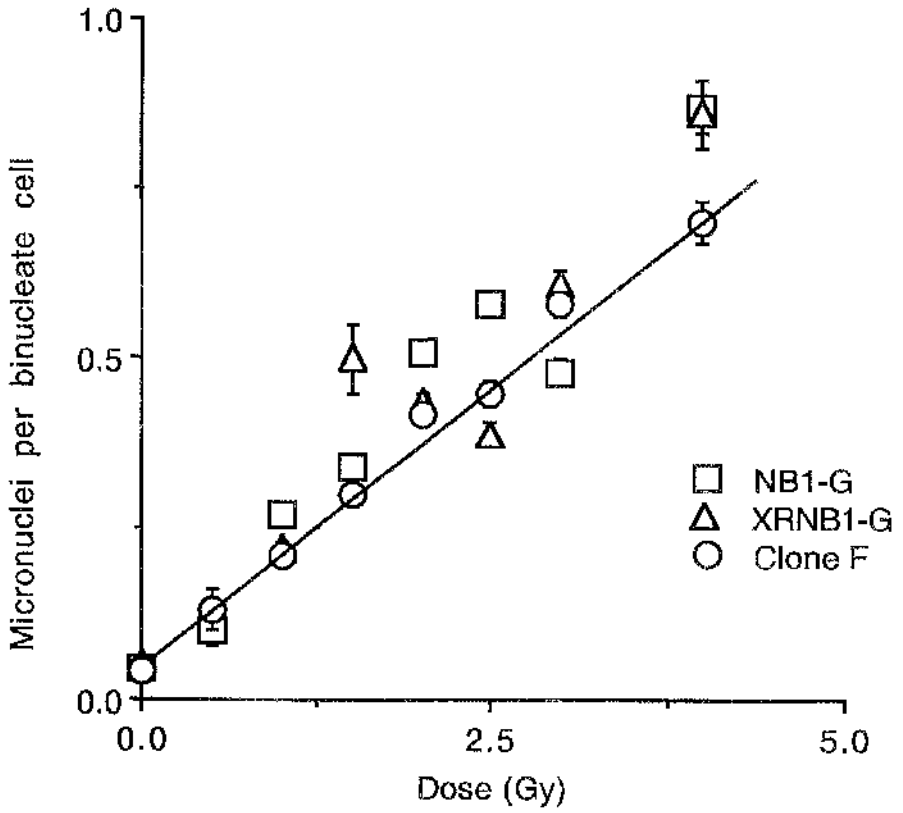


Figure 4.26 Micronucleus formation as a function of lethal lesion numbers. Dose was converted to mean number of lethal lesions per cell, using the linear-quadratic survival parameters, on the basis that one natural log of cell kill corresponds to an average of one lethal event per cell. The broken line represents the condition where there is a one-to-one relationship between lethal events and micronucleus formation. Heavy lines were fitted to the data by linear regression. Error bars represent standard errors of the mean.

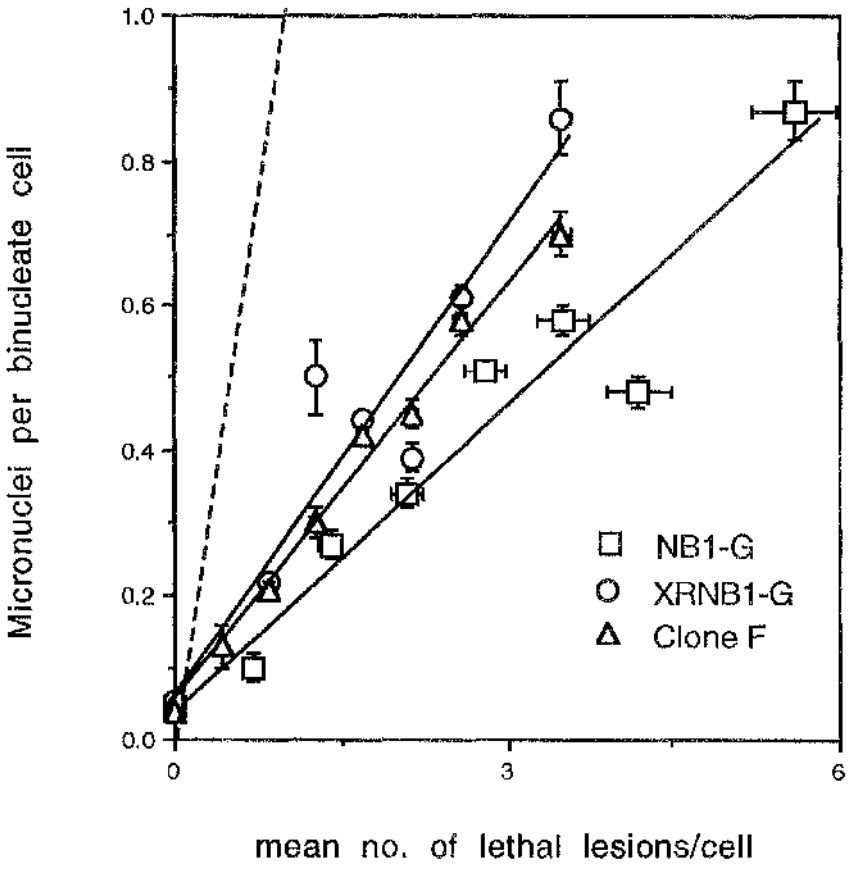


Figure 4.26 shows that the numbers of micronuclei per binucleate cell are considerably less than the mean number of lethal lesions per cell, though the mis-match is greater for NB1-G cells than for the resistant lines. The slopes and 95% confidence limits of the dose response curves shown in figure 4.26 are:

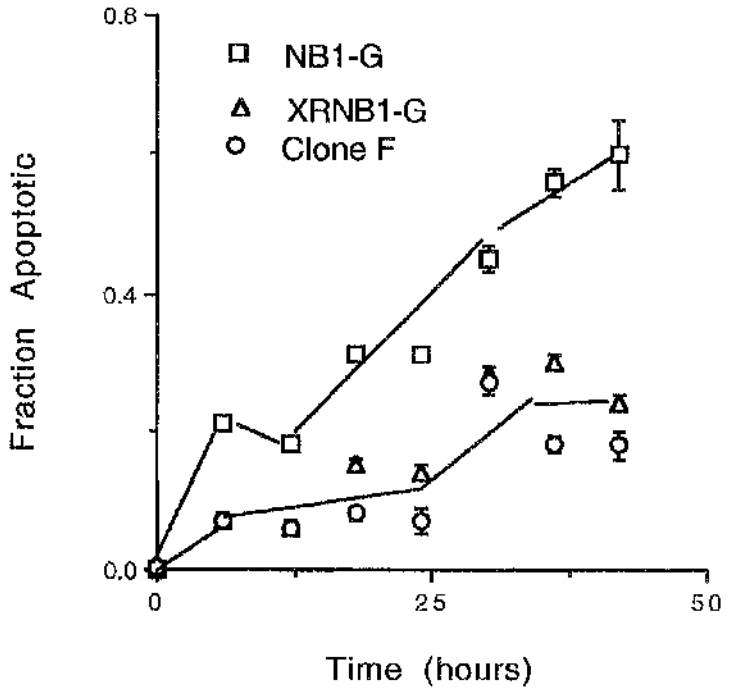
NB1-G	0.132 ± 0.036
Clone F	0.193 ± 0.019
XRNB1-G	0.213 ± 0.083

This implies that other forms of lethality are operating, and that they are more significant for NB1-G cells than for the resistant lines.

While conducting these experiments, apoptotic cells were clearly visible on the slides. Figure 4.24b shows an HL60 cell exposed for 4 hours to 0.15 μM camptothecin, a treatment known to induce apoptosis (Solary *et al.*, 1993). Figure 4,24c shows an NB1-G cell 24 hours after a dose of 5 Gy. The morphology of these cells is similar to published photographs of apoptotic cells (Barry *et al.*, 1993). To quantify radiation-induced apoptosis, cells were irradiated with a dose of 5 Gy and fixed at various intervals post-irradiation. Figure 4.27 shows that apoptosis is much reduced in the resistant lines compared to the parent line.

At all time points except zero, there are highly significant differences between each of the two resistant lines and the parent line ($p < 10^{-4}$, as measured by the Pearson Chi-squared test), though the two resistant lines do not differ significantly from each other. The dose used (5 Gy), reduces the

Figure 4.27 The apoptotic fraction following a dose of 5 Gy. Each point is derived from counts of at least 800 cells, counted over three independent experiments. Error bars represent the standard error of the mean. Where no error bar is shown, the error bars were smaller than the symbol.



surviving fraction of NB1-G cells to approximately 0.001 and that of the resistant lines to 0.01. Consequently, for all three lines the great majority of the cells are non-clonogenic and the differences in the apoptotic fraction cannot simply be the result of there being more survivors among the resistant cells.

It would also have been possible to use different doses which produced exactly equal levels of survival in the different lines. There were two reasons for adopting an iso-dose protocol. The cells responded differently to the same dose of radiation in the clonogenic assay, and it was this differential dose-response which needed explanation. Moreover, it seems from the DNA damage studies (figure 4.22), that parental and resistant cells suffer the same amount of double strand breakage for a given dose. Therefore, the apoptosis experiments were designed to test whether the same levels of damage were more likely to initiate the apoptotic pathway in NB1-G cells as compared to the resistant lines. However, it is also possible that other forms of DNA damage can trigger apoptosis and it might be valuable to test for levels and repair of single strand breaks and DNA-protein crosslinks.

The obvious conclusion is that the radioresistant cells are less likely to enter apoptosis following irradiation. If this is so, then the reduced levels of apoptosis may explain the enhanced clonogenic survival. However, there is an alternative explanation which would suggest that the differences in the apoptotic fraction are trivial and do not explain the resistant phenotype. In this scheme, the resistant and sensitive cells are just as likely to enter apoptosis, but after doing so, the resistant cells would lyse more rapidly. To discount this possibility, ideally one would measure how long apoptotic cells remain intact. However, this is difficult since these cells are free-floating and individual cells

cannot be observed over time. As an alternative, total cell numbers were monitored after 5 Gy to determine whether the resistant populations declined faster than the parental. Figure 4.28, despite considerable scatter in the data, shows that over the first 24 hours after irradiation, there are similar increases in the total cell number (as counted on a haemocytometer) in both NB1-G and the resistant cells. After 24 hours there may be a reduction in NB1-G cell numbers which is not seen in the resistant cells. This trend, though it may not be significant, is the opposite of that which would be required in the above scenario. This suggests that the low apoptotic fraction measured in the resistant lines is not due to rapid lysis of apoptotic cells.

The differences in apoptotic frequency between the cell lines may be linked to differences in cell cycle kinetics between the cell lines, if the rate of entry into apoptosis is determined by the rate at which cells pass through the cycle. Unirradiated NB1-G cells have a faster growth rate than the resistant cells (figure 4.12). In this scheme, the influence of cell cycle kinetics should be minimal just after irradiation, but increase with time. However, inter-line differences in the apoptotic fraction appear rapidly after irradiation. At the six hour time point, the fraction of apoptotic cells was 0.21, as compared to 0.07 in the XRNB1-G and clone F cells - a threefold difference. More importantly, the NB1-G cells show a post-irradiation G_1 arrest, (McIlwrath *et al.*, 1994), and it may not be possible to predict the kinetics of irradiated cells on the basis of their unirradiated behaviour. It is very important to extend these studies on post-irradiation kinetics to the clone F cells. This is a major point which will be returned to in the discussion.

Figure 4.29 shows how membrane integrity is lost following 5 Gy

irradiation. For all three lines, the dye-permeable fraction started to rise six hours after radiation, suggesting a lag between entering apoptosis and membrane destruction. By the Pearson chi-squared test, the dye permeable fraction was significantly lower in XRNB1-G cells than in NB1-G cells, at all time points beyond six hours. (Even at twelve hours, where the differences between the lines were slight, $p < 0.05$.) The same applies to the clone F cells, except for the 18 hour point. It is possible to compare the complete data sets, using the Mantel-Haenszel statistic. By this measure both XRNB1-G and clone F cells differ from NB1-G ($p < 10^{-4}$) but not from each other.

Figure 4.28 Total cell numbers following a dose of 5 Gy. Data were obtained by haemocytometer counts of at least 200 cells per point, over two independent experiments. Error bars represent the standard error of the mean. For the purpose of comparison, the numbers are expressed relative to the initial cell count.

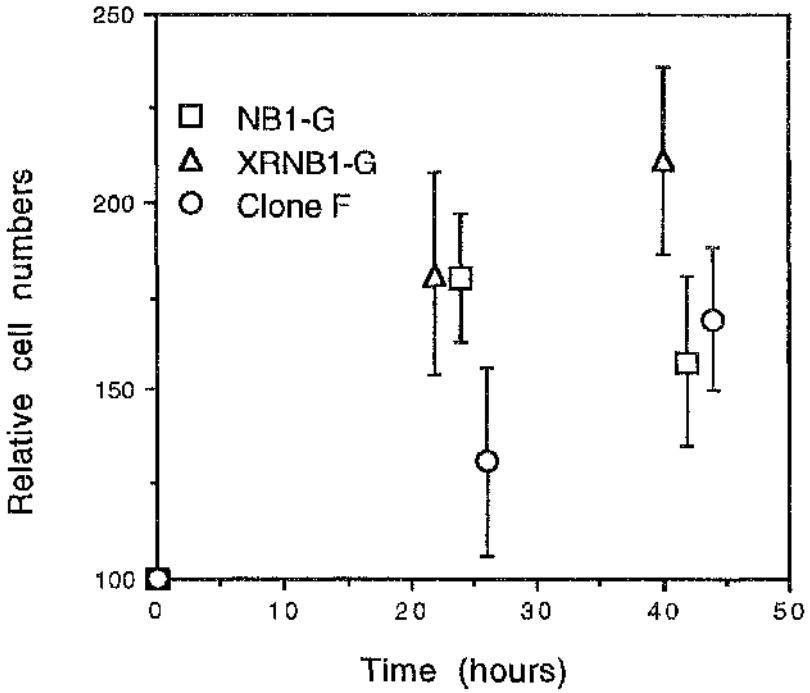
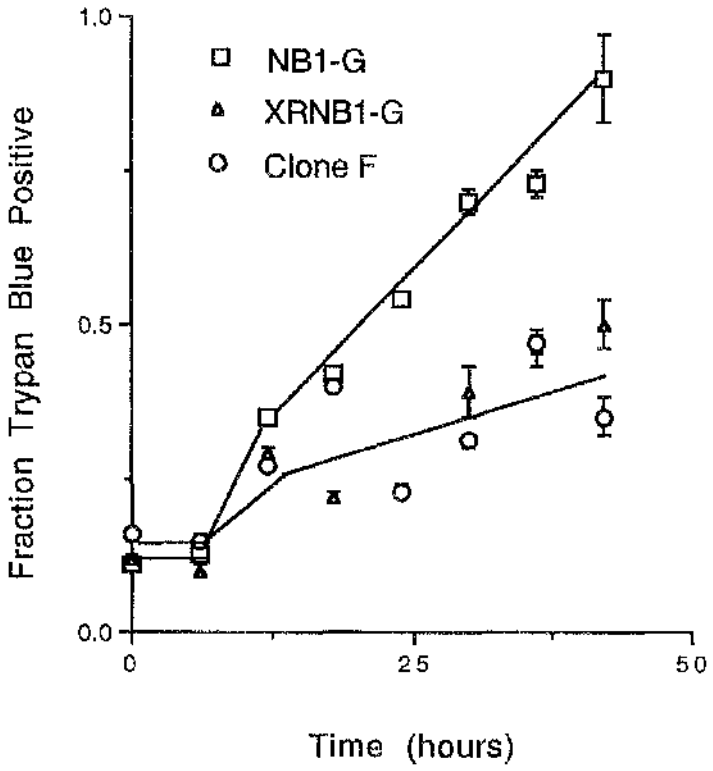


Figure 4.29 Loss of membrane integrity following a dose of 5 Gy. Each point is derived from counts of at least 200 cells, made in at least two independent experiments. Error bars represent the standard error of the mean. Where no error bar is shown, the error bars were smaller than the symbol.

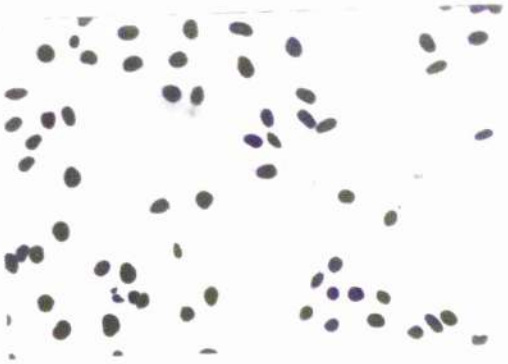


4.3.6. p53 status of NB1-G and clone F cells.

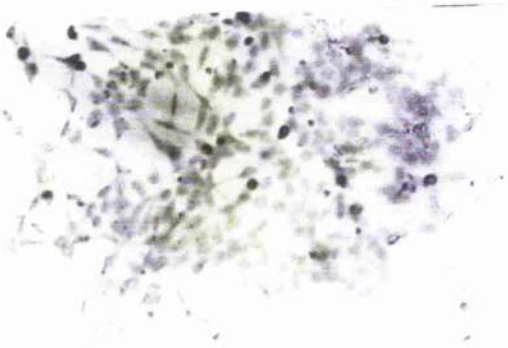
p53 is known to play an important role in mediating radiation induced apoptosis (Merrit *et al.*, 1994; Lowe *et al.*, 1993). The majority of reported mutations in the p53 gene are missense mutations. These seem to occur almost exclusively within the region covered by exons 5 to 8 (Friend, 1994) which codes for the DNA binding region of the protein (Vogelstein and Kinzler, 1994). Such mutations frequently give rise to a protein with an extended cellular half-life, resulting in accumulation of the protein (Levine *et al.*, 1991). In contrast, the wild-type protein is normally expressed at significantly lower levels. Immunostaining ordinarily only detects cells expressing mutant p53 (Hall *et al.*, 1991). Figure 4.30 shows NB1-G and clone F cells stained using the antibody PAb 1801, which detects wild-type and mutant p53. The cell line SCC4, which is known to express mutant p53 (Burns *et al.*,1993) is shown for comparison. There is no detectable p53 product in the neuroblastoma cells.

To confirm these results, the p53 transcripts from NB1-G and clone F cells were amplified using reverse transcriptase-PCR, and sequenced. Sequencing was carried out over codons 115-315, which covers exons 5-8. A sample sequence is shown in figure 4.31. Figure 4.32 shows the same sequence matched against the published sequence (Harlow *et al.*, 1985), as obtained from the Genebank (accession, K03199). Mismatches between the sequences were resolved either by comparison with the antisense sequence or by re-sequencing. No mutation was detected in this region. However, nonsense and frameshift mutations have been reported outside of exons 5-8. In breast cancer, a recent study found that such mutations accounted for over 20% of the

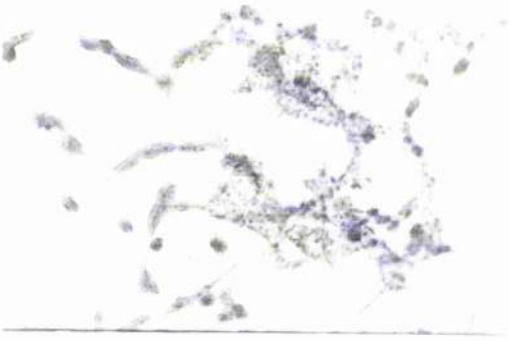
Figure 4.30 Immunostaining of cells with anti-p53 antibody. a) SCC4 b) NB1-G c) clone F cells.



A SCC4 cells



B NB1-G cells



C Clone F cells

Figure 4.31 Sample histogram obtained from the automatic sequencer. The sequence shown was obtained from amplified NB1-G p53 product. The sequencing primer was GTTTCCGTCT GGCTTCTTGC, which hybridises at codons 108-115.

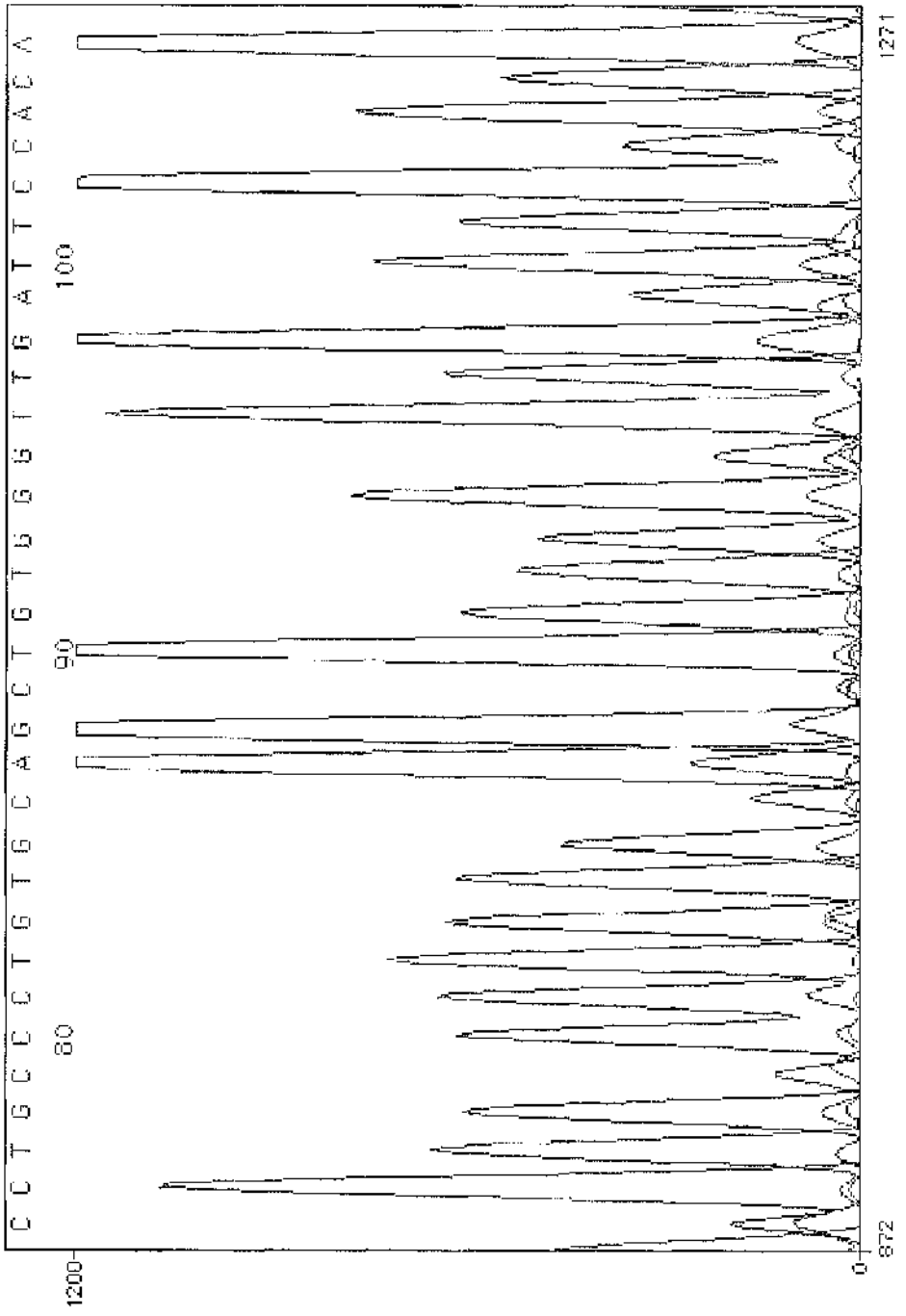
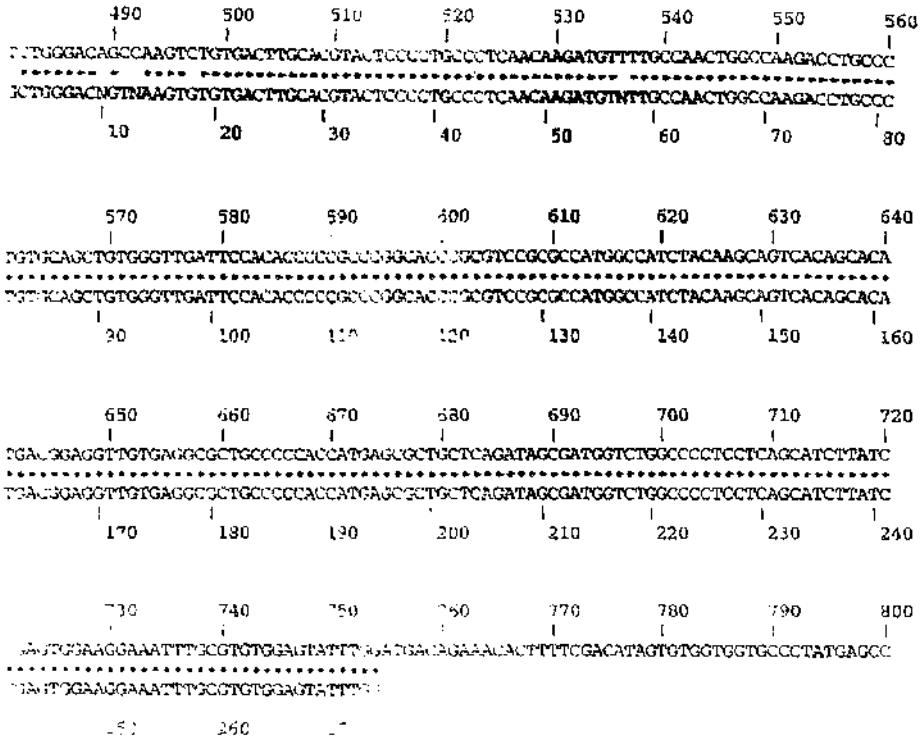


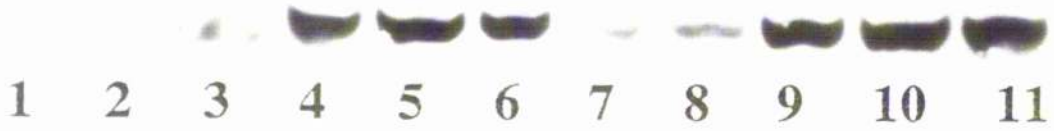
Figure 4.32 A sample NB1-G p53 cDNA sequence, matched against the published wild-type sequence.



total (Hartmann *et al.*, 1995), and the sequencing results obtained here do not exclude this possibility.

In normal cells, p53 expression rises following irradiation (Kastan *et al.*, 1991). p53 expression was studied in NB1-G and clone F cells by western blotting. Figure 4.33 shows that for both lines, levels of the protein increase substantially following radiation. Densitometry revealed that eight hours after irradiation, cells of both lines had a four-fold increase in p53 protein.

Figure 4.33 p53 protein expression at 0 or 8 hours after 5 Gy irradiation. Lanes 1-3 NB1-G, 0 hours; lanes 4-6 NB1-G 8 hours; lanes 7-8 clone F, 0 hours; lanes 9-12 clone F 8 hours. Each lane represents an independent protein sample.



5 DISCUSSION

5.1 THE CONTACT EFFECT

A Chinese hamster cell line, known to form gap junctions between cells, (figures 4.2 and 4.3) was found not to display a contact effect (figure 4.1, table 4.1). (This line was selected for these studies since a sub-clone, DON 6 had been shown to lack gap junctions (Slack *et al.*, 1978).) Equally, a neuroblastoma line NB100 also displayed gap-junctional communication, without showing any contact effect (figures 4.6 and 7). These findings seem to contradict Dertinger's hypothesis (Dertinger *et al.*, 1982, 1983), namely that those cell lines that form gap junctions also possess the contact effect. In fact, gap junctional formation is a feature of most cell lines, normal or malignant, whereas the contact effect has been found in only a minority of the cell lines that have been studied (see chapter 2). For these reasons it seems unlikely that the contact effect is entirely the result of intercellular communication. However, it is possible that gap-junctions are necessary but not sufficient for the contact effect.

In a study of six human ovarian carcinoma cell lines, Rofstad and Sutherland (1986) only observed a contact effect in the most sensitive cell line. This led them to suggest that the contact effect might be found mainly in radiosensitive cell lines, perhaps because the underlying mechanism, whatever

it might be, is normally operational in radioresistant cell lines, regardless of their culture conditions, but is only switched on in radiosensitive cells when they are grown as spheroids. Some support for this comes from published data (reviewed in chapter 2). The nine cell lines in this data base that displayed a contact effect had a mean SF_2 of 0.31 when grown as monolayers, compared to 0.5 for the other lines.

To extend these observations, three human neuroblastoma lines were tested for a contact effect. Neuroblastoma cell lines are generally radiosensitive (Fertil and Malaise, 1981, 1985; Deacon *et al.*, 1984; Steel and Peacock, 1989): published data suggest a mean SF_2 of 0.11. Of the three neuroblastoma lines studied here (figures 4.4 to 4.6) IMR 32 and NB1-G were typically radiosensitive with an SF_2 for cells grown as monolayers of 0.12 and 0.085. However, NB100 was more radioresistant with an SF_2 of 0.5. Schwachofer *et al.* (1992) found an SF_2 of approximately 0.2 for NB100, though these authors obtained values for D_o and n similar to those presented here. This suggests that the difference lies in the initial slope of the survival curve, a parameter that is distinct from D_o and n.

None of the lines displayed clear evidence of a contact effect. This finding in itself does not rule out the hypothesis that radiosensitive lines are more likely to display a contact effect. However, it was shown (figure 2.1) that all the cell lines with monolayer SF_2s below 0.2 exhibited a contact effect. The results obtained here with IMR 32 and NB1-G cells suggest that a contact

effect is not universal, even in very radiosensitive cell lines. Therefore it seems that even if the contact effect occurs primarily in radiosensitive cell lines, it is not exhibited by all such lines. Further in depth studies of the relationship between radiosensitivity and contact effect would require the generation of a large number of survival curves covering a spectrum of radiosensitivity in monolayer and spheroid cultures. Such an investigation was beyond the scope of the present study.

5.2 THE EFFECT OF TRANSFECTION WITH THE ONCOGENES H-*ras* AND c-*myc* ON THE RADIOSENSITIVITY OF A MINK EPITHELIAL CELL LINE.

The effect of the oncogenes, H-*ras* and c-*myc*, on cellular radiosensitivity was examined using the mink cell line Mv1Lu as a model. Two transfected lines were derived from Mv1Lu, each containing one of the two oncogenes. As shown by Khan *et al.*, (1991) using immuno-peroxidase staining, both sub-lines expressed the transfected sequences, whereas the parent line was negative for both oncogene products. Western blotting (figure 4.8) indicated over-expression of c-*myc* and H-*ras* in the transfected lines. The *ras* transfectants have decreased doubling times, and their growth is unaffected by low serum concentrations (table 4.3). The transfected lines are both significantly more tumourigenic in nude mice than the parent line (Khan *et al.*, 1991). The *ras*-transfected cells also show greater resistance to doxorubicin and vincristine, while both transfected lines show increased sensitivity to etoposide (Wishart *et al.*, 1991).

The survival curves for the transfected lines are shown in figure 4.9. Compared to the vector controls, the *ras*-transfected cells were the most radiosensitive as judged by the D_o values. However the 95% confidence limits on the D_o values all overlapped, so the differences cannot be considered

significant. As shown in table 4.4 the same applies to the other parameters, n, α and β. Nor was there any large difference in the SF_2 values. Analysis of variance failed to separate either of the oncogene transfected lines from the vector controls, though it should be noted that there was no appropriate vector control available for the *c-myc* transfected cells. It is unlikely that the difference in D_o between the *ras*-transfected cells and the vector controls is real, since previous work with this line revealed a slight trend for *ras* transfected cells to be more resistant to radiation (Russell *et al.*, 1992).

One problem with studying monoclonally derived transfected sublines is that the presence of any clonal heterogeneity in the parent line may mask the effects of the transfected sequences. However, variation in radiosensitivity between five vector control lines was slight, and it seems reasonable to conclude that there may not be much radiobiological heterogeneity in the Mv1Lu cells. Despite this, it would still have been appropriate to examine the radiosensitivity of a number of *ras* transfectants, rather than one, since the possibility exists that the *ras* transfectant derives from an unrepresentative clone. In retrospect, a superior experimental design could have eliminated this possibility, which cannot, at present, be totally excluded.

Ras mutations are common in many cancers (Bos, 1989; Rodenhuis, 1992); consequently it is important to know whether they affect cellular response to cytotoxic agents. Fitzgerald *et al.* (1985) initiated the approach of transfecting mutated *ras* into recipient cells and measuring their radiosensitivity

and there have been a number of similar subsequent studies, recently reviewed in Cassoni (1994). However, the situation is complicated since some workers report that *ras*-transfection increases radioresistance, while others find no effect. It seems that the recipient cell line influences the outcome: in this regard epithelial cells may be a more appropriate model than fibroblasts, since carcinomas are derived from epithelial cells. The only other study to use epithelial cells was that of Alapetite *et al.*, (1991), who found that activated *ras* had no effect on radiosensitivity. It is possible to induce fibroblastoid cells to adopt an epithelial morphology and vice-versa (reviewed in Birchmeier and Birchmeier, 1994). Such a strategy might help determine whether the effects of activated *ras* on radiosensitivity are specific for fibroblasts.

A further issue is the possible requirement for *ras* and *myc* to co-operate in generating radioresistance. In rat embryo fibroblasts, *ras* transfection only affects radioresistance if accompanied by c-*myc* (McKenna *et al.*, 1990). Both oncogenes are also needed for transformation of rat embryo cells (Land *et al.*, 1983), raising the possibility that radioresistance is linked to transformation. However Khan *et al.*, (1991) showed that Mv1Lu cells were transformed by either oncogene alone. Clearly transformation does not lead to enhanced radioresistance in this system. This result also suggests that Mv1Lu cells (an immortalized line), are a poor model for oncogenesis, since activated *ras* on its own will not transform primary epithelia (Kumar *et al.*, 1990). Presumably the cells are in a pre-malignant state as the result of some

unknown extra lesions. These experiments would be easier to interpret if performed on primary cultures, which should be a closer approximation to a well-defined system.

If *ras* mutations confer resistance to cytotoxic therapy, then tumours with such mutations should have a poorer prognosis. However, *ras* mutations have been found to predict poor outcome for patients receiving surgery alone (Mitsudomi *et al.*, 1991). This suggests that *ras* mutations lead to increased tumour aggressiveness, besides any possible increased resistance to cytotoxic therapy. If *ras* mutations do sometimes lead to increased radioresistance, then compounds such as Lovastatin, (Schafer *et al.*, 1989) which interfere with p21 function, might specifically radiosensitise cells with *ras* mutations.

5.3 SELECTION OF A RADIORESISTANT VARIANT BY REPEATED EXPOSURE TO FRACTIONS OF X-RADIATION

5.3.1 Establishing a radioresistant subline

A radioresistant variant of NB1-G was obtained by exposing the cells of the parent line to repeated fractions of 2 Gy, with 5 - 7 days recovery allowed between each fraction. This resulted in a statistically significant decrease in the radiosensitivity of the exposed cells, as measured by the clonogenic assay (figure 4.10, table 4.5). The linear-quadratic parameters presented in table 4.5 suggest that while the NB1-G and clone F survival curves display little or no curvature, there is considerable bending in the XRNB1-G survival curve. This may be an artefact of the fitting process, as there is some uncertainty attached to the estimates of α and ß. However, at all dose points beyond 1 Gy, there were statistically significant differences in the surviving fractions between the resistant lines and the parent line, though there were no such differences between the two resistant lines. This is shown in figures 4.10 and 4.13, where the error bars represent 95% confidence intervals. Increased radioresistance was also shown in the XRNB1-G cells using the spheroid growth delay assay. This supported the clonogenic data (figures 4.15 to 4.17). (Spheroid growth delay was not performed on clone F cells.)

One explanation for the increased radioresistance in the irradiated cultures might be that NB1-G cells mount an adaptive response to radiation, as has been observed in human lymphocytes (Olivieri *et al.*, 1984). However, the fact that a stably resistant clone was obtained (figures 4.13 and 4.14) suggests that this is not an adaptive response. Moreover, previous work on the response of NB1-G cells to fractionated radiation delivered over a three day period produced no evidence of induced radioresistance (Wheldon *et al.*, 1987). We therefore believe that the selective pressure of repeated irradiation had favoured the emergence of a radioresistant clone.

Regarding the phenotypic instability of the XRNB1-G cells, the simplest explanation is that the XRNB1-G population contained a small proportion of wild-type cells that succeeded in overgrowing the culture when the periodic irradiation was stopped. Consistent with this is the fact that the NB1-G cells had a faster doubling time (22 hours) than the XRNB1-G cells (33 hours) (figure 4.12) and that we isolated a stably resistant clone. It is possible that the XRNB1-G population may be composed of clones of differing degrees of stability. To test this fully, radioresistance would have to be studied in a range of clones.

5.3.2 Cytogenetic studies

Karyotypic analysis of NB1-G and XRNB1-G cells (figures 4.19 and 20) revealed that both populations shared five chromosomal abnormalities: namely trisomy of chromosome 1; a deletion on chromosome 9p; chromosomes 15 and 16 both abnormal, carrying extra material on the p and q arms respectively, and only one sex chromosome (X). This implies that the radioresistant line is indeed derived from NB1-G, rather than being the product of inadvertent cross-contamination by another cell culture (e.g., Nelson-Rees and Flandermeyer, 1977), especially since these aberrations are not common in neuroblastomas. (For a review of this subject, see Brodeur and Moley, 1991.) In addition to these aberrations, the XRNB1-G cells analyzed carried a further 3 to 7 identifiable chromosomal changes (table 4.7). These probably accumulated throughout the course of the radiation treatment used to select the radioresistant cells.

5.3.3 The basis for enhanced radioresistance in the XRNB1-G and clone F cells

The enhanced clonogenic survival of the resistant cells is accompanied by a reduced frequency of radiation-induced apoptosis (figure 4.27), suggesting that the two events are causally linked. Depressed levels of apoptosis might be due to the enhancement of some general radioprotective mechanism - for example, if the resistant cells sustained less initial damage compared to the parental line. However, induction and repair of DNA double strand breaks, as measured by neutral filter elution, were similar in NB1-G and XRNB1-G cells (figures 4.22 and 23, table 4.8). Chromosomal aberrations are believed to represent the link between double strand breaks and mitotic death. The dose-response curve for micronucleus formation was the same for the parent and resistant lines (figure 4.25), implying that the difference between the lines is confined to the apoptotic pathway.

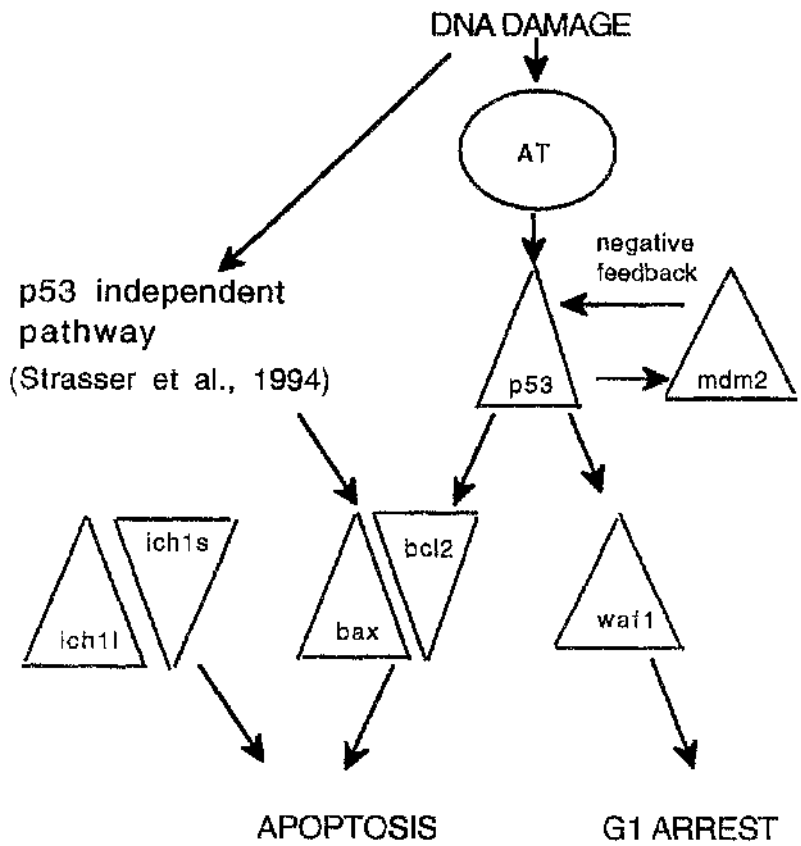
However, interpreting the micronucleus data is complicated by the presence of radiation-induced apoptosis. There are two possible problems. The first is that yields of micronuclei in all the lines may be underestimated. This would happen if the presence of micronuclei caused cells to enter apoptosis. If the two events are unrelated, then apoptosis would remove the same proportion of cells with and without micronuclei. The second is that the greater amounts of apoptosis found in NB1-G cells might lead to the underestimation

of the yield of micronuclei relative to the resistant cells. This could only happen if there were a greater probability of micronucleus formation leading to apoptosis in the NB1-G cells. This hypothesis thus requires two differences between the NB1-G and resistant lines. The resistant cells must be less susceptible to micronucleus formation and less prone to activate the apoptotic pathway in response to micronuclei. It is difficult to devise an experiment that could conclusively settle this issue and this explanation remains a possibility. However, the simpler hypothesis is to take the data at face value, and assume that there is only one difference between the lines, namely the reduced tendency of the resistant cells to undergo radiation-induced apoptosis.

With these caveats in mind, the results indicate that for NB1-G cells at least, radiation-induced apoptosis contributes to their elevated radiosensitivity. Intuitively, a causal link between apoptosis and radiosensitivity seems obvious. However, an alternative hypothesis is that apoptosis might represent a separate mode of death for cells that were in any case doomed to mitotic failure. This point has been made by Hickman, (1992) and Smets (1994). There is one report of cells acquiring radiation-induced apoptosis through c-*myc* transfection, without becoming more radiosensitive (Chen *et al.*, 1994).

The events that lead from radiation insult to apoptosis are partially understood. For a recent review see Oltvai and Korsmeyer (1994). A simplified version of the process is shown in figure 5.1. The tumour suppressor gene p53

Figure 5.1 A schematic representation of some of the steps which lead from DNA damage to apoptosis or G_1 arrest.



is necessary for radiation-induced apoptosis in gastro-intestinal crypt cells. (Merritt *et al.*, 1994), bone marrow cells (Lotem and Sachs, 1994), fibrosarcoma cells (Lowe *et al.*, 1994) and non-cycling thymocytes (Lowe *et al.*, 1993a), though cycling thymocytes and some other lymphocyte subpopulations can undergo radiation-induced apoptosis in the absence of p53 (Strasser *et al.*, 1994; Seki *et al.*, 1994). A variety of forms of DNA damage lead to increases in p53 expression (Khanna and Lavin, 1993; Nelson and Kastan, 1994), implying that p53 is a target for several damage- recognition systems. Upstream of p53 is the AT gene. Cells from AT individuals induce p53 after UV radiation, though not after ionising radiation (Khanna and Lavin, 1993). p53 activity is also controlled by the gene *mdm2*, in a negative feedback loop. The *mdm2* product is up-regulated by p53 (Juven *et al.*, 1993) and *mdm2* binds p53 and inactivates it (Momand *et al.*, 1992). Constitutive overexpression of *mdm2* through gene amplification is common in sarcomas (Oliner *et al.*, 1992), where *mdm2* amplification seems to act as a substitute for p53 mutation.

Downstream of p53 is the *Bcl-2/ bax* system; *Bcl-2* is a membrane bound protein that can override p53 induced apoptosis (Chiou *et al.*, 1994). *Bcl-2* forms a heterodimer with *Bax*; the ratio of the two proteins helps determine whether cells enter apoptosis or not (Oltvai *et al.*, 1993). p53 alters the ratio by inducing the synthesis of *Bax* and repressingthe synthesis of *Bcl-2* (Selvakumaran *et al.*, 1994). Dual control is also exercised by the *Ich-1S* and *Ich-1L* proteins; *Ich-1L* promotes and *Ich-1S* represses apoptosis (Wang *et al.*,

1994). These proteins are homologues of the protease, interleukin-1β converting enzyme (Thornberry *et al.*, 1992), which promotes apoptosis.

p53 also induces G1 arrest as well as apoptosis (Kastan *et al.*, 1991). How cells decide between the two fates may depend on whether the stop signal is over-ridden by signals for cell cycle progression (Fisher, 1994). Lowe *et al.* (1993b) found that apoptosis and G_1 arrest were mutually exclusive. Fibroblasts expressing wild-type p53 would arrest in G_1 after irradiation. However, if the cells were transfected with either the viral gene E1A, or mutant H-*ras*, the cells continued into S phase, and died through apoptosis. Similarly, the transcription factor *E2F-1*, which positively regulates entry into S-phase co-operates with p53 to induce apoptosis (Wu and Levine, 1994).

Following irradiation, NB1-G cells arrest in G_1 and G_2/M (McIlwrath *et al.*, 1994). In view of the findings of Lowe *et al.*, (1993b) it is surprising that this G_1 arrest does not suppress the apoptotic response. The G_1 arrest studies have not yet been extended to the resistant cells and this is clearly a very important question. Making comparisons between the cell cycle distributions of the lines following irradiation will be complicated by the differences in the apoptotic fractions and will require the combination of standard flow cytometric techniques for measuring G_1 arrest (Kastan *et al.*, 1991) with the measurement of apoptosis (Darzynkiewicz *et al.*, 1992). These experiments would have to be done to determine the role that cell cycle kinetics play in the resistant phenotype.

After radiation, both NB1-G and clone F cells showed similar increases in the levels of p53 protein (four-fold, at eight hours post-irradiation; figure 4.31), implying that the cell lines do not differ with regard to the proteins upstream of p53. However, to analyze fully the p53 response would require measuring levels at several different time-points. Both the wild-type and resistant cells seemed to have normal p53, as judged by immunostaining (figure 4.30) and sequencing over exons 5-8. This region which spans the DNA binding region is where most p53 mutations are found (Levine at al, 1991; Friend 1994). It is possible that the cells carry a mutation outside exons 5-8. Such mutations are generally nonsense or frameshift mutations (Hartwell *et al.*, 1995), and do not lead to overexpression of p53, as judged by immunostaining. Normal p53 function could be confirmed by the induction of proteins such as *p21* (El-Deiry *et al.*, 1993) or *GADD45* (Kastan *et al.*, 1994), which are upregulated by p53.

The investigations have not revealed the molecular basis for the radioresistant phenotype but it seems that the answer may lie downstream of p53. A particularly attractive candidate is *Bcl-2*, since this gene is implicated in neuroblastoma. High expression correlates with other markers of poor prognosis (Castle *et al.*, 1993) and renders neuroblastoma cells resistant to cis-platin and etoposide induced apoptosis (Dole *et al.*, 1994). Another possibility is that cells differ with regard to *mdm2* levels, though *mdm2* amplification seems to be absent in neuroblastomas (Waber *et al.*, 1993) .

It is also possible that differences in lesion repair might be involved, since G_1 and G_2 arrest have yet to be investigated. However, the results suggest that any repair differences would have to act on lesions that are involved only in apoptosis and are not responsible for micronucleus formation. An important point is whether repair of DNA damage can impede progress towards apoptosis or whether p53 induction initiates an irreversible pathway. Transfection experiments have shown that wild-type p53 can produce apoptosis in undamaged tumour cells (Yonish-Rouach *et al.*, 1991), suggesting that DNA repair cannot completely determine events downstream of p53.

5.3.4. The emergence of XRNB1-G cells as a model for the evolution of tumour radioresistance.

In these experiments, the radioresistant phenotype was selected by repeated exposure to 2 Gy. In this respect the experiments modelled a clinical radiotherapy regime. Do the results imply that irradiated tumours also may evolve under selective pressure? The cells acquired radioresistance through partial suppression of the apoptotic pathway, loss of which is an important step in oncogenesis (Symonds *et al.*, 1994). Therefore the changes observed in the irradiated cells *in vitro* may well correspond to *in vivo* processes. The ideal approach would be to address this question directly. This could be done by comparing tumour cells from biopsies taken before and after radiotherapy. Some groups (e.g. Davidson *et al.*, 1990), use biopsy material for predictive assays of tumour radiosensitivity; however there might be ethical problems in performing biopsies on relapsed patients unless the procedure offered some benefit to the patient.

The evolution of tumour radioresistance has been mathematically modelled by Yaes (1989), who showed that radioresistant subpopulations could determine the outcome of therapy, even if they comprised only a small fraction of the initial tumour cell population. This approach assumes that the only factor governing the ratio of resistant to sensitive cells is their differential

survival after radiation. Work by Leith *et al.* (1989) has questioned this assumption. Irradiating tumours results in damage not only to the tumours but also to the surrounding normal tissue, which in consequence becomes less able to support tumour growth (Milas *et al.*, 1986). This is known as the tumour bed effect. By xenografting mixtures of two clones derived from a human colon adenocarcinoma into previously irradiated mice, Leith *et al* (1989) have shown that the tumour bed effect acts to eliminate the minority clone. The effect may be based on competition for a limited supply of nutrients between the two populations, where the majority population always has some advantage. Whether this "competitive exclusion" is a universal phenomenon is unknown, nor is it clear whether it would be sufficient to suppress the emergence of a radioresistant clone during the course of radiotherapy. The clone F/NB1-G system would provide a good model to answer these questions.

6 FUTURE WORK

6.1 THE CONTACT EFFECT

Although the contact effect was discovered twenty years ago, its cause is still not fully understood. From alkaline unwinding experiments, Olive and Durant (1985) concluded that differences in DNA conformation exist between V79 cells grown as monolayers and spheroids, and suggested that this might influence the rate of DNA repair. Surprisingly, the rate of repair in V79 monolayers and spheroids has not been measured directly and so the question remains open.

The evidence from published data, collated here, suggests that the contact effect is most frequently found in radiosensitive human tumour cell lines. Although the results obtained from two radiosensitive neuroblastoma lines weaken this hypothesis, it cannot yet be discounted. It is well known that cell lines cultured from radioresponsive tumour types are generally radiosensitive, at least in monolayer culture (Fertil and Malaise, 1981, 1985; Malaise *et al.*, 1987; Deacon *et al.*, 1984). Any concentration of the contact effect in radiosensitive cells would undermine this relationship. This possibility is important enough to justify further studies, since it is widely assumed (Steel *et al.*, 1989a) that sufficient variation in cell radiosensitivity exists to explain the clinically observed variation in tumour response. However if the contact

hypoxia and repopulation may have a greater effect on clinical outcome than is currently recognised.

6.2 ONCOGENES AND RADIORESISTANCE

Many groups have worked on the relationship between *ras* expression and radiosensitivity. However, activated *ras* only enhanced radioresistance in some lines. Although the cells used here, Mv1Lu, were not affected by *ras* transfection, it is possible that *ras/myc* cotransfection would lead to increased radioresistance. Failing this, it is difficult to see how the Mv1Lu model can be used for any further investigations in this field.

6.3 THE RADIORESISTANT NEUROBLASTOMA CELL LINES.

The most interesting aspect of this work was the selection of a radioresistant variant XRNB1-G and the cloning of a stably resistant subline, clone F. The cells acquired resistance to radiation by becoming less prone to radiation induced apoptosis. This might be due to superior repair, though the implication is that the repair could only act on lesions relevant for apoptosis. Alternatively, there could be some difference in expression of the genes that promote or repress cell death. One way of approaching this would be to transfect the resistant and parent cells with a temperature sensitive p53 mutant. Wild-type p53 can induce apoptosis in tumour cells in the absence of DNA damage (Yonish-Rouach *et al.*, 1991). Exposing the neuroblastoma cells to high levels of p53 separate from any radiation exposure, would show whether the cells differed in their ability to cope with DNA damage or with p53 initiated apoptosis.

Other investigations would follow on naturally from here. If DNA repair is implicated, then studies on G_1 delay would be a logical first step. If the cells differ in their response to p53, there are several proteins downstream that can be studied. Of particular importance concerning apoptosis is the *Bcl-2/bax* system. However, p53 induces other proteins that are not yet identified (Buckbinder *et al.*, 1994). Studies on protein expression using two dimensional

gels could provide useful information. If the difference between the resistant and parent cells cannot be explained in terms of a known protein, subtractive hybridisation would be an important investigation. This might allow the effects of any isolated cDNA to be studied through transfection. However, as the transfected gene(s) would not be under its normal control, anti-sense transfections that block gene function might be more informative.

The evolution of radioresistance in a cell line raises the question of whether a similar process occurs in tumours undergoing radiotherapy. As discussed above, the best approach to this problem would be to take biopsy material from tumours before and after radiotherapy. However, simply applying the selection procedure used here to different lines also would be valuable. It may be that radioresistance is most easily acquired through changes in the apoptotic response. This could be studied by comparing lines with and without a tendency to radiation-induced apoptosis.

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