

ADAPTIVE RESPONSES TO IONIZING RADIATION
IN NORMAL HUMAN SKIN FIBROBLASTS

by

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To Sonia, Milène and André

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ABSTRACT

Plateau-phase normal human skin fibroblasts (AG1522) pre-exposed to low-dose-rate ionizing radiation (IR) became less susceptible to the lethal effect of a subsequent acute challenge dose of radiation. A threshold and an optimum adapting dose were observed. This adaptive response (AR) at the survival level was accompanied by a decreased number of chromosomal breaks due to the challenge dose, as indicated by a reduction in the frequency of micronucleus formation. The frequency of micronucleus formation was further reduced when an incubation period at 37°C separated the challenge dose and the adapting dose delivered at either low- or high-dose-rate. The rate of reduction of micronucleus formation was higher for the low dose-rate as compared to the high dose-rate adapting dose, suggesting that the ability of human cells to adapt to radiation increases with decreasing rates of damage. A certain amount of damage per unit time with which the cell can cope appears to be necessary to trigger the AR to IR. The rate of repair of DNA double-strand breaks, as indicated by the frequency of micronucleus formation, was higher in adapted cells, suggesting that the mechanism of adaptation could include increased repair capacity and/or an increased ease of access of repair enzymes to the lesion. Adapted cells also showed a much longer delay in reaching the binucleate state than non-adapted cells, suggesting a second mechanism of adaptation which may increase the time available for DNA repair. The analysis of RNA from adapted cells showed a decreased level of cyclin A and cyclin B transcripts consistent with a mechanism leading to a delay in the progression of the cells in the cell cycle. The transcript levels of other genes possibly involved in the cellular response to IR were also altered.

Rodent C3H 10T½ cells showed a similar adaptation when assayed for micronucleus formation. The adapted cells were also protected against transformation to malignancy by a subsequent high dose of radiation. Transformation frequency was reduced about two-fold by low-dose-rate adapting doses ranging from 0.1 to 1.5 Gy.

Flow cytometric measurements showed that the cell cycle distribution of the plateau phase cells used was unaltered during the various treatments, indicating that the observed AR cannot be attributed to selection of cells at a radioresistant stage of the cell cycle.

RÉSUMÉ

Les fibroblastes de peau humaine normale en phase plateau (AG1522) préexposés à un rayonnement ionisant (RI) à faible débit de dose sont devenus moins sensibles à l'effet léthal d'une dose de rayonnement aiguë postérieure de provocation. On a observé une dose d'adaptation seuil et une dose optimale. Cette réaction d'adaptation (RA), au niveau de la survie, a été accompagnée d'une diminution des cassures chromosomiques dues à la dose de provocation, comme l'indique la réduction de la fréquence de formation des micronoyaux. La fréquence de formation des micronoyaux a été réduite davantage lorsque, par une période d'incubation à 37°C, il y a eu séparation de la dose de provocation et de la dose d'adaptation délivrée à un débit soit faible soit élevé. La vitesse de réduction de la formation des micronoyaux a été plus élevée à la suite d'exposition à une dose adaptative délivrée à un débit faible par rapport à un débit élevé - ce qui laisse supposer que la capacité des cellules humaines de s'adapter au rayonnement augmente lorsque la vitesse de détérioration diminue. Il semble qu'un certain degré de détérioration par unité de temps, que peut supporter la cellule, soit nécessaire pour déclencher la réaction d'adaptation au RI. La vitesse de réparation des cassures des doubles chaînes de l'ADN, comme l'indique la fréquence de formation des micronoyaux, a été plus élevée chez les cellules adaptées - ce qui laisse supposer que le mécanisme d'adaptation pourrait comprendre l'augmentation de la capacité de réparation et/ou l'augmentation de la facilité d'accès des enzymes de réparation à la lésion. En outre, les cellules adaptées ont manifesté un retard beaucoup plus grand à atteindre l'état binucléé que les cellules non adaptées - ce qui laisse supposer un deuxième mécanisme d'adaptation qui pourrait augmenter le temps disponible pour la réparation de l'ADN. L'analyse de l'ARN de cellules adaptées a montré une baisse de la teneur en messagers de la cycline A et de la cycline B qui coïncide avec le retard de division observé. La teneur en messagers d'autres gènes, qui entrent probablement en jeu dans la réaction des cellules au RI, a changé aussi.

Les cellules 10T½ du rongeur C3H ont également manifesté une adaptation semblable lorsqu'on les a analysées pour déterminer la formation des micronoyaux. En outre, les cellules adaptées ont été protégées contre la transformation en tumeur maligne par une forte dose de rayonnement postérieure. On a réduit la fréquence de transformation d'environ 50% par une dose d'adaptation à faible débit variant entre 0,1 et 1,5 Gy.

La cytométrie du flux a montré que la distribution, dans le cycle cellulaire, des cellules en phase plateau utilisées, n'a pas changé au cours des divers traitements - ce qui indique qu'on ne peut pas attribuer la RA observée à la sélection de cellules à un stade radiorésistant du cycle cellulaire.

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INTRODUCTION

For the purposes of radiation protection, the deleterious effects of radiation are assumed to have no dose threshold and to show a linear dose response, with low-dose-rate exposures resulting in reduced effects by about a factor of two. The effects of sequential doses are assumed to be additive (UNSCEAR 1988). One consequence of a linear, no threshold hypothesis is the assumption that exposure to any dose of radiation, however small, can potentially result in detrimental human health effects. Increasing experimental evidence in human and other mammalian cells, however, shows that exposure of lymphocytes (Olivieri et al. 1984), fibroblasts (Ikushima 1987, Azzam et al. 1992a, 1992b), bone marrow cells and germ cells (Cai and Liu 1990) to doses as low as 0.01 Gy induces a process for the repair of chromosomal breaks which reduces the amount of chromosomal damage caused by a subsequent exposure. This phenomenon, termed adaptive response, has been found to be dependent upon the adapting dose, dose rate, expression time (Shadley et al. 1987, Shadley and Wiencke 1989), culture conditions (Olivieri and Bosi 1990, Olivieri et al. 1992), pH (Bosi et al. 1991) and stage of the cell cycle (reviewed in Shadley 1994). An adaptive response to ionizing radiation has been observed for several biological end points including chromosomal aberrations (Khandoguina et al. 1991), chromatid aberrations (Olivieri et al. 1984), sister chromatid exchanges (Moquet et al. 1989), micronucleus formation (Ikushima 1987), and gene mutations (Sanderson and Morley 1986). These observations in mammalian cells mirror the evidence for the existence of radiation-inducible DNA repair systems in procaryotes and lower eucaryotes (Samson and

Cairns 1977, Boreham et al. 1991a). However, investigation of the adaptive response in human lymphocytes indicates an individual variability in the response (Olivieri and Bosi 1990). Several investigators found donors whose cultured blood lymphocytes did not express an adaptive response or even expressed a synergistic response, i.e., a higher frequency of chromosome aberrations in adapted cultures than in non-adapted ones (Bosi and Olivieri 1989, Hain et al. 1992). An explanation for these discrepancies may be that the radiation response of immune system cells is known to be dependent on many variables such as: genetically determined individual radiosensitivity, stress, immune state, age, gender, stage of differentiation, lifestyle factors such as smoking, alcoholic beverages consumption, viral illness, caffeine intake, contraceptive use, etc. (Anderson et al. 1991).

Clearly, induction of resistance to radiation by a prior exposure has significant implications in fractionated dose radiotherapy, for radiation risk assessment and for radiation protection. Therefore, validation of the presence of an adaptive response to ionizing radiation, as has been shown in cultured human lymphocytes (reviewed in Wolff 1992a, 1992b and in Shadley 1994) and Chinese hamster cells (Ikushima 1987, 1989), and an understanding of the molecular mechanisms involved will contribute to our understanding of the cellular effects of low doses of radiation, in particular those which cannot be explained by the conventional mechanisms extrapolated from high dose studies. The approach of our laboratory has been to study the phenomenon of the adaptive response to ionizing radiation in normal human fibroblasts maintained in culture under controlled conditions. This

constituted the first extension of such studies to a cultured human cell line, eliminated the effects of cellular heterogeneity and intra-donor variability that were encountered in studies with human lymphocytes, and examined the adaptive response in a cell type potentially at risk of radiation-induced cancer. Plateau-phase fibroblasts, at a specific passage number, cultured according to a rigorously defined protocol of growth medium constitution, trypsinization, seeding density and feeding regimen were used in the various experiments. Only cell populations at a specific time after seeding and at more than 90% in G_0/G_1 were used. This contrasts to experimentation with human lymphocytes that are a mixed population with varying subsets. The use of lymphocytes, in experiments, requires stimulation with a mitogen to which different subsets respond at a different rate, hence resulting in a variability in their progression in the cell cycle. Most of the cells in the body, however, are relatively quiescent, entering an active cell division cycle infrequently or in some cases not at all during the life span of an individual. The study of G_0/G_1 populations of cells may therefore be more relevant to the majority of human cells *in vivo* and hence to human risks of radiation exposure.

The effects of chronically delivered (adapting) radiation doses on cell survival and micronucleus formation resulting from an acute challenge dose were evaluated in the normal human skin fibroblast cell line AG1522, grown to stationary phase. The dependence of the adaptive response in these cells on both dose and rate of the adapting dose was studied. Changes in the frequency of micronucleus formation in adapted cells were correlated, for the

first time, with altered levels of cellular survival. The amount of damage per unit time to trigger the adaptive response was investigated. The rate of repair of DNA damage in adapted cells was compared with non-adapted cells. The extent of induction of division delay following the various treatments was measured, to investigate whether pre-exposure of cells to an adapting dose altered their progression in the cell cycle following a challenge dose of radiation. A checkpoint where the cellular division process was delayed would potentially provide more time for repair of cellular (DNA) damage. The effect of the radiation priming dose on the expression of various radiation responsive genes was studied.

The effects of an exposure to an adapting dose of radiation, on the carcinogenic risk of a subsequent radiation exposure was studied using the C3H 10T½ cell 'transformation' assay. Recent measurements of *HPRT* mutant frequency and analysis of the molecular nature of the mutants, in human lymphoblastoid cells pre-exposed to a low dose of γ rays and then treated with a high dose of radiation, showed that the low dose pre-treatment affected the number of mutants obtained, and suggested that an error-free repair capacity was induced (Rigaud et al. 1993). An induced error-free DNA repair capacity implies a decreased probability of tumour initiation and hence carcinogenic risk, whereas induced error-prone DNA repair implies a corresponding increase in risk.

LITERATURE REVIEW

Around the time period that the double helical structure of DNA was elucidated, it was widely believed that the genetic material was intrinsically very stable and stood isolated from the routine metabolism of the cell. Whereas RNA and protein 'turned over', DNA was not thought to be subject to any sort of non-replicative biochemical change. Atoms were thought to be permanently incorporated into DNA (Mazia 1952) in order to maintain the high degree of fidelity required of a master blueprint (Friedberg 1985). Subsequent research, however, confirmed the hypothesis of H.J. Muller, when he wrote in 1954 that "perhaps a continual turnover and replacement of at least some of the gene parts is normally occurring... and that in the course of this replacement missteps occasionally occur, whereby a new gene part is substituted which is different, or which becomes arranged and connected up differently, from the old one that it replaces" (Muller 1954). The existence of transposable elements in eukaryotes, including humans (Calos and Miller 1980) and the phenomenon of gene transposition in prokaryotes (Kleckner 1981) proved that the primary structure of DNA is in fact quite dynamic and subject to constant change. Current evidence indicates that organisms continually monitor the integrity of their genetic material, replacing those portions that have been damaged by either exogenous or endogenous events. The efficiency with which these events occur masks their action and contributes to the view that

repair of DNA damage is a very important event, as essential to cellular vitality as are replication and transcription (Bohr and Hanawalt 1984).

It is the accuracy of the repair processes which permits the short term survival of species. While the long term survival of a species may be enhanced by changes in its genetic inheritance, the accuracy and fidelity of the repair processes and their extensive checking procedures ensure that the residual mutation rates are extraordinarily low (Lindahl 1982, Friedberg 1985). The non-deleterious residual mutations became the source of genetic variation upon which natural selection operates in evolution, and leads to the survival of the fittest cells at the expense of the rest of the organism (reviewed in Alberts et al. 1989; Hanawalt and Sarasin 1986). In the extreme case however, uncontrolled cell proliferation or cancer can result. The evidence indicates that these cancers are initiated largely by the accumulation of changes in the DNA sequences of somatic cells. Thus, both for germ-cell stability and for the prevention of cancer resulting from mutations in somatic cells, eucaryotic cells depend on the remarkable high fidelity with which DNA sequences are maintained.

What are the various sources of DNA damage and the ensuing categories of lesions? How are they caused and what are the ways which allow cells to cope with the lesions? The following will be an attempt to review briefly the topic of DNA damage with an

emphasis on those damages induced by ionizing radiation on DNA and chromosomes. The various repair systems implicated in the repair of such damage will be discussed. Following a review of excision-resynthesis and recombinational-type DNA repair mechanisms, radiation-induced radioprotective processes will be emphasized. In particular, this section will summarize our knowledge of the adaptive response to ionizing radiation in mammalian cells, and the state of our understanding to-date of the induced processes in this response.

1. DNA DAMAGE

Damage in DNA is an abnormal alteration in DNA structure, which if misrepaired can lead to a mutation. A mutation is a change in the DNA nucleotide sequence in which normal base pairs are substituted, added, deleted, or rearranged. A mutation does not interrupt the sequence of standard nucleotide pairs, and therefore can be replicated and inherited. On the other hand, the abnormal structure in damaged DNA prevents its replication and therefore its inheritance. However damaged DNA can be repaired.

DNA damage can be introduced either by intrinsic processes or by extrinsic agents like chemicals and radiation. Even without exogenous damage, studies (Eastman and Barry 1992) show that DNA is disintegrating at a rate estimated to exceed 100,000 bases per cell

per day. However, it is well established that most of the damage in DNA can be, and is, repaired by the cell, and all types of DNA damage are not equal in terms of their biological significance (Travis 1989).

1.1 ENDOGENOUS DNA DAMAGE

Endogenous changes in DNA sequence arise as a consequence of errors introduced during DNA replication, recombination and repair itself. Without the intervention of any cellular factors, the difference in free energy for the stable pairing of a complementary base relative to that for a non-complementary base during DNA synthesis is estimated to be only 1 to 3 Kcal/mol (Pitha et al. 1968). Based on this estimate and in the absence of other influences, Loeb and Kunkel (1982) calculated that such a free energy difference would translate into a potential error frequency of 1 to 10 percent per nucleotide. Obviously the spontaneous mutation frequency is considerably less than that predicted from the above energetic considerations. A significant contribution to this much lower frequency stems from the action of specific components of the replication machinery and/or repair machinery. Base selection and proofreading of newly inserted nucleotides are among the factors that increase the accuracy of DNA synthesis (Lindahl 1982).

Base alterations in the DNA may also arise from the inherent instability of specific chemical bonds that constitute the normal chemistry of nucleotides under physiological conditions of temperature and pH (Friedberg 1985). Tautomeric shifts, deamination of bases, as well as loss of bases can occur (Friedberg 1985). Table I lists the estimated rates of occurrence of DNA damage in mammalian cells, and shows that DNA is a very unstable molecule that, even without exogenous damage, is disintegrating at a very high rate. The major causes of the DNA damage listed in Table I arise from spontaneous hydrolysis and interaction with reactive oxygen species (Bernstein and Bernstein 1991). These causes of damage are likely to be omnipresent in nature.

A summation of the events listed in table I below results in about 72,000 events/cell/day. Undoubtedly, intricate pathways of DNA repair must exist to ensure the survival of the cell, and to enable the passing of accurate and complete genetic information to daughter cells. Tice and Setlow (1985) estimated that the maximum rate of repair, in human cells, of single strand breaks in DNA is 2×10^5 /cell/h. Based on data of Waldstein et al. (1982), the maximum rate of repair of O⁶-methylguanines in human lymphocytes is estimated to be 10^4 - 10^5 /cell/h. Compared with the respective rates of occurrence of single strand breaks and O⁶-methylguanines, these rates of repair suggest that repair activity is adequate to cope with these damages. However, experimental results (Bernstein and Bernstein 1991, chapter 4) also show that long-lived, nondividing,

differentiated cells have a relatively low DNA repair capacity, suggesting that these cells may accumulate DNA damage with age.

Table 1. Estimated Rates of Occurrence of Endogenous DNA Damages in Mammalian Cells

Damage	Events per Cell per Day	Reference
Depurination	12,000. 13,920.	Lindahl 1977 Tice and Setlow 1985 Lindahl 1977
Depyrimidination	600. 696.	Lindahl 1977 Tice and Setlow 1985
Cytosine deamination	192.	Tice and Setlow 1985
Single-strand break	55,200.	Tice and Setlow 1985
O ⁶ -methylguanine	3,120.	Tice and Setlow 1985
Glucose-6-phosphate adduct	2.7	Bucala et al. 1985
Thymine glycol	270.	Saul et al. 1987
Hydroxymethyluracil	620.	Saul et al. 1987
8-Hydroxydeoxyguanosine	unknown	Richter et al. 1988; Ames 1989
Unidentified methyl adduct	unknown	Park and Ames 1988a, 1988b
Double-strand break	8.8	*
Interstrand cross-link	8.0	*
DNA-protein cross-link	unknown	*

* Reviewed in Bernstein and Bernstein, 1991
After Bernstein and Bernstein 1991, chapter 2.

Many of the constitutively occurring damaging events such as single-strand breaks, double-strand breaks, base damage and crosslinks within the DNA molecule or from one molecule to another can also occur as a result of exogenous physical damage to DNA.

1.2 RADIATION EFFECTS ON DNA AND OTHER CELLULAR TARGETS

In addition to the endogenous structural alterations, the cellular genome is continually subjected to environmentally induced structural alterations. Our environment contains a multitude of DNA damaging agents which have always been present as an ubiquitous ingredient of nature. However, clear evidence indicates that exposure to high doses of these agents can result in adverse effects. In many cases carcinogenesis can result as a consequence of DNA damage. An obvious source of damage, which living organisms on this planet had to contend with since the beginning of biological evolution, is ionizing radiation. Background radiation has changed little since the emergence of humans four million years ago, but early life forms evolved when earth's radiation was about ten times greater than it is now (Luckey 1991). As a result of modern technology, however, we can become additionally exposed to various sources of man-made radiations. Most of the dose from these artificial sources of exposure is the result of medical diagnosis and treatment, but discharges of radionuclides to the environment and miscellaneous sources such as consumer products and air and space travel also contribute to the dose received.

Unlike UV radiation at approximately 260 nm, which is preferentially absorbed by nucleic acids, ionizing radiation creates highly reactive oxidizing species and can cause damage to all cellular components including DNA. Radiation has been observed

to cause chain breakage in carbohydrates (Phillips 1968), structural changes in proteins (Jayko and Garrison 1986), alteration in the activity of enzymes (Hexum and Fried 1979), and oxidation of lipids (Köteles 1979). Permeability of the cell membrane is altered after irradiation, affecting the transport function of the membrane (Ashwell et al. 1986). Alteration of membranes by radiation can also affect organelles in the cell that are membrane-bound, e.g., mitochondria and lysosomes (Petkau 1980). However, because of their obvious importance to the life of the cell, much attention has been, and continues to be, focussed on chromosomes and in particular on DNA (Ward 1975, reviewed in Tubiana et al. 1990). Moreover, it has been established beyond reasonable doubt that the most sensitive sites to radiation are in the nucleus as opposed to the cytoplasm. Chinese hamster cells irradiated with a dose of α -particles in excess of 250 Gy delivered to the cytoplasm maintained a normal cell proliferation. By contrast the penetration of a few α -particles a distance of a micron or two into the nucleus proved to be lethal (Munro 1970). Compiled circumstantial evidence (reviewed in Hall 1978) strongly indicates that the DNA of chromosomes constitutes an important target for radiation-induced lethality:

- a) Mammalian cells are killed by the β -rays resulting from intracellular incorporation of tritiated thymidine. Since the radioactive thymidine is incorporated in the DNA, this results in a radiation dose almost totally restricted to the nucleus.

- b) Cells grown in a culture medium in the presence of halogenated pyrimidines in the place of thymidine, incorporate these analogues in their DNA. This substitution dramatically increases the radiosensitivity of the cells. Radiosensitivity increases as the level of analogue incorporation increases.
- c) Factors which modify cell lethality, such as a variation in the type of radiation used, the dose rate or the oxygen concentration, also affect the production of chromosome damage in a qualitatively and quantitatively similar fashion.
- d) The radiosensitivity of a wide range of plants has been correlated with the mean interphase volume, which is defined as the ratio of nuclear volume to chromosome number.
- e) A correlation has been established between the radiosensitivity of different cellular species and their DNA content. The mean lethal dose or D_0 is, to a first approximation, inversely proportional to the DNA content of various cells. The D_0 of viruses, bacteria and mammalian cells being on the average 1500, 100 and 1 Gy respectively (Kaplan and Moses 1964).

What are the initial interactions of radiation in the cell?

The following will describe briefly the initial changes that lead to the response of the cell.

Energy deposited from ionizing radiation results in the formation of excited molecules and ionized species in critical biological targets (e.g. DNA) or in the medium in which the targets are suspended (e.g. water). Such products are either fairly rapidly deactivated to the starting substrate or result in free radical formation (reviewed in Singh and Singh 1982). Based on the site of these interactions, the action of radiation on the cell has been described as either "direct" or "indirect" (Hall 1978, Ward 1988).

Direct action occurs when radiation deposits its energy directly in the organic target (Figure 1), resulting in ionization, excitation or free radical formation of this target. Such action is more likely to occur after exposure to high linear energy transfer (high LET) radiations, such as particles and neutrons, than after exposure to sparsely ionizing (low LET) radiation, such as X-rays. Highly ionizing particulate radiations have appreciable mass and/or charge, and have a greater probability of interacting with matter than low LET radiations. They lose energy rapidly, producing many ionizations in a very short distance. Scission is recognized to be quite probable after an ionization because the amount of energy received is large.

Figure 1. Direct and indirect effects of X- and γ -rays on DNA (from Tubiana et al. 1990).

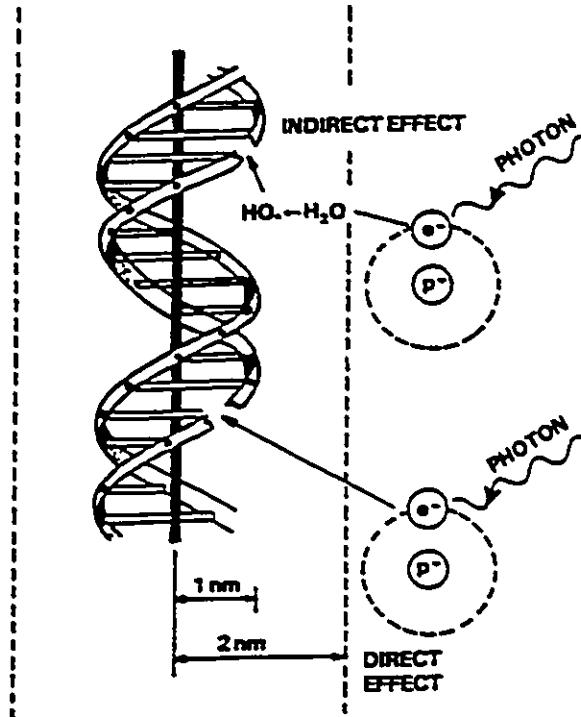
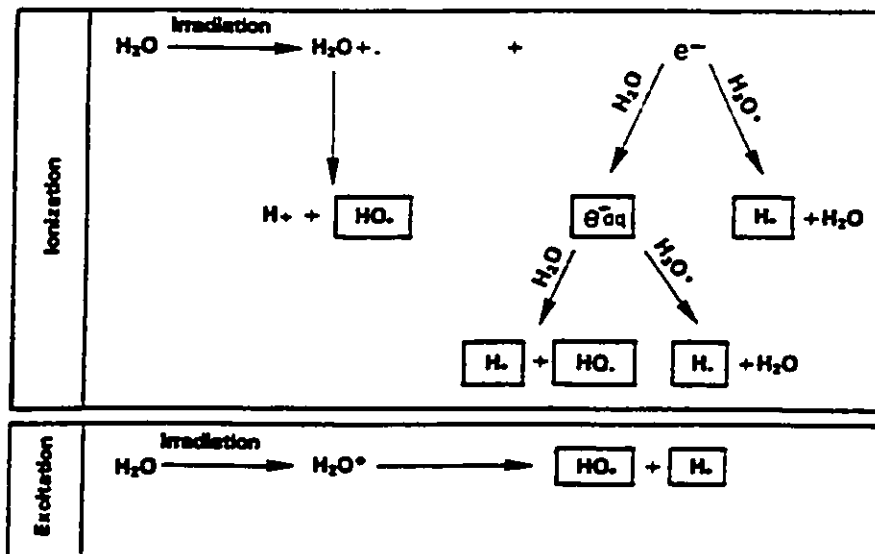


Figure 2. Representation of the stages in the radiolysis of water (from Tubiana et al 1990).



With the indirect type of damage, the organic molecule is damaged by reactive species produced by ionizations elsewhere in the cell. Since 80% of the cell consists of water, indirect action primarily occurs through ionizations of water molecules. The interaction of radiation with water (Figure 2) results in the production of highly reactive diffusible species termed free radicals. Free radicals, symbolized by a dot, contain a single unpaired electron in their outer shells, a state which confers to them a high degree of reactivity.

Free radicals can undergo a number of reactions, a few of which are:

1. Recombining with each other and producing no damage, e.g., $\cdot\text{H} + \cdot\text{OH} \longrightarrow \text{H}_2\text{O}$
2. Joining with other free radicals, possibly forming a new molecule that may be damaging to the cell, e.g.:
 $\cdot\text{OH} + \cdot\text{OH} \longrightarrow \text{H}_2\text{O}_2$ (Hydrogen peroxide is an agent that is toxic to the cell. Along with organic hydroperoxides, it is the most likely source of continuing damage in the post-irradiation period (Singh and Singh 1982).
3. Reacting with normal molecules and biological macromolecules in the cell, forming new, reactive or damaged structures, e.g.:
 $\text{H}\cdot + \text{O}_2 \longrightarrow \text{HO}_2\cdot$ ($\text{HO}_2\cdot$ is a new free radical)
 $\text{RH}_2 + \text{H}\cdot \longrightarrow \cdot\text{RH} + \text{H}_2$ ($\cdot\text{RH}$ is an organic free radical)
 $\text{R} + \cdot\text{OH} \longrightarrow \cdot\text{ROH}$

The effects of free radicals in the cell are compounded by their ability to initiate chemical reactions, and therefore potential damage, at distant sites in the cell. The distance that free radicals can travel is dependent on their reactivity. For example, OH^\cdot is extremely reactive and will react with the first molecule it encounters. The superoxide radical O_2^\cdot is about 5 orders of magnitude less reactive and can move considerable distances. While indirect damage occurs primarily from radicals formed by the ionization of water, the ionization of other cellular components, such as fat, also can result in free radical formation (Singh and Singh 1982, Petkau 1987).

Regardless of whether the mechanism of damage is direct or indirect, all cellular components are potentially a target upon which radiation can act. While some molecules, like ribosomal proteins and RNAs, are present in millions of identical copies per cell; others, like DNA, are present as only one or two. Moreover, the instructions for the cell to grow, divide and ultimately to produce all other cellular molecules are encoded in the sequence of the DNA. It is for these reasons that DNA is considered to be the critical molecule in the cell.

Radiation damage to DNA can be divided into five categories (reviewed in Tubiana et al. 1990):

1. Single-strand break: A single-strand break (ssb) can be as simple as a cleavage of the phosphate diester

bond, between the phosphate and the deoxyribose. Such a break would be easily religated. However, a ssb may also be complex and result in gaps in one strand of the DNA. Frequently, the ends have altered structures resulting from damage to the deoxyribose moiety in the backbone or from covalent cross-linking to a protein. This prevents a simple religation of the ends. A large proportion of single strand breaks are produced through the indirect action of $\text{OH}\cdot$ as has been shown by the use of compounds which specifically trap this radical (reviewed in Greenstock 1981). Following breakage of the sugar backbone, the two strands of DNA separate allowing water to penetrate between the strands, breaking hydrogen bonds between the bases. The efficiency of radiation-induced single-strand breaks was found to decrease with increasing linear energy transfer (LET) (Ritter et al. 1977). Hypoxia and exogenously supplied thiol compounds reduced the yield of these breaks in irradiated mammalian cells (Edgren et al. 1981).

2. Double-strand break: A double-strand break (dsb) involves breakage of the two adjacent strands of DNA at points less than three nucleotides apart. It can be produced either by a single event, or by the combination of two single strand breaks in complementary strands if the second event in the same

region occurs before the first break has had time to be repaired. A dsb is homologous if it occurs at the same pair of bases, otherwise it is heterologous. While the number of ssb is directly proportional to dose over a wide range (0.2-60 000 Gy), the relationship between dose and dsb in mammalian cells is disputed. Some authors find that it is linear and others that it is linear quadratic (in Tubiana et al. 1990). Blöcher and Pohlit (1982) showed that cell survival curves could be interpreted on the basis of one unrepaired double-strand break being a lethal event. The dsb is the only molecular lesion known whose yield increases with increasing LET of the radiation applied (reviewed in Frankenberg-Schwager 1989). It has been estimated that following low LET radiation damage to DNA, single- and double-strand breaks would occur in ratios of about 50 to 1 (Singh and Singh 1982).

3. Base damage: The bases can be partially destroyed, chemically modified or lost entirely, resulting in an alteration of the coding sequence. Hydroxylation can occur with the formation of hydroperoxide in the presence of oxygen. Pyrimidine bases have been found to be more radiosensitive than purine bases in their response to radicals produced by the indirect effect. It is estimated that after a given dose, the total

number of damaged bases in human lung fibroblasts is twice the number of DNA single-strand breaks (reviewed in Frankenberg-Schwager 1989)

4. Destruction of sugars: 0.2-0.3 alterations of deoxyribose have been estimated to occur per 10 ssbs. The mechanisms of such alterations are still not well understood. The sugars are oxidized, then hydrolysed with liberation of the base, with or without breakage of the phosphodiester bond.
5. DNA inter- and intra-strand cross-link and DNA-protein cross-links: Interstrand DNA cross-links prevent DNA strand separation, and therefore can block DNA replication and transcription. They are a minor product of ionizing radiation. DNA-protein cross-links are also known to occur as a result of the exposure of cells to ionizing radiation. Experiments with Chinese hamster V79 cells and exponentially growing leukemia mouse cells have shown that DNA-protein cross-links are generated linearly with radiation dose. The yield of these cross-links was reduced in the presence of cysteamine, an OH scavenger, indicating an indirect process. Treatment of the cells with dimethylsulphoxide also resulted in a decreased yield of DNA-protein cross-links, again suggesting a role for OH radicals in the generation of this type of damage (Oleinick et al. 1986).

Some of these radiation induced changes in the DNA molecule do not necessarily result in major changes in chromosome structure. However, it is well established that radiation induced DNA lesions such as double-strand breaks can lead to gross chromosomal abnormalities with serious consequences for the cells.

1.3 RADIATION EFFECTS ON CHROMOSOMES

Radiation-induced chromosome breaks can occur in both somatic cells and in germ cells, and can be transmitted during mitosis and meiosis respectively. They can be observed microscopically during post-irradiation cell division and are evident during metaphase and anaphase. At these stages the chromosomes reach their greatest condensation, become shortened and are visible by light microscopy. Various fixation, staining and banding techniques, as well as dynamic techniques have been developed to analyze chromosomes (reviewed in Tubiana et al. 1990).

The interaction of radiation with a chromosome can result in breakage of the chromosome, thereby producing two or more chromosomal fragments. The ends of the broken chromosomes can undergo the following steps: 1) they can be restituted to their original state, therefore resulting in no apparent damage to the cell, or 2) proceed to give rise to an acentric fragment following the loss of part of the chromosome or chromatid at the next

mitosis. This process is called a deletion. 3) The broken ends can get rearranged to produce a distorted chromosome such as a ring chromosome, a dicentric chromosome or an anaphase bridge. 4) The broken ends can get rearranged resulting in chromosomal translocations and inversions. The latter changes do not result in microscopically visible chromosomal damage, and may not be immediately lethal to the cell. However they result in rearrangement of the genes on the chromosomes, and therefore cause a change in the heritable character of the cell. New techniques based on recombinant DNA technologies such as fluorescence in-situ hybridization have contributed to detecting and quantifying the broad spectrum of DNA and chromosomal lesions. It is becoming possible to describe the radiation lesions responsible for specific endpoints.

Depending on the position of the cells in the mitotic cycle at the time of irradiation, various types of aberrations are observed. While a chromosome aberration denotes a lesion which occurred prior to the duplication of chromosomal DNA in S phase, chromatid aberrations are those produced in individual chromatids when the cell is irradiated after DNA synthesis. When one chromatid of a pair is damaged, only one daughter cell will be affected. However, if the repair of a chromosomal lesion is not completed prior to DNA synthesis, then both chromatids will exhibit the damage, and both daughter cells will inherit a damaged chromatid. If the cells are irradiated during S phase, at a time

when the chromosomes are only partly duplicated, a mixture of chromosome and chromatid aberrations is produced. Finally, if the cells are exposed to ionizing radiation during prophase, sub-chromatid aberrations can be seen involving subunits of the chromatids.

A series of mechanisms might be involved (reviewed in Therman and Susman 1993) in the process leading to the formation of aberrant chromosomes. Many of these still remain unknown. However, it is clear that the primary event is damage to the DNA which has not been repaired or has been misrepaired. Hittleman (1990) showed that the frequency of remaining breaks decreases through G_2 , since the majority of the breaks are restituted.

One approach to monitor chromosome damage and repair is through the enumeration of micronuclei in cultured cells (Countryman and Heddle 1976). This approach was utilized in the course of this study to evaluate the effects of a priming dose of radiation on chromosomal damage due to a subsequent challenge dose of radiation. The method is simple and rapid and is an alternative to classical cytogenetic methods; it is statistically more precise than karyotypic analysis for quantitation of chromosomal damage. For the same time needed to analyze 100 metaphase spreads for chromosomal aberrations, several hundred cells can be scored for micronucleus formation. Moreover, the micronucleus technique has the potential to be automated (Schreiber et al. 1992). It is being

evaluated as a biological dosimeter following *in vivo* exposure to ionizing radiation (Fenech et al. 1991) and it was shown to be a useful tool to study the effects of radiation dose-rate and split dose effects in a variety of cells (Vral et al. 1992). Micronuclei contain acentric chromosome fragments or whole chromosomes which are not incorporated into the main nucleus at mitosis (Miller et al. 1992) and which consequently appear in micronuclei in those cells that have undergone a nuclear division. Transmission electron microscopy (Schiffman and De Boni 1991) showed that micronuclei exhibit structural details typical of interphase nuclei. Specifically, micronuclei exhibited morphological evidence of a nuclear lamina and segregation of karyoplasm into euchromatic and heterochromatic regions. Examined micronuclei were enclosed by a nuclear envelope of normal morphology and showed nuclear pore complexes. Recent development in the technique (Fenech and Morley 1985, 1986) using a cytokinesis blocking agent, allows the scoring of micronuclei specifically in those cells that have completed one nuclear division. The use of kinetochore labelling, as well as telomeric and centromeric probes, can further distinguish micronuclei containing whole chromosomes and those containing acentric fragments. Also it has been shown that, independently of the dose and time after irradiation, about 60% of radiation-induced micronuclei arise from acentric fragments and a dose-dependent relationship has been observed in various cell systems exposed *in vitro* to ionizing radiation (Miller et al. 1992).

2. REPAIR OF DNA DAMAGE

The various types of radiation-induced DNA damage differ in their biological significance. Some may cause an early death of the cell. Others may take years to express their effects. The evidence from research with prokaryotes and lower eukaryotes, however, indicates that much of the damage in DNA can be, and is, repaired by the cell. The isolation of DNA repair deficient mutants in microorganisms contributed to the elucidation of many of the molecular processes underlying repair pathways in prokaryotes and lower eukaryotes (reviewed in Friedberg 1985). The adaptation of standard techniques of microbial genetics to somatic cell genetics has contributed to the isolation of many mutant rodent cell lines sensitive to ionizing radiation (reviewed in Collins 1993). These mutants and cell lines derived from patients showing sensitivity to DNA-damaging agents are helping to unravel the repair mechanisms operating in mammalian cells. Various mechanisms for repairing lesions in DNA have been described (reviewed in Tubiana et al. 1990). The various schemes have been postulated to include sets of enzymes that survey the damage in DNA, and repair it by restoring the correct nucleotide sequence, ordinarily depending on the replacement of damaged information by intact information from a redundant copy (Bohr et al. 1987). The enzymatic pathways involved in repair vary with the type of damage introduced. However, our knowledge of the molecular mechanisms of repair of ionizing radiation damage in mammalian cells remains

incomplete. Nevertheless, with the application of molecular techniques, progress is occurring at an accelerated rate, and last year we saw a succession of novel and exciting discoveries in DNA repair. Genes for DNA repair are being cloned and associated with human diseases (Cleaver 1994).

It is well established now that the damage due to sparsely ionizing radiations such as x-rays and γ -rays is repaired. This phenomenon has been demonstrated in many systems, including cells in culture, and in normal tissues and malignant tumours in experimental animals (reviewed in Travis 1989). A positive correlation of mammalian life span with DNA repair capacity has been found (reviewed in Bernstein and Bernstein 1991). The more effective a cell is in preventing or repairing DNA damage, the more slowly it ages. Based on the type of experiment performed, two types of repair have been defined, and are known by the operational terms of **sublethal damage repair** and **potentially lethal damage repair**. Sublethal damage repair occurs when two doses are separated by time. It was first described by Elkind and Sutton (1960). Their investigations with cells in culture have shown that when a given dose of radiation was divided into two equal doses separated by various intervals of time, the surviving fraction of cells was larger than if the same total dose were given as a single dose. Sublethal radiation damage and its repair have been measured for almost every cell that can be grown *in vitro*, and for every normal tissue *in vitro* for which a quantitative endpoint is

available (reviewed in Travis 1989). In general, the amount of sublethal damage repair agrees well with the size of the shoulder on the survival curve. It is considered that this type of repair takes place with a half-time of 0.5-1.5h, depending on the type of the cell (reviewed in Tubiana et al. 1990)

Potentially lethal radiation damage repair (PLDR) was first demonstrated by Phillips and Tolmach (1966). It refers to the repair of radiation damage caused by a single dose of radiation, and which can occur only if the post-irradiation conditions are conducive for repair. Some post-irradiation conditions (reviewed in Azzam and Raaphorst 1985) that permitted the repair of PLD were: incubation with metabolic inhibitors, exposure to temperatures around 20°C, incubation in balanced salt solutions, and holding in stationary growth phase. The repair of PLD was correlated with increased survival levels, and a loss of chromosomal aberrations (Dewey et al. 1971), sister chromatid exchanges (Nakatsugawa et al. 1978), and a decreased giant cell formation (Hetzel and Kolodny 1976).

While the phenomena of sublethal and potentially lethal damage repair are widely described, little is known about the molecular mechanisms which eliminate the radiation-induced lesions, reconstitute the original structure of the DNA, and result in restoration of viability. Survival, however, does not always imply restoration of the DNA because there can be induction of genetic

mutations or chromosomal aberrations that are compatible with viability. While error-free repair mechanisms restore the DNA to its original state, error-prone repair, such as the SOS mechanism in bacteria, increase the frequency of mutations (reviewed in Tubiana et al. 1990).

Several pathways have been implicated in the repair of DNA lesions in general. At the molecular level, they all take place under the control of genes which govern repair by the production of enzymes. Constitutive and damage-inducible DNA repair systems have been described in a variety of organisms. The following will be a description of various DNA repair systems.

2.1 REPAIR SYSTEMS

2.1.1 Excision-resynthesis

Excision-resynthesis (Figure 3) is considered to be a major DNA repair mechanism, and is the one that has been most extensively investigated. It is responsible for the removal of many types of lesions, including UV-induced cyclobutane pyrimidine dimers, bulky chemical adducts and a variety of alkylation and base damage (Hoeijmakers 1993). It takes place in DNA molecules which are not in the replication phase. A lesion in one strand of the DNA molecule is recognized, excised and the missing segment is

synthesized using the complementary strand as a template. As described in Figure 3, the sequential steps in this system include: (a) pre-incision recognition of the damage; different glycosylases are known to recognize different types of damage; (b) incision of the damaged DNA strand at or near the site of the defect; (c) excision of the defective site and localized degradation of the affected strand; (d) repair replication to replace the excised region with corresponding stretch of normal nucleotides; and finally (e) ligation to join the repair patch at its 3' end to the contiguous parental DNA strand. Specific enzymatic mechanisms control the different stages of this type of repair (Bohr et al. 1987). For the excision of certain bases, altered by X-rays, UV or alkylating agents, a specific glycosylase cuts the bond between the damaged base and the sugar. At least seven DNA glycosylases have been identified in mammalian cells (Wallace 1988). The resulting abasic site is recognized by a specific endonuclease which cuts the phosphodiester bond at that point. An exonuclease excises the segment of the strand containing the lesion and the repair is completed by the action of a polymerase and a ligase (Bohr et al. 1987).

Single-strand breaks and DNA-DNA cross-links, in mammalian cells, are believed to be repaired by the mechanism of excision-resynthesis, as well as by recombinational repair. A large number of independent genes act in the various mechanisms of excision-resynthesis. About 15 genes have been found to be implicated in *E. coli* (Friedberg 1985). The RAD3 epistasis group defines the genes

The Three Steps in Excision-resynthesis DNA Repair

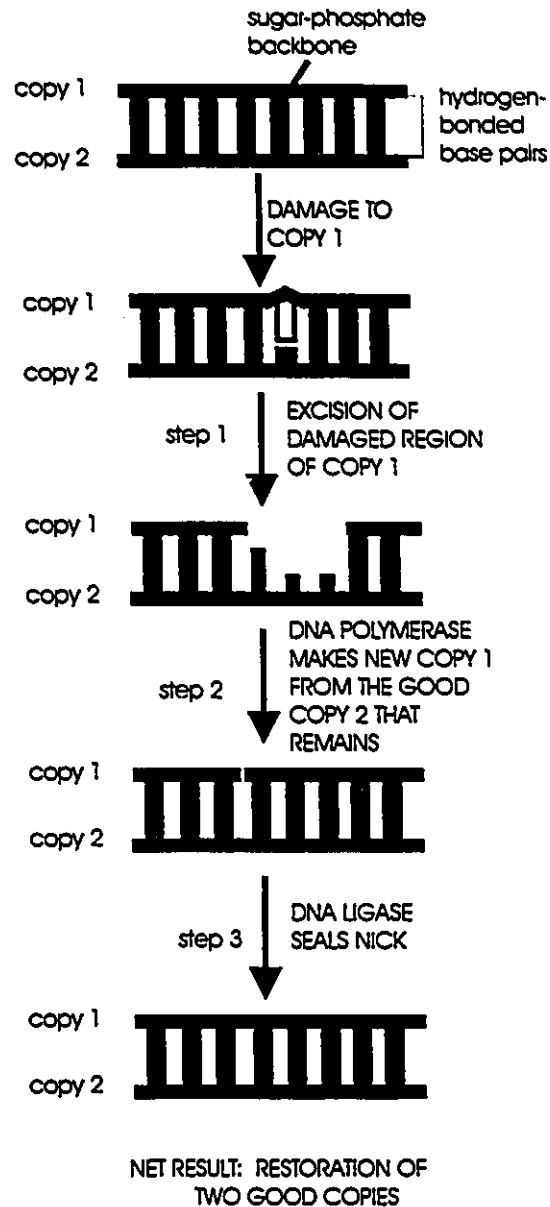


Figure 3. The three steps in Excision-resynthesis DNA repair. In step 1 the damage is excised; in steps 2 and 3 the original DNA sequence is restored. DNA polymerase fills in the gap created by the excision events (step 2) and DNA ligase seals the nick left in the repaired strand (step 3). (From Alberts et al. 1989).

involved in excision repair in yeast (reviewed in Hoeijmakers and Bootsma 1990). Seven *S. cerevisiae* excision-repair genes have been recently sequenced [RAD1, RAD2, RAD3 (human homolog ERCC2), RAD4, RAD7, RAD10 (human homolog ERCC1), ERCC3^{Sc} (human homolog ERCC3)]. A high proportion of the predicted gene products harbour acidic regions that are thought to confer ability to bind chromatin, mediated by electrostatic interactions with basically charged histones. Analysis of excision-deficient mammalian mutants has revealed the existence of a large number of genes involved in nucleotide excision. The mutants are either from patients suffering from excision repair disorders or from laboratory-produced excision-deficient rodent cell lines. Xeroderma pigmentosum patients which suffer from a disorder in excision repair (Cleaver 1968) show >1000-fold increased incidence of skin cancer, and a 10- to 20-fold increased incidence of neoplasia in other organs and tissues. Nine known complementation groups have been assigned to this disease (Hanawalt and Sarasin 1986, Cleaver and Kraemer 1989). Cockayne syndrome is another hereditary disease where patients appear to suffer from a defect in the repair of transcriptionally active genes, which is considered to be one of the excision repair sub-pathways. Three complementation groups are known to exist for this disease (Venema et al. 1990). The greater removal of transcription-blocking DNA damage from the transcribed strand compared with the nontranscribed strand in mammalian genes

is of particular interest, because it suggests that the chromatin structure of active sequences may be in more open conformation, which allows the DNA to be more accessible to repair enzymes, or, alternatively, transcription is directly coupled with DNA repair (Selby and Sancar 1993). The identification of several excision repair genes with transcription factors enhances this thinking. A subunit of the human transcription initiation factor TFIIH is now known to be encoded by ERCC-3, a gene previously linked to DNA repair (Schaeffer et al. 1993). More recently, it has been established that both ERCC2 and ERCC3 are components of TFIIH (Drapkin et al. 1994). Table 2, below, lists cloned human genes involved in excision repair and their properties.

2.1.2 Recombinational Repair

Excision repair is presumably ineffective in removing double-strand damage. As noted above, informational redundancy is essential for the repair of damaged and lost information. In the case of single-strand damage in DNA, the needed redundancy is available in the undamaged complementary strand. In the case of double-strand damage however, the source of redundancy must be a second DNA molecule with intact information homologous to that lost in the first molecule. Repair consists of the exchange of information between the two molecules, whereby the undamaged DNA

Table 2. Properties of Cloned Human Genes Involved in Nucleotide Excision Repair. (From Hoeijmakers and Bootsma 1990)

Gene	Chromosomal Location	Size of Gene (kb)	Encoded Protein ^a	Yeast Homolog	Protein Features
XPAC	9	~ 25	31 kD; 40-50 kD ^b	unknown ^c	DNA binding
ERCC -1	19q13.2	15-17	297 amino acids	RAD10	DNA binding? ^d . homology to <i>E. coli</i> UvrA and UvrC
ERCC -2	19q13.2	~ 20	760 amino acids	RAD3	nucleotide, DNA binding? DNA helicase? essential function
ERCC -3/ (XPB C)	2q21	~ 45	782 amino acids	ERCC-3 ^c	nucleotide, DNA binding, helicase?, essential function? acidic
ERCC -5	13q	~ 32	unknown	unknown	
ERCC -6	10q .11	~ 100	>1000 amino acids	unknown	

- a Predicted on the basis of the nucleotide sequence.
- b The cloned cDNA that confers a partially corrected phenotype predicts a molecular mass of 31 kD; the HeLa and calf thymus protein that specifically corrects the XP-A defect upon microinjection has an apparent molecular mass of 40-45 kD and binds to ss, ds and UV-irradiated DNA.
- c Protein appears to be highly conserved, since a microinjection protein preparation from *Xenopus* still complements the human defect.
- d (?) A property of function that has been postulated on the basis of amino acid sequence homology to known functional domains in other proteins; direct proof at protein level is lacking.

molecule donates a single-stranded section to the damaged DNA duplex to permit replacement of its lost information. This process of exchange of information between two DNA molecules is referred to as recombinational repair. The repair of DNA double-strand breaks is believed to involve a recombinational activity (Saeki et al. 1981). Recombinational repair was discovered, in 1947, by Luria in studies of bacteriophages T2, T4 and T6. When he irradiated these phages with UV, he observed that their ability to produce progeny was much higher when bacteria were infected by two or more phages than when they were infected by a single phage (Luria and Dulbecco 1949). The higher survival rate was interpreted as being due to recombination between the damaged parental phage chromosomes, and much of the knowledge of recombination molecular events comes from genetic studies.

Two broad classes of genetic recombination are recognized: general recombination and site-specific recombination. In general recombination, genetic exchange takes place between homologous DNA sequences, usually located on two copies of the same chromosome. In site-specific recombination, DNA homology is not required. Instead, exchanges occur at short, specific nucleotide sequences on either one or both of the two nucleotide molecules that are recognized by site-specific recombination enzymes. Most of the knowledge about the biochemistry of (genetic) recombination has come from studies with *E. coli* and its viruses.

The general recombination pathway shown in Figure 4 ensures that the two regions of DNA double helix undergo an exchange reaction only if they have extensive sequence homology. At the site of exchange (known as Holliday junction), a strand of one DNA molecule becomes base-paired to a strand of the second DNA molecule to create a staggered joint called a heteroduplex joint. The heteroduplex region can be thousands of base pairs long. The events of this type of recombination occur so precisely that not a single nucleotide is lost, gained, or changed (error free) (reviewed in Alberts et al. 1989).

Unlike general recombination, site-specific genetic recombination is guided by a recombination enzyme that recognizes specific nucleotide sequences present on one or both of the recombining DNA molecules. Base pairing between the recombining DNA molecules need not be involved. Only a short region of identical DNA sequence, on the two regions of DNA helix to be joined, is required. This type of recombination is used to mediate genetic mobility and gene assembly, and to regulate gene expression and plasmid copy number. In vertebrates, the only site-specific recombination system that has been identified functions in developing lymphocytes to assemble the variable portions of the genes that encode immunoglobulin and T cell receptor molecules using component V (variable), D (diversity), and J (joining) gene segments (Chun et al. 1991).

A Recombinational-type DNA Repair Model

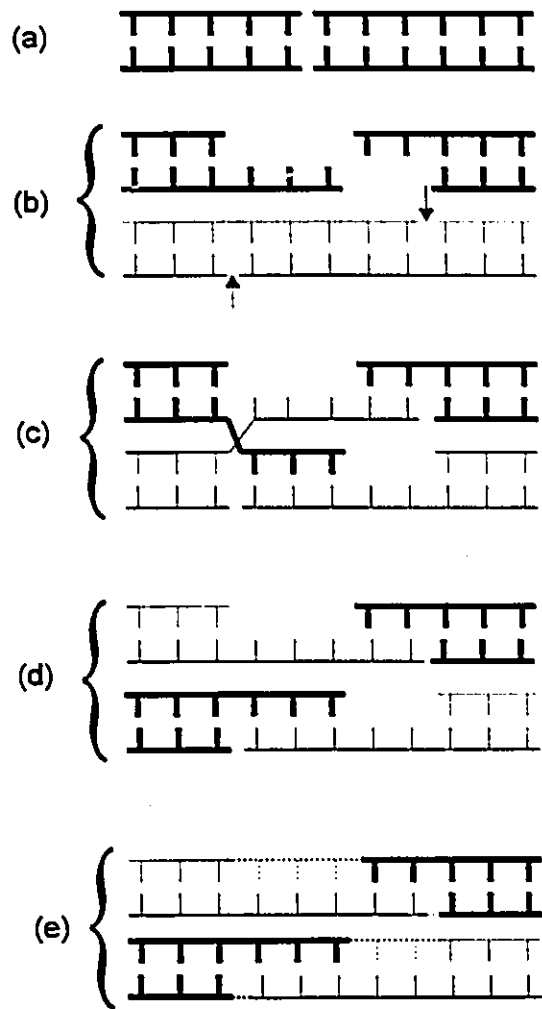


Figure 4. A recombinational-type model for the repair of DNA double-strand breaks.

(a) Double-strand break of DNA molecule (thick line);

(b) enzymatic excision of each strand, one on each side of the breakage point;

(c) the terminal unjoined segments of each strand 'interact' with the homologous segments of the other intact DNA molecule (fine lines) in such a way as to allow repair (by recombination or postreplication).

For this purpose the intact double-strand molecule must be cleaved at two sites by an endonuclease;

(d) reciprocal exchange between the double strands of DNA; two single-strand breaks remain, together with two gaps;

(e) the breaks are repaired by means of a ligase; the gaps are repaired (dotted lines) by resynthesis.

(From Tubiana et al. 1990)

Most of what is known about homologous pairing and strand exchange comes from studies of the *recA* protein of *E. coli*. Biochemical and physical studies have shown that *recA* binds to DNA to form long polymeric filaments within which DNA-DNA pairing occurs. Studies have also shown that the *recA* protein structure has been conserved throughout the bacterial kingdom, in yeast, and most likely in humans (reviewed in West 1994).

In a review of DNA repair in microorganisms (Bernstein and Bernstein 1991), recombinational repair has been observed in viruses in the form of multiplicity reactivation and postreplication repair. In bacteria, there is evidence that double-strand breaks, interstrand crosslinks and gaps opposite thymine dimers are repaired by recombinational events. In the wildtype yeast, *S. cerevisiae*, exposed to ionizing radiation, an average of about 35 double-strand breaks are required to produce one lethal hit, whereas in a *rad-52* mutant, lacking recombinational repair, only 2.2 double-strand breaks were required to produce a lethal hit. Mutant *Drosophila melanogaster* defective in allelic recombination was also found to be sensitive to several DNA damaging agents, including X-rays. Gatti et al. (1980) presented evidence that the normal products of the genes *mei-9* and *mei-41* function to remove X-ray induced damages that, if unrepaired, can lead to the formation of chromosome aberrations. Song et al.

(1985) have shown that double-strand breaks enhance homologous recombination in mammalian cells and extracts, and that the breaks may be the initiation sites for the recombination event. Coogan and Rosenblum (1988) measured repair of double-strand breaks following X-irradiation in rat spermatogenic cells during different stages of germ cell formation. These stages were spermatogonia and preleptotene spermatocytes, pachytene spermatocytes, and spermatid spermatocytes. Pachytene spermatocytes demonstrated the greatest repair capability. Because pachytene is the stage of meiosis in which recombination occurs, these findings may reflect meiotic recombinational repair of the double-strand breaks. Most importantly, a human homolog of *S. cerevisiae* RAD51 gene, whose product is structurally similar to recA, has been cloned (Shinohara et al. 1993), and a mammalian Holliday Junction RESOLVASE that appears similar to *E. coli* RuvC protein has been characterized (Hyde et al. 1994).

The above shows that recombinational-type DNA repair is an important process in the repair of DNA damage caused by ionizing radiation. Cell survival to ionizing radiation has been observed to be diminished, in procaryotes and lower eucaryotes, when the gene(s) necessary for the process of recombinational repair is/are inactivated. Yeasts mutant at the rad51, rad52 and rad54 loci were found to be extremely sensitive to ionizing radiation. The mutants

were found to be almost completely defective in meiotic and mitotic recombination (reviewed in Friedberg 1988). Cells from patients suffering from Ataxia telangiectasia (AT) are three or four times more sensitive to ionizing radiation than normal subjects. Immunodeficiency is a known characteristic of AT patients, and a deficit in genetic recombination has been considered as one of many possible factors (e.g. lack of division delay) that may contribute to their hypersensitivity to radiation (reviewed in Paterson 1978, McKinnon 1987). Unfortunately, the molecular events underlying recombinational-type DNA repair in mammalian cells remain obscure. Several groups are engaged in purifying and characterizing the proteins that interact specifically with the recombining strands and those that promote recombination in human cells (Kucherlapati et al. 1985; Hsieh et al. 1986). In this past year analogs of some of the bacterial recombination proteins were found to exist in human cells, suggesting that the essential cellular processes required for the recombination of DNA are conserved from microorganisms to humans (West 1994).

Excision resynthesis and recombinational repair are multistep reactions involving a number of different enzymes. Other molecular mechanisms have been also implicated in the repair of DNA damage, whereby a single enzyme catalyses a single reaction that restores the structure of the genome to its normal state (reviewed

in Friedberg 1985). The repair of single-strand breaks, as a result of exposure to ionizing radiation, by direct rejoining of the ends by polynucleotide ligase, is an example of direct repair. DNA purine insertase is responsible for the repair of apurinic sites formed as a consequence of base loss due to ionizing radiation. Reversal of DNA damage in single-step reactions is highly economical in terms of the use of genetic information.

3. RADIATION-INDUCED EFFECTS

3.1 Adaptive Responses in Prokaryotes

The aforementioned repair mechanisms are among those known to be constitutively operational to ensure genomic stability as cells are continually subjected to spontaneous structural alterations. In bacteria, 13 different multigene systems were shown to be induced in response to a variety of stress stimuli (Neidhardt 1987), when damaging species due to various stresses overwhelm the basal level of damage-scavenging and repair of the cell. In *E. coli*, additional damage to DNA has been shown to result in the induction of a diverse set of physiological responses such as the SOS response and the adaptive response (reviewed in Walker 1985). Each of these responses has been found to be controlled by specific sets of proteins and 21 genes have been

found to be induced in the two responses. The SOS network controls the expression of genes whose products are known to play roles in excision repair, daughter-strand gap repair, double-strand break repair, methyl-directed mismatch repair, and SOS processing. The expression of the genes in the SOS regulatory circuit is controlled by the LexA and RecA gene products. The adaptive response network controls the expression of proteins with roles in the direct removal of methyl and ethyl groups from DNA, in the excision repair of alkylated bases, and perhaps in other repair processes. One of the processes involved in this response is controlled by the *ada* gene, whose product O⁶-methyl guanine-DNA methyl transferase is a repair enzyme. Exposure of *E. coli* cells to non-toxic concentrations of DNA methylating agents results in expression of the *ada* gene and translation of its message. After synthesis, sufficient amounts of the enzyme remain available for a limited period to repair DNA lesions that occur after exposure to a toxic concentration of the methylating agent (Samson and Cairns 1977). Recently, the O⁶-methylguanine-DNA methyltransferase has been also shown to be induced, in rodent cells, by exposure to ionizing radiation (Fritz et al. 1991).

Active oxygen species resulting from exposure to ionizing radiation are thought to be responsible for most of the DNA damage (Chapman et al. 1973). Oxidatively stressed bacteria were shown to

respond by invoking two inducible systems known as the *oxyR* and *soxR* responses, the first being mediated by hydrogen peroxide stress and the second by superoxide stress. Each of these responses is regulated by more than 30 proteins. In the peroxide stimulon of *E. coli*, 8 proteins are positively regulated by the *oxyR* locus. At least 6 proteins of the superoxide stimulon are known to be regulated by the products of the *soxR* and *soxS* genes. Deletion of these regulatory genes in bacteria results in failure to induce these responses (reviewed in Farr and Kogoma 1991). In both cases, bacteria pretreated with low concentrations of peroxide or superoxide generating compounds displayed enhanced resistance to subsequent challenge doses of these compounds. Evidence indicates that these two oxidative stress responses are distinct, and the proteins that are induced by the superoxide radical are, for the most part, different from those induced by the peroxide stress. Some of the proteins, such as the heat shock proteins GroEL and GroES, are induced by either stimulon as well as other types of stress, such as heat shock, starvation and SOS. Another heat shock protein, DnaK, has been shown to be induced by treatment with H₂O₂, but not by superoxide radical. In certain microorganisms, the genes for these proteins were found to have cis-acting heat shock elements (Morimoto 1993). It is interesting that an SOS protein, RecA, can be induced by both types of oxidative stress but not by heat shock (reviewed in Farr and Kogoma 1991). Farr and Kogoma

(1991) and others (e.g. Adams et al. 1992) argued that these observations indicate that several stress responses overlap and that the extent of the overlap varies in different responses. In support of this idea, it has been shown that deletion of a major heat shock protein, *rpoH*, renders *E. coli* SOD⁺ and SOD⁻ cells extremely sensitive to both peroxide and superoxide stress (Farr and Kogoma 1991). Also, Privalle and Fridovich (1987) observed an induction of superoxide dismutase by heat shock in *E. coli*. *Salmonella typhimurium* cells pretreated with H₂O₂ developed an increased resistance to heat shock. Glucose-starved stationary phase cells of *E. coli* develop enhanced resistance to several forms of stress such as heat shock, oxidation, osmotic shock and starvation. Thirty proteins are induced in starved *E. coli*, including several oxidative stress and heat shock proteins (Matin 1991). Brawn and Fridovich (1985) proposed that the superoxide radical induces the SOS response. Treatment with low but not high concentrations of H₂O₂ induces the *recA* SOS gene (reviewed in Farr and Kogoma 1991).

While a repair function of the heat shock proteins remains to be tested, similarly to prokaryotic cells, a number of DNA-damaging agents were shown to induce in eukaryotes genes that are members of the heat shock regulatory network. Following irradiation of rat embryos in utero, the expression of a heat shock

gene labelled Hsp 70 and of the c-myc proto-oncogene is increased 4 or 5 days after treatment (Higo et al. 1989). Other DNA damaging agents such as chemical teratogens were found to enhance the induction of low molecular weight heat shock proteins in rat embryos cultivated *in vitro* and of ubiquitin (a 7 to 8 kd Hsp) in mammalian cells (reviewed in Smith-Sonneborn 1992). In yeast, the Rad6 DNA repair enzyme is an ubiquitin-conjugated protein essential for normal growth, sporulation and repair, which suggests that ubiquitin may be a key regulatory molecule in the stress response. Work by Boreham and Mitchel (1994) has shown that the heat shock protein HSP104 may be involved in regulating the mechanism of stress-induced radiation resistance in *S. cerevisiae*. Therefore, as in bacterial cells, it has been suggested, that the above defence mechanisms against DNA damage can be activated by the same agents, and that they are elements of a complex defence system which is available to cells undergoing stress (Fornace 1992). It is of interest to note that in the case of heat shock of eukaryotic cells, activation and binding of the heat shock factor (a transcription factor) to the heat shock element (a specific DNA recognition sequence located in the 5'-flanking sequences of heat shock-responsive genes) occurs within minutes of temperature elevation (Morimoto 1993).

3.2 Increased Cellular Proliferation at Low Radiation Dose Rates

Several reports have shown that chronic exposure at very low dose rates to various agents including ionizing radiations, heavy metals and antibiotics, stimulate cell division rates, accelerate maturation time and acclimation (reviewed in Smith-Sonneborn 1992). Experiments with *Paramecium tetraurelia* and the Cyanobacteria *Synechococcus lividus* (Planel et al. 1992) have shown a reduced growth rate when the cells were grown in a shielded device. The growth inhibition disappeared when the shielded cultures were simultaneously irradiated by a ^{232}Th source at a dose rate close to background (7mGy/year). A 3-day irradiation by a ^{60}Co source at doses ranging from 0.015 to 0.04 mGy resulted in an enhanced cell growth rate. This stimulatory effect was dependent on the experimental culture conditions, the dose and dose-rate and on photo-oxidative stress. Increases in the enzyme activities of superoxide dismutase, glutathione reductase and glyceraldehyde P-dehydrogenase were noted. Since the only variable in Planel's experiments was the level of radiation, his data point to a radiation-induced stimulatory effect in cells exposed to low doses. Several other studies confirm Planel's results, and an increased proliferation of microorganisms exposed to low-level radiation have also been observed in yeast, algae and other bacteria and protozoa (reviewed in Luckey 1991).

The term radiation hormesis has been applied to describe the stimulatory effects of ionizing radiation. In a summary of the literature between 1976 and 1991, Luckey (1991) lists about a thousand reports on the hormetic effects of ionizing radiation in animals and human populations. Recently, Satta et al. (1992) measured the amount of damage in *Saccharomyces cerevisiae* exposed to the radiomimetic agent methyl methanesulphonate. Two sets of cultures were maintained either at background radiation levels of about 5 μ Sv per day or below background levels (about 0.6 μ Sv per day) respectively. Reciprocal recombination was used as an index to measure damage induced by the chemical mutagen. Their data showed that in spite of the same exposure to the DNA-damaging agent used, the cells grown in a lower than normal background radiation environment behaved as if they were more heavily damaged than those grown in the presence of the normal environmental conditions. It is possible that environmental background radiation contributes to the maintenance of a reservoir of enzymes with detoxifying and DNA repair capacity which act against the induction of DNA damage.

3.3 Effects of Ionizing Radiation on Plants

The use of ionizing radiation to stimulate plant growth dates back to 1898, when soon after the discovery of X-rays by Roentgen, Atkinson reported that algae grew faster after exposure

to X-rays. Since, several reports were published suggesting that the stimulatory effect is quite general when sufficiently low doses are given (reviewed in Luckey, 1991). The stimulation of seed with radiation has been measured in different ways, such as the effect on germination, root development, stem number, number of blossoms per plant, time of ripening and yield. However, the results have been variable. A clear evidence of a relationship in the growth pattern between the effects of irradiation and other factors such as the seed type, seed lot, the presence of light and humidity of the atmosphere surrounding the seeds had not been made. In their attempt to explain the mechanism of growth stimulation by X-rays, Petkau and Geisel (1985) proposed a physico-chemical process. While studying the effect of X-ray treatment of seed potatoes on yield, the above authors noted increases in total and water-soluble proteins, both of which increased with X-ray dose up to 0.5 Gy, after which they remained high, even up to 3 Gy. They hypothesized that free radicals produced due to the interaction of X-rays with water in the seed lead to increased permeability of cell membrane; as a result, increased influx of water, oxygen and minerals occurs. Increased enzyme activity within cells leads to increased oxidation of growth inhibitors and growth enhancement occurs.

3.4 Effect of Radiation in Lower Organisms and Mammals

Increases in the genetic fitness of x-irradiated organisms was shown as early as 1958. Wallace (1958) found that mutations, induced in the second chromosome of *Drosophila* by an x-ray dose of 5 Gy, brought about a significant net increase in viability. Similar experiments (Crenshaw 1965) in the flour beetle *Tribolium confusum* confirmed Wallace's results, and female progeny of irradiated males produced more viable offspring than control female progeny of non-irradiated males.

A life prolonging effect of ionizing radiation was noted by Lorenz (1950) in laboratory mice exposed to 0.11 cGy per 8 hour day from about 2 months of age until natural death. Similarly, chronic intermittent ionizing radiation increased the life span of rats exposed to 0.1 cGy/h for 8 hours of each day during a year of adult life and to the additional stress of cold exposure (Carlson and Jackson 1959). Experiments by McGregor and Newcombe (1972) showed a decreased mortality among embryos of rainbow trout as a result of exposure of the sperm to 25 cGy and 50 cGy of γ -rays. Higher doses of 2 and 4 Gy resulted in increased mortality. The beneficial effect of radiation was attributed to a hereditary change, since it persisted over many cell generations.

3.5 Induced Repair in Eukaryotic Cells

A radiation-induced repair model, in eucaryotic cells, was first proposed by John Calkins in 1967. While irradiating protozoa with fractionated x-ray doses, he observed that at a specific dose level, a small increment of radiation produced a net increase in the survival level of the irradiated cells. He attributed this effect to the activation of a radiation- (or some equivalent stimulus) induced repair mechanism, which operates in addition to constitutive repair. Mitchel and Morrison (1982) have reported the first proof that a transient increase in resistance to γ -radiation can be induced by heat shock in *Saccharomyces cerevisiae*. Subsequently, they have demonstrated that radioresistance in yeast can be induced by a variety of stresses including γ -irradiation (1984). They have also shown that multiple DNA-repair systems can be induced in yeast (e.g. recombinational repair system and the inducible error-prone system) which can influence the processing of DNA lesions by different DNA-damaging agents (1987). In a subsequent study, Boreham and Mitchel (1991) have shown that DNA lesions produced by low LET γ -rays at doses greater than about 20 Gy given in oxygen are more efficient, per unit dose, at inducing radioresistance to killing than were lesions produced by neutrons (high LET radiation). Oxygen-modifiable lesions produced by γ -rays were particularly efficient as inducing signals. Of particular

importance, their results provide evidence that DNA damage is a signal to induce the mechanisms responsible for radioresistance. Data by the same investigators indicated that the systems which confer resistance to radiation in yeast are independent of the activities of topoisomerase I and II and DNA polymerase I. However, they suggested that the topoisomerases may have a regulatory role during the signaling of these mechanisms (Boreham et al. 1990). In a review of radiation and stress response in *Saccharomyces cerevisiae*, Boreham et al. (1991) summarized evidence that radioresistance induced by ionizing radiation exposure results specifically from induction of an error-free recombinational-type DNA repair. However, clear molecular evidence for a similar induced repair system in higher eucaryotes has not been demonstrated. Our understanding of the regulation of DNA-damage-inducible responses in mammalian cells is certainly less complete than in bacteria and lower eukaryotes, but the evidence suggests that this regulation almost certainly involves multiple mechanisms (Fornace, 1992).

Support for repair induced by low X-ray doses in mammalian cells came from subsequent work by Calkins et al. (1990). Calkins proposed a model in which it was implied that the cellular radiation response is not monotonic and that it is impossible to predict the behavior across a threshold from observations on the

other side, and that there can be more than one threshold for repair induction. While investigating the survival of V79 cells to X-rays, Joiner and Johns (1988) and Marples and Joiner (1993) showed that the rate of killing in these cells is reduced in the dose range of 0.10-0.25 Gy. These results were previously observed in our own laboratory while characterizing various aspects of the response of V79 cells to radiation (Azzam and Raaphorst 1985). The chemical ara-A eliminated such a response, implicating DNA repair mechanisms. More recent work by Calkins et al. (1991) has shown that both radiation-induced lethality and transformation frequency have been observed to plateau or diminish abruptly at relatively low doses and then increase with increasing doses, but at a reduced incremental rate. These results suggest the induction of an "error-free" repair mechanism, in contrast to the "error-prone" nature of induced repair in prokaryotic cells (Walker 1984), but consistent with the error free-system demonstrated in yeast (Friedberg 1988). Studies by Koval (1986, 1988) showed that split dose irradiation or holding in plateau phase of γ -irradiated TN-368 lepidopteran insect cells results in substantial increases in survival. An inducible recovery mechanism(s) was suggested to be responsible for this enhanced recovery from radiation damage. Addition of non-toxic concentrations of cycloheximide or actinomycin D following irradiation, but prior to cell dilution and plating (PLD-type experiments), or during the interval between

split radiation doses (SLD experiments), inhibited the increases in cellular survival. These results indicate that such increases are dependent upon transcriptional and translational activities which are not present in unirradiated cells. In a later study, Rand and Koval (1994) presented evidence that shortly after irradiation the protein synthesis profile is altered. Many proteins have their synthesis decreased or totally eliminated, many others have enhanced synthesis and many new proteins appeared. Also, data was presented indicating that the radiation-inducible response of lepidopteran cells is genetically determined. The transfection of TN-368 cell DNA into XR-1 radiation-sensitive hamster cell mutants resulted in the restoration of radiosensitivity to nearly that of the wild type parental hamster cells. Evidence for the induction of a competent repair system was recently demonstrated by Mothersill and Seymour (1993). Data obtained in their laboratory show that deficient mutant progeny of XRS-5 Chinese hamster cells exposed to a fractionated dose of ionizing radiation express shouldered survival curves, as compared to the progeny of cells which received the same dose in a single fraction. It was concluded that a competent repair system is induced after 2 h following the first dose of radiation. A methylation event was suggested to be responsible for the induction of this system as demethylation using 8-azacytidine eliminated the effect observed.

3.6 Adaptive Responses to Ionizing Radiation in Mammalian Cells

Experimental evidence accumulated over several decades supports the hypothesis that exposure of cells to non-toxic concentration of a mutagen can mitigate the severity of the deleterious effects of a subsequent exposure to a toxic concentration of that mutagen (reviewed in Frosina and Abbondandolo 1985, Wolff 1992b). In recent years, repeatable experiments have been carried out showing that low doses of radiation can induce a chromosome-break repair mechanism in cells that, if in place, will render the cells less susceptible to the consequences of a second dose. Such a response has been labelled the adaptive response (Olivieri et al. 1984). Using chromosomal aberrations as an end point, Olivieri et al. (1984) have described the existence of this response in human lymphocytes cultured in the presence of [³H]dThd concentrations that would give rise to one disintegration per cell in the course of the experiment prior to being challenged by a dose of 1.5 Gy. The yield of chromatid aberrations in cells which received the combined treatment was significantly less than the sum of yields of the aberrations induced by the two separate exposures. Subsequently, it was demonstrated that this adaptive response could be induced by pre-exposure to doses of x-rays as low as 0.01 Gy delivered during G₁ (Shadley and Dai 1992, Wang et al. 1991) or S-phase (Shadley et al. 1987, Cai and Liu 1990) of the cell cycle.

These observations mirror what had been observed initially with chemical agents in bacteria (Samson and Cairns 1977) for survival and mutagenic effects as end-points, and subsequently extended to the killing and cytogenetic effects of chemicals in rodent and human cells (Samson and Schwartz, 1980), and to the chromosome aberrations induced in plants (Rieger et al. 1982). Similarities are also observed with the SOS response in bacteria (Walker 1985), although SOS repair leads to more rather than fewer mutations.

Olivieri et al. (1984) have postulated that the inducing dose of radiation activates a repair mechanism which, if in place at the time the cells are exposed to a challenge dose, causes the restitution of some chromosomal damage, so that fewer chromatid aberrations are observed than in cells receiving the challenge dose only. This hypothesis was supported by the finding that the adaptive response was inhibited by cycloheximide (Youngblom et al. 1989), suggesting a role for de-novo protein synthesis, and by 3-amino-benzamide (Wiencke et al. 1986), an inhibitor of poly (ADP-ribose) polymerase which is known to be induced during the repair of DNA strand breaks. The adaptive response in human lymphocytes was found to be dependent upon both the adapting dose and dose rate (Shadley and Wiencke 1989), suggesting that a certain amount of damage needs to occur within a fixed time scale in order to initiate the signal for expression of the adaptive response. It

appears that high inducing-doses must be given at a low dose-rate to induce the adaptive response, whereas a low dose must be given at a high dose-rate. It has been argued that for the response to be induced, a certain amount of lesion, perhaps of a specific type, may have to be produced within a certain time span. The induction of the effect by a narrow range of doses has also been found to occur when the effect is induced by radiomimetic chemicals such as bleomycin (Wolff et al. 1989; Vijayalaxmi and Burkart 1989). Wolff argued (1992b) that it is this relationship of expression of the adaptive response and the inducing dose which may have been a reason why this phenomenon eluded discovery for some time, in spite of numerous attempts. Once characterized, it was found that the adaptive response takes place approximately 4 to 6 hours after exposure to the adapting dose and remains effective for 3 cell cycles of the cultured human lymphocytes (Shadley et al. 1987). Experiments were done to examine the yield of chromatid damage at various fixation times after the challenge dose. The results indicated that the reduction in the expected yield of chromosomal aberrations in the adapted cells was not attributable to radiation-induced mitotic delays, which could result from the sampling of metaphase cells that were challenged with X-irradiation in different parts of the cell cycle, where sensitivity to X-rays changes dramatically. Olivieri et al. (1984) described evidence which indicates that differential stage sensitivity did not account

for the decline in aberration numbers. Experiments were done in which the cells were fixed 5, 7, 9, or 11 hours after the challenge X-ray treatment. In all cases the observed yields were smaller than would be expected from the sum of the yields induced by the adapting and challenge doses separately. Also, experiments were done where ^3H -labelled female cells were co-cultured with unlabelled male cells, and which showed that labelled and unlabelled cells progressed to metaphase equally (Wiencke et al. 1986). Therefore, it was concluded that the adaptive response is not the result of selection against a radiosensitive population of cells that have incorporated [^3H]dThd. Measurements of chromosomal aberrations induced in the labelled female cells and unlabelled male cells that had been co-cultured showed that the adaptive response is restricted to those cells exposed to radiation from the incorporated [^3H]dThd. Therefore, it was also concluded that diffusible factors are not involved.

The discovery of the adaptive response to ionizing radiation in cultured human lymphocytes confirmed previous indications that chromosome aberrations were less frequent than expected in lymphocytes from persons who had been exposed to high ambient amounts of radiation from radium (Pohl-Rüling and Fisher 1979). Also, in a study of DNA excision repair in lymphocytes from persons occupationally exposed to low-dose radiation from ^{222}Rn , Tuschl et

al. (1980) observed a stimulatory effect of repeated low-dose irradiation on repair enzymes. As early as 1950, Cronkite et al. reported an increased tolerance of mice to lethal x-irradiation as a result of previous sublethal exposures. Their interest to alter the lethality of total body x-ray exposure of mice was triggered by a yet earlier report by Raper (1947) in which the LD₅₀ of total surface β -radiation was increased by a previous exposure to β -rays.

Following the discovery of the above adaptive response to ionizing radiation in human lymphocytes, several laboratories confirmed its existence in these cells and used different parameters to describe it (reviewed in Wolff 1992a, 1992b, Shadley 1994). While most studies (Wiencke et al. 1986, Bauchinger et al. 1989, Shadley et al. 1987, Bosi and Olivieri 1989, Sankaranarayanan et al. 1989) have measured chromosomal aberrations, specifically chromatid deletions, Moquet et al. (1989) used sister chromatid exchanges and Sanderson and Morley (1986) used gene mutations as end-points to measure the adaptive response. Sanderson and Morley (1986) showed that pre-exposure of human lymphocytes to 0.1 and 1.0 μ Ci/ml [³H]thymidine for 6 hours, prior to X-irradiation in G₀, produced a significant decrease in the number of mutations at the hypoxanthine phospho-ribosyltransferase locus induced after both 1.5 and 3 Gy. However, there were no effects on cell survival. They concluded that the most likely interpretation of their data is

that ionizing radiation induces a system which repairs pre-mutational lesions in DNA rather than potentially lethal damage. Also, they proposed that the types of lesions leading to mutation and lethality are different, and that differential repair of pre-mutational and potentially lethal lesions is possible.

3.6.1 Adaptive Response Studies With Radiomimetic Chemicals For Either The Adapting or Challenge Dose

A number of studies have substituted chemicals for either the adapting or challenge dose (Vijayalaxmi and Burkart 1989, Wolff et al. 1989, Cortés et al. 1990b, Shadley and Dai 1993b). Moquet et al. (1989) have shown that human lymphocytes pre-exposed to 0.01 Gy of X-rays become protected against sister chromatid exchanges induced by the DNA cross-linking agent mitomycin C. Other studies have shown that low doses of bleomycin (which like X-rays can generate free radicals and induce DNA strand breaks) and hydrogen peroxide (which like X-rays induces oxygen radicals) can result in significant reductions in X-ray induced aberrations (Vijayalaxmi and Burkart 1989, Wolff et al. 1989, Cortés et al. 1990b). Again, it was necessary that the doses of mutagens used for adaptation as well as the time interval between the two treatments be within a

certain range to observe a pronounced adaptive response. When compared to low doses of ionizing radiation, the two chemicals yielded equivalent reductions. However, as bleomycin and hydrogen peroxide produce a variety of lesions, their use did not improve our knowledge of which, if any, DNA lesions are involved in the adaptive response. The free radicals that they both generate can interact with DNA and all other cellular constituents, including membranes, which play a role in signal transduction. The effect of free radical scavengers or signal transduction inhibitors on induction of the adaptive response is an interesting topic not yet studied.

As the major lesions responsible for the induction of chromosome breakage are double-strand breaks in DNA, Wolff et al. (1988) initiated a study in an attempt to see if the repair mechanisms induced by low-dose exposure in human lymphocytes can affect various types of clastogenic lesions induced in DNA by chemical mutagens and carcinogens. The results showed that a significant adaptive response was observed in human lymphocytes pre-exposed to 0.01 Gy of X-rays or to low doses of tritiated thymidine and subsequently challenged with high doses of tritiated thymidine, bleomycin or mitomycin C. On the other hand, when the cells were challenged with the alkylating agent methyl

methanesulphonate (MMS), approximately twice as much damage was found as was induced by MMS alone. It was concluded that prior exposure to 0.01 Gy of X-rays reduces the number of chromosome breaks induced by double-strand breaks and by cross-links in DNA, but has the opposite effect on breaks induced by the alkylating agent MMS. Wolff et al. (1988) concluded that the induced repair mechanism is different from that observed in the adaptive response that follows exposure to low doses of alkylating agents. Mitchell and Morrison (1984) had shown that radiation-induced adaptation in yeast protected against a variety of alkylating agents, but not MMS. Therefore, this lack of adaptation to MMS may be due to an inability of the induced processes to protect against the specific lesions induced by this chemical. Alkylating agents have been shown to induce an alkyltransferase (reviewed in Frosina and Abbondandolo 1985). Any role for this enzyme in the repair of radiation-induced damage has not been substantiated. In the case of ionizing radiation, the major lesions responsible for the induction of chromosome aberrations are DNA double-strand breaks (Savage 1990).

3.6.2 Identification of Induced Proteins in Human Lymphocytes exposed to Low Doses of Ionizing Radiation

Soon after the discovery of the adaptive response, it was clear that identification of induced proteins following the low dose exposure may shed light on the identity of candidates which may be involved in the recovery processes. To verify if proteins responsible for the response were being induced by the very low dose of 1 cGy, Wolff (1992b) carried out two-dimensional gel electrophoresis experiments. Proteins were extracted from unirradiated control cells and from lymphocytes exposed to 1 cGy. The proteins were separated on equilibrium gels and scanned. In many cases, the irradiated samples on the average had either five times more or five times less protein in a spot than did the average of the controls. One protein was consistently present in the samples exposed to 1 cGy, but was absent in the controls 75% of the time. As two-dimensional gels have a low loading capacity which makes it difficult to extract an analyzable amount of protein from a given spot, Wolff (1992b) resorted to one-dimensional gel electrophoresis to obtain proteins that might be involved in adaptation. Upon electrophoresis, southwestern blotting and exposure to a nick-translated ³²P-labelled probe, three proteins

(105 kDa¹, 35 kDa and 14-18 kDa) were found to reproducibly bind to the probe. Binding could not be decreased by competition with a 25-fold excess of the respective non-radioactive probe, indicating that the binding occurs only with the radioactively damaged probe as ³²P produces radiation-induced DNA strand breakage. When blood cultures were irradiated with 1 cGy of X-rays, the binding of the DNA probe to the 30-35 kDa and 14-18 kDa bands was increased to twice the level found in non-irradiated cells. An induction of binding began 1 hour after irradiation and seemed to reach its maximum 6 hours later. The response was induced by 1 cGy of X-rays more strongly than by high doses. The study of proteins induced by ionizing radiation is currently an intensive area of research (Boothman et al. 1989, Teale et al. 1992). A coordinated expression of several proteins was found to be induced by Boothman et al. (1989) following the X-irradiation of confluence-arrested human malignant melanoma cells. A strong correlation was found between the induction of one of these proteins (XIP269) and potentially lethal damage repair capacity. Rather than analysing cellular extracts for radiation induced proteins, several laboratories, including ours, have opted for molecular techniques to qualitatively and quantitatively analyze radiation responsive genes at the level of mRNA expression (Fornace 1989, 1992).

¹ Recently, hsp 104 has been implicated in the control of induced radiation resistance in yeast (Boreham and Mitchel 1994).

3.6.3 Adaptive Response to Ionizing Radiation and Cross-adaptation in Chinese Hamster Cells Maintained in Culture

To verify whether the adaptive response noted in human lymphocytes was also induced in proliferating mammalian cells in culture, Ikushima (1987) performed experiments to test the presence of the adaptive response to ionizing radiation in cultured Chinese hamster cells (V79). When actively growing V79 cells were internally exposed to low doses of ^3H -dThd for longer than one cell cycle duration, they became more resistant to the induction of chromosomal damage by subsequent higher acute doses of γ -rays. The yield of sister-chromatid exchanges (SCEs) in the ^3H -dThd-pretreated cells was less than the yield induced by γ -rays alone, and the micronucleus frequency was less than the sum of the induced frequencies by ^3H -dThd and γ -rays separately. The weaker effect observed with micronucleus formation was thought to reflect a different process in the formation of micronuclei and SCEs. The radioadaptive response in these cells was also inhibited by 3-aminobenzamide. The protective effect disappeared after 1 cell division following the adapting dose, while it persisted for 3 cell cycles in human lymphocytes (Shadley et al. 1987). In a subsequent study Ikushima (1989) presented data indicating that there is an optimum dose range of the adapting ^3H β -rays or γ -rays for induction of the adaptive response in V79 cells. Similar to human

lymphocytes, full expression of the response occurred 4 hours after the adapting dose. V79 cells adapted with γ -rays displayed cross-resistance to SCE lesions produced by UV-B and mitomycin C (MMC), but not to those by ethyl methanesulphonate (EMS) or cis-platinum (II) diamine dichloride (cisplatin) suggesting that the radio-adaptive response in these cells mechanistically couples to the repair network which copes with chromatin lesions induced by MMC and UV-B. Cross-resistance to UV has also been reported in yeast (Mitchel and Morrison 1984). It is of interest to note that radio-resistant lepidopteran cells were also resistant to the lethal effects of UV (Koval et al. 1977a and 1977b, Koval 1991).

3.6.4 Variability in the Expression of the Adaptive Response to Ionizing Radiation

Although many laboratories have confirmed the initial observations of Olivieri et al. (1984), the adaptive response was not observed in lymphocytes from all donors (Bosi and Olivieri 1989, Sankaranarayanan et al. 1989, Schmid et al. 1989, Bauchinger et al. 1989, Aghamohammadi and Savage 1991). Bosi and Olivieri (1989) suggested that the human population exhibits a heterogeneity in the adaptive response to ionizing radiation which might be, in part, genetically determined. They postulated that the individuals that fail to express the adaptive response are constitutively

deficient in the repair function which mediates the adaptive response. Recent reports by Bosi et al. (1991) showed that the pH of the medium greatly affects the yield of induced chromatid aberrations. Olivieri and Bosi (1990) proposed that the failure to exhibit an adaptive response is a consequence of the physiological state of the cells at the time of the low dose exposure. They were able to alter the response in the cells of some individuals by addition of adrenal/thymus extracts or growth factors at the time of the low-dose exposure. In an abstract presented at the Radiation Research Society meeting in 1993, Shadley and Dai (1993a) have shown that the response of mitogen stimulated lymphocytes to high-dose-induced cytotoxicity is correlated with endogenous levels of lymphokines at the time of the exposure. The subject of the effects of cytokines on radiation responses has been recently the subject of a report of a forum of the Medical Research Council's Committee on the Effects of Ionizing Radiation (Wilson 1993). Cytokines are being shown to influence the proliferative characteristics of stem cells and therefore represent a potentially valuable tool in the post-irradiation management of radiotherapy patients and radiation accident victims.

Knowledge of the prevalence of the adaptive response in individual donors and throughout the human population remains sparse. Data presented by Hain et al. (1992) suggest that a high

frequency of human lymphocyte cultures do not display an adaptive response to ionizing radiation.

3.6.5 Cell Cycle Effects on the Expression of the Adaptive Response

While actively investigated, much remains to be learned about the cell cycle dependence of the adaptive response to ionizing radiation. The initial experiments to characterize the adaptive response were done with lymphocytes in S or G_2 phase of the cell cycle. More recently, experiments were done with lymphocytes at other stages of the cell cycle. Conflicting results have been published. Early investigations by Shadley et al. (1987) failed to see an AR for cells pre-exposed to the low dose in G_0 and challenged in late S/early G_2 . Also, Wang et al. (1991) did not observe an effect in cells pre-exposed in G_0 and challenged in G_1 . As well, Moquet et al. (1989) did not find an AR to sister chromatid exchanges by mitomycin C with a 1-cGy treatment in G_0 cells, but observed a response when the adapting dose was given in G_1 . However, Cai and Liu (1990) and Khandogina et al. (1991) observed a cytogenetic AR when G_0 phase cells were treated with 0.01 Gy and challenged with 1.5 Gy in the late S/early G_2 phase. As noted above, Sanderson and Morley (1986) observed a reduction in the mutation rate when G_0 lymphocytes were pre-exposed to 1 cGy, but, they failed to observe a survival AR in these cells. Further

studies are needed to establish the effect of exposure of human lymphocytes to an adapting dose in G_0 . It is of interest to note that Tuschl et al. (1983) found a significant decrease in the number of sister chromatid exchanges and an increase in unscheduled DNA synthesis in lymphocytes of individuals occupationally exposed to low doses of radiation.

The initial report of Olivieri et al. (1984) suggested that the AR acts in G_2 and in part of the S phase. It was not determined if the response acted throughout the entire S phase. As noted above, Shadley et al. (1987) did not observe induction of the cytogenetic adaptive response when lymphocytes were given 5 cGy in G_0 and 150 cGy in late S/early G_2 . It has not been established whether the lack of response is due to exposing the cells in G_0 , or to delivering the challenge dose in late S/early G_2 .

The studies of Wang et al. (1991) and Shadley and Dai (1992) showed an adaptive response when both the adapting and challenge doses were given in G_1 . However, reported data of Wang et al. (1991) indicate that the AR in the G_1 phase occurs only if the cells are challenged in the interval between 5 and 9 hours after the adaptive dose. Hain et al. (1992) attempted to investigate in detail whether the AR to IR undergoes fluctuations during different phases of the cell cycle. For this purpose, they gave an adaptive

dose in the G₁ phase and the challenge doses in late G₁, S or G₂ phase. Unfortunately, none of the blood cultures from five different donors expressed a protective AR.

In studies of the AR to IR in human lymphocytes, staging in the cell cycle was assigned relative to the time after PHA stimulation, and no actual identification of the position in the cell cycle at the time of treatment was done. Heterogeneity of the sample population, as well as the continuous changes in chromatin replication and packaging status that occur in stimulated lymphocytes during S and G₂ phase may be complicating factors in analysing the data of such a cell population and may have contributed to the variability of the response. Identification of the phase of the cell cycle at the time of the low and high dose exposures are possible by using a method based on a BrdU (bromodeoxyuridine) pulse double-labelling (Aghamohammadi and Savage 1991). This technique ensures that cell mixtures from a heterogeneous, asynchronous population can be scored and compared. However the technique is tedious and published experiments have not made use of it.

The finding of donors whose cultured blood lymphocytes did not express an adaptive response or even expressed a synergistic response, and the inter/intra donor variability observed by various

laboratories, remains to be addressed. An investigation of whether this variability occurs in other cell types will further characterize the mechanism(s) underlying the adaptive response to ionizing radiation and contribute to our ability to measure this response in large populations. Extension of the AR studies to a uniform cell population which exhibit a consistent AR, and where various culture variables can be controlled, will facilitate characterization of the AR at the molecular and biochemical levels. The extension of the AR studies to normal human skin fibroblasts maintained in culture, where many variables controlling cell growth can be monitored, was the focus of the studies in this thesis.

3.6.5 Review of Recent Reports on the Adaptive Response to Ionizing Radiation in Mammalian Cells

Due to the important consequences of exposure to sequential doses in radiotherapy, the establishment of lower exposure standards for atomic radiation workers and the general public, and the pursuit of space habitation, research into the AR to IR is proceeding at an accelerated rate. Several groups are now engaged in this research. While our laboratory focusses on the characterization of the AR to IR in a normal human skin fibroblast, reports are continuously being published on the AR to IR in human lymphocytes.

3.6.6(a) Radiation-induced Radioresistance to Lesions From High LET Radiations

Experiments were carried out in Wolff's laboratory (Wolff et al. 1991) to investigate if pre-exposure to low doses of X-rays would also decrease the yield of chromatid breaks induced by a subsequent exposure to α -particles from radon. Human lymphocytes exposed to 2 cGy of X-rays before radon exposure contained approximately one-half the number of chromatid deletions when compared with lymphocytes treated with radon alone, and analyzed at the same time. Thus, it was concluded that the putative chromosomal repair mechanism induced by low doses of sparsely ionizing radiation is also effective in reducing chromosomal aberrations induced by high LET- radiations, which had been thought to be independent of repair processes.

3.6.6(b) Adaptive Response to Ionizing Radiation Measured by Multiple End-points

Most studies of the AR to IR were performed on different donors, each looking at a single biological end-point. This approach limits the conclusions that can be drawn concerning the number of mechanisms involved in the AR and their relationships to each other. The measurement of several biological end-points in the same experiment would certainly elucidate the mechanisms

involved and their inter-relationships. As will be described later, this has been our approach. Our report (Azzam et al. 1992a) on the presence of an AR to IR in normal human fibroblasts for micronucleus formation and cellular survival, coincided with the report of Shadley and Dai (1992) on chromosomal and survival adaptive responses in G₁-phase human lymphocytes. As mentioned earlier, prior to these studies Sanderson and Morley (1986) measured survival and mutation in G₀-exposed lymphocytes. While reductions in X-ray-induced 6-thioguanine resistance were observed, there was no effects on survival. Subsequently, Shadley and Dai (1993b) reported on a radiation-induced AR to the clastogenic action of a high dose of X-rays, as well as sister chromatid exchange (SCE) induction by three drugs, etoposide (VP16), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), and cisplatin, which induce specific forms of DNA damage. Significant reductions in SCEs were found more often for VP16 than the other 2 drugs. It was thought that this may be related to the type of lesion formed by VP16, which consists of a complex of drug and enzyme trapped on DNA (Chen et al. 1984), as opposed to the base modifications caused by BCNU and cisplatin.

3.6.6(c) Action of the Adaptive Response on Induction of Mutations

Kelsey et al. (1991) carried out experiments to confirm whether the induction of adaptation to X-ray-induced clastogenic damage in lymphocytes plays a role in inducing protection against mutations. Previously, Sanderson and Morley (1986) delivered the low adapting dose of radiation with 0.1 or 1 $\mu\text{Ci/ml}$ ^3H -thymidine in the culture medium of non-cycling lymphocytes, while Kelsey et al. (1991) exposed human lymphocytes to 1 cGy of X-rays 24 hours after PHA stimulation. The yield of mutations, at the HPRT locus, induced by a 300 cGy X-ray dose given 16 hours later was reduced by approximately 70% from the control level of X-ray-induced mutations. Approximately 50% or more of radiation-induced mutations at the HPRT locus are deletion mutations. It was concluded that an inducible enzymatic process which repairs chromosomal lesions might be expected to repair induced DNA deletions which are pre-mutagenic. Consistent with the above reports, Zhou et al. (1993) also showed that cultured mouse SR-1 cells exposed to a low dose of γ -rays become less susceptible to the induction of mutagenesis by radiation as well as by bleomycin. Their data indicate that the AR in these cells is expressed at a later time than the 4-6 h after the low dose exposure observed in

human lymphocytes.

Rigaud et al. (1993) extended these studies, and also observed that 0.02 Gy of γ -rays protects against mutations induced by 4.0 Gy in human lymphocytes. A 70% reduction in HPRT mutants was observed in pre-exposed cells; however cell survival was unaffected. It is of interest to note that the low-dose pre-treatment not only affected the number of mutants obtained, but also the type of molecular alterations observed in the mutant cells. Mutant cells which arose from low-dose-pretreated cells had a decrease in partial deletions/rearrangements, indicating that the mutational specificity differed, depending on whether or not the cells were adapted. The authors suggested that the low-dose pre-exposure leads to a reduced susceptibility to the mutagenic effect of a high dose of γ -rays by inducing an error-free repair system. Furthermore, it was found that in the pre-exposed cells 4 out of 5 mutations occur in the 3' end of the HPRT gene indicating that the distribution of mutations differs from that induced by a single high dose. Recently, Rigaud and Moustacchi (1994) extended their molecular analysis and found that 86% of the adapted mutants were still expressing mRNA, whereas only 56% of the non-adapted mutants expressed HPRT mRNA.

3.6.6(d) Recent Evidence for an Adaptive Response to Ionizing Radiation in vivo

Following their report on the induction of a cytogenetic AR in rabbit lymphocytes irradiated *in vitro* (Cai and Liu 1990), Liu et al. (1992) demonstrated the induction of a cytogenetic AR by exposure of rabbits to very low dose-rate γ -radiation (92.6 μ Gy/min). It is of interest to note that, similarly to our results with human skin fibroblasts (Results Section), a rather high total dose [e.g. 1.5 Gy in the study of Liu et al. (1992)] delivered at a very low dose rate could induce significant protection against the formation of chromosome aberrations by an acute challenge dose. These results are consistent with the findings of Shadley and Wiencke (1989) that for a relatively high dose to be effective in inducing an AR, the dose rate must be quite low. The fact that an AR was observed when the challenge dose of 1.5 Gy was given *in vitro* in either the G_0 or the G_2 phase of the cell cycle implies that chronic-irradiation-induced protective mechanisms can protect the cell for an extended period of time. In fact, the AR induced in the above studies of Liu et al. (1992) remained effective when the challenge dose was given 12 days later. It is of interest to determine how long such an AR persists, when it is induced by an even lower dose rate than the 0.1 mGy/min in the above study. Such studies would be of interest in risk estimate analyses of

sequential doses delivered at high and low dose rates.

Wojcik and Tuschl (1990) reported on an AR induced in C57BL/6 mouse lymphocytes irradiated *in vivo* by 0.05 Gy of γ -rays delivered at 1.25 mGy/min on 4 consecutive days. Lymphocytes isolated from the spleens of pre-exposed mice, up to 26 days later, were exposed to UV radiation, or to mitomycin-C. The results showed that a protective effect, measured by higher rates of unscheduled DNA synthesis and lower sister chromatid exchanges, was observed in the pre-exposed mice than in the non-preexposed mice. The protective effect was observed to be effective for approximately 12 days.

In a follow-up study Wojcik et al. (1992a) investigated the AR to low doses of X-rays in pre-implantation embryos and spleen lymphocytes of an inbred mouse strain (Heiligenberger) and in peripheral lymphocytes of human donors. Embryos were adapted to 0.05 Gy X-ray 50h post-conception either *in vitro* or *in vivo* and challenged with 2 Gy X-ray dose 6h later. Chromosome aberrations of the 8 to 16 cell stage mitoses were scored. No AR was seen in the embryos. Of 14 female mice studied, an AR was seen in spleen lymphocytes of only one mouse. In human peripheral lymphocytes, an AR was seen in all 3 donors tested. It was concluded that the inbred mouse strain was deficient in the AR.

In a later study, Wojcik et al. (1992b) used chromosomal aberrations as an endpoint to compare the ability of pre-implantation embryos and spleen lymphocytes of the Heiligenberger and C57BL/6 mouse strains to express an AR to low doses of X-rays. An AR was not detected in the embryos of either strain whether the adapting dose was given *in vivo* or *in vitro*. While spleen lymphocytes of the Heiligenberger strain failed to express an AR, the investigators observed an AR in spleen lymphocytes of 4 out of 11 C57BL/6 female mice adapted with 0.1 Gy of X-rays after 32 hours of culture and challenged with 1.5 Gy at 48h of culture. However, when the frequencies of chromosomal aberrations from parallel cultures of lymphocytes originating from the same mouse were analyzed, a high variability was seen. It was concluded that the AR observed in some C57BL/6 mice was perhaps a result of this variability rather than of induced radiation resistance.

The absence of an AR in mouse embryos was later confirmed by Müller et al. (1992) who used, in some experiments, conditions of dose, dose-rate and radiation equipments that are similar to those used in experiments with human lymphocytes where an AR was observed. Caution has to be exercised in comparing data between human and mouse experiments. Biochemical differences which can affect the AR exist between mouse and human lymphocytes, and altered levels of thiol and glutathione are found (Lawrence 1992).

Cortés et al. (1990a) presented evidence for an AR to IR damage in plant roots conditioned with X-rays or incorporated thymidine. A reduction in X-ray-induced chromosomal damage from 1.5 Gy of X-rays was observed when the roots were pre-exposed to 0.26 Gy of X-rays. Lower adapting doses (0.06 Gy) did not induce an AR. In cells grown in the presence of [³H] TdR, the AR was evident after both doses given.

3.6.6(e) Induction of an Adaptive Response to Ionizing Radiation in Mouse Germ and Bone Marrow Cells After Chronic or Acute Exposure to γ -Rays in vivo

In a recent report, Cai et al. (1993) confirmed an earlier study for the induction of a cytogenetic AR in mammalian germ cells (Cai and Liu 1990). They showed that a whole-body pre-exposure to chronic doses of X-ray up to 200 mGy could induce a significant reduction in the chromatid and isochromatid breaks found in spermatocytes 4 h after a 1.5 Gy challenge dose, and for reciprocal translocations 60 days after the challenge dose. They also found that pre-treatment of germ cells with 50 mGy could also induce a significant AR to radiation-induced dominant lethality. However, while pre-exposed pre-meiotic cells (stem spermatogonia, differentiated spermatogonia and spermatocytes) showed the AR,

post-meiotic cells (spermatid and spermatozoa) did not. It was thought that this could be due to differences in metabolism between the pre-meiotic and post-meiotic cells.

Cai and Liu (1990) also observed an AR to IR in bone marrow cells of male Kummung mice pre-exposed *in vivo* to 0.1 Gy and challenged 2.5 to 3 hours later with 0.75 Gy of X-rays. Bone marrow cells which originated from mice that received the combined treatment had smaller numbers of chromatid aberrations than cells that originated from mice which received the challenge dose only. Farooqi and Kesevan (1993) confirmed the presence of an AR in mouse bone marrow cells. Swiss albino male mice were pre-exposed to priming γ -doses of 0.025 and 0.05 Gy (1.67 Gy/min) and challenged with 1 Gy γ -dose (2.7 Gy/min). When the time-interval between the conditioning dose and the challenging dose was 2 h, both priming doses induced the adaptive response as evidenced by reduced frequency of micronuclei in polychromatic erythrocytes and chromosomal aberrations in bone marrow cells. However, when the challenge dose was given 24 hours later, only the lower dose was effective in inducing the AR. Jacobsen-Kram and Williams (1988) also failed to observe an AR when the challenge dose was given 24 h after the adapting dose, a time-span possibly too long for the induced activity to remain effective in rapidly dividing bone marrow cells and to protect against the damaging effects of the

challenge dose. Jiang et al. (1992) demonstrated that an AR to chronically delivered ^{60}Co γ -rays and β -rays from tritiated water, *in vivo*, can be induced in the bone marrow of mice. Gaziev et al. (1992) also observed a radio-adaptive response in SHK male mice pre-exposed chronically to 0.12-1.0 Gy γ -rays. The frequency of micronuclei in polychromatic erythrocytes of the bone marrow was significantly reduced in cells from mice pre-exposed to the chronic dose prior to receiving the challenge dose of 1 Gy, as compared to cells from mice that received the challenge dose only. Moreover, they have presented data indicating that the activity of DNA-polymerase β was increased in the nuclei of liver and lung tissue of mice exposed chronically to 1.7 Gy.

The above results indicate that an AR to IR can be induced in bone marrow cells after acute or chronic pre-exposures to low LET radiations *in vivo*, provided the challenge dose is delivered within a few hours after the pre-exposure.

3.6.7 Further Characterization of the Adaptive Response

Reports on the individual variability in the adaptive response to ionizing radiation continue to appear. Aghamohammadi and Savage (1991) suggested that the variability may be due to the

use of chromatid-type aberrations to demonstrate the adaptive response in human lymphocytes. As mentioned earlier, Olivieri and Bosi (1990) have proposed that the failure to exhibit an adaptive response is a consequence of the physiological state of the cell at the time of the low dose exposure. They have shown that the response could be induced in cultured lymphocytes from donors who had not previously displayed the AR, by addition of adrenal/thymus extracts, or growth factors at the time of the low-dose exposure. Shadley and Dai (1993a) have shown that the response of mitogen-stimulated lymphocytes to high-dose-induced cytotoxicity is correlated with endogenous levels of lymphokines at the time of the exposure. Recent experiments by Bosi et al. (1991) indicate that if the pH of the medium is lowered, even supposed nonresponders now show the effect. Variability in the levels of endogenous cytokines (Shadley and Dai 1993a, Wilson 1993), physiological state of the cell (Olivieri and Bosi 1990) as well as the heterogenous cellular composition of peripheral blood (Aghamohammadi and Savage 1991) undoubtedly contribute to this inter and intra-donor variability in low-dose-induced responses.

Several reports further characterizing the AR to IR have been published recently. A report by Fan et al. (1990) showed that the amount of chromatid damage in human lymphocytes exposed to a

challenge dose of radiation was similar when the cells were pre-exposed to two inducing doses of 0.01 Gy between 4 and 24 hours after PHA stimulation, the second dose thus providing no additional protection against the damage caused by the challenging dose. However, a more recent report by Bai and Chen (1993) showed that a 0.005 Gy dose, which in itself did not induce an adaptive response, did so when given twice within the same cell cycle. The magnitude of the cumulative effect was strongest when there was an interval of 6 hours between the two adaptive doses and between the second adaptive dose and the challenge dose. Delivering two 0.01 Gy doses had no greater effect than did a single 0.01 Gy dose. These results indicate that the induced repair system is maximally induced at 0.01 Gy.

Using micronucleus formation as an end-point, Domínguez et al. (1993) confirmed earlier reports (Wolff et al. 1989, Cortés et al. 1990b) of a reduction in chromosomal aberrations by high doses of X-rays in human lymphocytes pre-exposed to low doses of H_2O_2 , which like X-rays, produces oxygen radicals. The molecular basis for this apparent protective effect of low doses of H_2O_2 against oxidative damage remains to be elucidated.

Osmak and Horvat (1992) investigated the response of Chinese

hamster V79 cells irradiated daily with 0.3 Gy of γ -rays 5 times per week for 12 weeks and challenged with an additional dose of 1.5 Gy γ -rays or treated with 5 $\mu\text{g/ml}$ of the DNA cross-linking agent mitomycin C. An insignificant decrease in the yield of chromatid aberrations and sister chromatid exchanges was observed when pre-exposed cells were challenged with γ -rays. In contrast, pre-irradiated cells became significantly more resistant to the induction of chromosomal damage when challenged with mitomycin C. It is of interest that in this study, pre-treatment with rather high, repeated-daily acute doses of 0.3 Gy induced the AR to mitomycin C.

OBJECTIVE OF THE STUDY

The aim of this study was to test whether pre-exposure to ionizing radiation induces a biological response which increases the resistance of normal human skin fibroblasts, maintained in culture, to a second dose of ionizing radiation. The end-points of cellular survival and micronucleus formation were used to test for the presence of this adaptive response to ionizing radiation in human fibroblasts.

Once the existence of this response was established in human fibroblasts, experiments were undertaken to characterize the possible mechanisms responsible for inducing the response.

The significance of the induced protective mechanism was investigated by studying the carcinogenic risk of a subsequent radiation exposure in adapted cells.

Most of the cells in the human body are relatively quiescent, entering an active cell division cycle infrequently or in some cases not at all during the life span of an individual. In contrast, most studies of radiation effects and DNA repair mechanisms in human cells are done with cultured cell populations maintained in an active, proliferative state. In the experiments described in this thesis, we have studied cultures of quiescent, confluence arrested cells.

OUTLINE OF RESULTS PRESENTATION

The results section consists of 4 chapters. Chapters 1,2 and 3 describe experiments where normal human skin fibroblasts (AG1522) were used. The materials and methods section described on page 89 applies to these chapters which address a specific objective of the thesis, namely, 'the existence of an adaptive response to ionizing radiation in human fibroblasts and the mechanisms involved in such a response'. The data reported in these three chapters is jointly discussed. Chapter 4 describes experiments where mouse embryo cells (C3H 10T½) were used to test the existence of a radiation-induced AR for protection against neoplastic transformation. This latter chapter is comprised of its own materials and methods, results and discussion sections. Appendix 1 to the thesis describes the modulation of expression of various genes following exposure to an adapting dose of radiation. It also consists of materials and methods, results and discussion sections. Appendix 2 is a list of the abbreviations used in this manuscript.

Chapter 1 is a characterization of the normal human skin fibroblast cell line (AG1522) used in these studies to test the hypothesis that "pre-exposure to low dose-rate irradiation induces an adaptive response to ionizing radiation which protects normal

human skin fibroblasts from the lethal and chromosomal damaging effects of a subsequent acute challenge dose of radiation".

- a) The growth of this cell line is characterized. The doubling time of the cells and their progression in the cell cycle as a function of time after initial seeding is described. The effects of refeeding on cellular growth, as well as acidification of the growth medium is also described.
- b) The effects on cell survival of high and low dose-rate irradiation of AG1522 cells is presented.
- c) The capability of AG1522 cells to recover from the lethal and chromosomal damaging effects of low- and high-dose-rate ionizing radiation is assessed.

Chapter 2 presents results which show for the first time the existence of an adaptive response to ionizing radiation in normal human skin fibroblasts. AG1522 cells in stationary phase of growth were pre-exposed to a range of adapting radiation doses delivered at variable dose rates and subsequently challenged with large acute doses of radiation. This chapter shows:

- a) The biological end-points of cellular survival and micronucleus formation used simultaneously for the first time to measure the extent of the AR in this cell line.

- b) Relationships between the end-points of cellular survival and micronucleus formation.
- c) The presence of a threshold and an optimum adapting dose delivered at a low dose-rate for induction of a survival adaptive response.
- d) The number of cells in colonies originating from surviving cells. (This end-point is used as a novel parameter to measure the AR to IR).
- e) The effects of the AR on the distribution of micronuclei in binucleate cells. (This end-point is also used as an indicator of the AR to IR).
- f) The expression time of the AR following pre-exposure to adapting doses delivered at variable dose rates.
- g) Effects on the cell cycle redistribution of the cells during the various treatments.
- h) Data testing for artifactual results as a consequence of any selective loss of cells due to the pre-exposure.

Chapter 3 addresses some mechanisms potentially leading to the expression of the AR in the AG1522 cell line.

- a) The amount of chromosomal damage per unit time needed to induce the protective effects of the AR is discussed.

- b) Experiments designed to study any enhancement of the rate of repair of DNA lesions due to a challenge dose of radiation in cells pre-exposed to an adapting dose of radiation are presented.
- c) The induction of division delay is examined as a mechanism leading to expression of the AR.

Chapter 4 addresses the consequences of the AR to IR. One mechanism postulated to lead to the AR to IR is the induction of a DNA repair mechanism. The effects of such an induced DNA repair mechanism on the carcinogenic risk of a subsequent radiation exposure had not been addressed. Using the C3H 10T½ cell line, the effects of an acute 4 Gy dose on cell survival, micronucleus formation and transformation to the malignant state is assessed in these cells pre-exposed to a chronic dose of radiation. The frequency of malignant transformation following exposure to a range of chronic doses of γ rays is described.

Appendix 1 examines the effects of pre-exposure to low dose-rate ^{60}Co - γ rays on the expression of specific genes that might be radiation responsive. The technique used in the study is described. The transcript levels in control and cells exposed to an adapting dose of 3.6 Gy (0.003 Gy/min) is reported.

HYPOTHESIS

A chronic or small acute dose of ionizing radiation, in human fibroblasts, induces a biological response which increases their resistance to a second challenge dose of ionizing radiation. The induced biological response enhances the rate of repair of DNA damage from the second dose and results in a greater division delay following the challenge exposure.

EXPERIMENTAL PROTOCOL

Plateau phase AG1522 cells in passage 10 or 11.

Exposure to adapting dose $\xrightarrow[\text{37}^\circ\text{C}]{\text{time at}}$ Challenge dose $\xrightarrow[\text{37}^\circ\text{C}]{\text{time at}}$ Trypsinize and assay

END-POINT ASSAYED FOR:

- Cellular survival
- Micronucleus formation
- Neoplastic transformation (using a rodent cell line 'transformation assay' as a test system).

MATERIALS AND METHODS

Glassware

Dedicated glassware was used throughout this project. Pieces of glassware were initially thoroughly cleaned with a solution consisting of 4% potassium dichromate in concentrated sulfuric acid, followed by exhaustive rinsing in water as below. Immediately after each use, they were washed with soap (Sigmaclean, Sigma), rinsed sequentially in distilled, double-distilled-deionized (14M Ω cm) and then in (18.2 M Ω cm) water. The glassware was then sterilized by dry heat (160°C, 2h) or by autoclaving in plastic bags. Although, toxic residues could be deposited through steam sterilization, at times this was the only option available.

Water

The water used throughout the experimental steps was double-distilled and deionized through a "Millipore Milli-Q UF Plus system for life sciences application", generating an ultrapure, pyrogen-free water with a resistivity of 18.2 M Ω cm.

Laminar Flow Cabinets

Handling of live cells was done in a laminar flow cabinet (Labgard, Minneapolis, MINN) equipped with 'Hepa 0.2 μ m filters'.

The hood was initially cleaned and decontaminated by circulating formaldehyde gas in the cabinet at a concentration of 50-75 ppm for two hours (Laboratory Biosafety: Principles and Practices), to fix any viruses or other pathogens that may have been present. Once decontaminated, the hood was dedicated to the culture of normal human cells, and only the cells used in the experiments pertaining to this project were cultured in it. The inner surfaces of the hood were routinely cleaned with a 70% ethanol solution.

Incubation

A water jacketed 'Hotpack' incubator (Waterloo, ON) was used to provide a controlled 37°C atmosphere for the growth of cultured cells. The stability of the temperature was routinely checked through a digital thermometer. It was observed that a combination of external CO₂ regulator and flow meters in addition to the built-in controller provided an excellent control of the CO₂ atmosphere as revealed by daily checks through a "Fyrite CO₂ meter" (Bacharach Instrument Company, Pittsburgh, Pa). Air supplied to the incubator was filtered through a 'Nalgene' 0.22 µm filter. Residues trapped by the filter were observed to have an adverse effect on the growth of the cells. The incubator used was equipped with a CO₂ atmosphere quick recovery system which restored the CO₂ concentration in the incubator atmosphere to the set conditions following opening of the

incubator. A good control of atmospheric CO₂ tension is crucial to the growth of the cells, as it directly regulates the concentration of dissolved CO₂. Dissolved CO₂ along with NaHCO₃ plays an important role in regulating the pH of the growth medium.

Illumination

The handling of cells, culture medium and serum was done in the absence of white fluorescent light to prevent damaging photo-oxidations, whereby superoxide radicals are generated from reduced riboflavin (Petkau et al. 1987). Light from standard fluorescent bulbs was found to be toxic and mutagenic to bacteria (Webb and Malina 1967) and to mammalian cells *in vitro* (Burki and Lam 1978). Fluorescent light also induced breaks in DNA strands (Bradley et al. 1978), cross-links, and chromosomal aberrations (Gantt et al. 1978) in cultured cells. A study by Kennedy et al. (1980) has shown that fluorescent light can transform cells *in vitro* and that the frequency of malignant transformation induced is related to dose. Pyrimidine dimers and thymine glycols have been implicated in the light-induced transformation process.

The use of gold fluorescent lights has been recommended (Kennedy et al. 1980) in tissue culture laboratories, and they have been used when necessary. Such lights do not emit wavelengths

below 5000 Å, and have been shown to be non-mutagenic to mammalian cells.

Culture Medium

The combination of different materials in a medium determines whether or not cells grow *in vitro*. The cell culture medium used throughout the experiments described in this project was designated as DF. It consists of a 1:1 mixture of Dulbecco's Modified Eagle Medium and Nutrient Mixture F-12 (D-MEM/F12) (Gibco). The powdered medium was dissolved in water according to the specifications of the manufacturer. It was supplemented with 1.2 g L⁻¹ NaHCO₃. Before filtration through a 0.22 µm pore size filter, the pH of the medium was adjusted to 7.45 with 5N NaOH solution. The combination of 8 mM NaHCO₃, 20 mM Hepes and 2% CO₂ during incubation resulted in a good pH control of the medium. The AG1522 and C3H 10T½ cells used throughout the experiments of this project had higher plating efficiencies when cultured in DF medium as compared to Ham's F12, Dulbecco's Modified Eagle (DME) or Basal Medium Eagle (BME) culture media (data not shown). A report by Weinstein and Mukherjee (1988) highlighted the importance of culture media variations as related to *in vitro* aging of human fibroblasts.

Usually enough DF medium was prepared for 1 week. In the event the medium used was more than 1 week old but less than 2 weeks old, it was then supplemented with 2 mM glutamine. Only medium from the same batch was used in a particular experiment.

The medium was routinely stored at 4°C. When needed it was warmed in a 37°C bath only for enough time to reach that temperature. Extended heating of the medium at 37°C prior to addition to the cells was avoided to prevent degradation of heat labile constituents such as glutamine. In addition to consistency of composition of the culture medium, other parameters such as cell density, nature of the growth substrate and the way the cells are handled during subculturing are also critical factors that can affect the culture performance of cells. The use of 18.2 MΩcm water substantially improved the plating efficiencies (from 11 to 17%) of the cells used in these experiments.

Serum

For the culture of the AG1522 cells, the DF medium was supplemented with 13% fetal calf serum (FCS). The FCS was purchased in 500 ml bottles and kept frozen at -20°C. For use, a bottle of FCS was thawed overnight at 7°C. The serum was then aliquoted in 45 ml quantities in 50 ml polypropylene tubes and re-

frozen at -20°C . The needed volume was then rethawed overnight at 7°C for medium supplementation. Handling of the serum in a consistent manner is crucial, for its various constituents are labile and the growth of cultured cells is known to be affected by modifications in the constitution of the growth medium. Such modification can result in variations in the response of the cells to various treatments. Every effort was taken to pursue the experiments of this project with serum from the same lot. Unfortunately, due to various financial and administrative factors this was not possible. Serum lots purchased from Sigma Chemical Co. (Missouri) and from Gibco (Grand Island, N.Y.) were used in the experiments. Before a serum lot was chosen, the growth, plating efficiency, gross morphology and number of cells at a certain time after plating a specific number of cells were monitored in medium batches supplemented with the various sera being tested. Some serum lots did not sustain the growth of the AG1522 cells.

Although proteins are a major component of serum, the functions of many of these, *in vitro*, remain obscure. Polypeptides such as platelet-derived growth factor (PDGF) which are released from the platelets have a mitogenic activity and are probably the major growth factors in serum (Antoniades et al. 1979). Other platelet-derived factors such as TGF- β may be inhibitory to growth and promote differentiation. Normal human bronchial epithelial

cells underwent irreversible inhibition of DNA synthesis in the presence of TGF- β (Masui et al. 1986). This inhibition was antagonized by epinephrine. Other growth factors such as fibroblast growth factor, epidermal growth factor, endothelial growth factor, insulin-like growth factors IGF-1 and IGF-2 also have various growth promoting activities and can modulate the response of the cells to various treatments including radiation (Witte et al. 1989, Haimovitz-Friedman et al. 1991). Some of these factors associate with their specific receptors, and modulate cellular activity via signal transduction pathways. Accumulating experimental evidence indicates that ionizing radiation initiates similar transduction pathways in exposed cells (Weichselbaum et al. 1991). Hence, it is of extreme importance to exercise utmost care in handling serum to obtain reproducible results which allow a measurement of radiation effects.

Serum also contains hormones with mitogenic activity such as insulin and growth hormone. The hormone hydrocortisone, which can promote cell attachment and cell proliferation, can be under certain conditions cytostatic and can induce cell differentiation. Serum also contains nutrients and metabolites such as amino acids, glucose, lipids, ethanolamine and phosphoethanolamine. Trace elements such as iron, copper and zinc are provided by serum. The mineral element selenium has been found to be essential for the

growth of cells probably due to its ability to act as a cofactor to glutathione synthetase, a free radical detoxifying enzyme (Freshney 1994). However, serum may also contain substances that can inhibit cell proliferation. Experiments by Loo et al. (1987) showed that the *in vitro* growth of mouse embryo cells initiated and maintained in serum-free medium is markedly inhibited by both serum and plasma, suggesting the presence in serum of circulating inhibitors that are distinct from platelet-associated factors.

While heat inactivation of serum removes complement from the serum and reduces the cytotoxic activity of certain compounds such as immunoglobulins, it reduces the activity of growth promoting substances. Due to this reason we opted not to heat shock the serum. All batches of serum used were triple filtered through a 0.1 μm pore-size filters which virtually eliminated potential mycoplasma contamination.

Clearly, serum is composed of components that not only have an effect on cellular growth, but on the cellular response to radiation (Raaphorst and Azzam, 1980). Hence, its careful handling in a consistent manner would eliminate or minimize potentially serious variations in the response of cultured cells to killing by radiation, repair of DNA damage and transformation to the neoplastic state. It would be ideal that experiments in a specific

project be done with the same serum lot [in 112 samples tested by Hyclone Laboratories, Inc., the progesterone range was 0.2-238 mg/dl showing more than a 793-fold variation; the endotoxin range was 0.003-0.90 mg/dl, showing more than a 300-fold variation (Hyclone Laboratories, Inc., 1986)].

Cells

The AG1522 skin fibroblasts, of apparently normal non-fetal human tissue were obtained from the N.I.A. Aging Cell Culture Repository at the Coriel Institute for Medical Research, Camden, NJ. These primary cells originated from a foreskin explant from a clinically unaffected 3 day old male. They proliferate in culture in an anchorage-dependent manner. When cultured in a plastic dish, they continue to proliferate until the dish is covered by a single layer of cells. Once the cells form a continuous sheet, they stop dividing, hence exhibiting the phenomenon of contact inhibition of cell proliferation. Upon the subculturing of the cells, during the course of these studies, the karyotype was verified to consist of 46 chromosomes.

Experiments were routinely done in cells at passage 9 or 10. It is very important to experiment with cells of the same passage number, as cells have been shown to accumulate mutations with

passaging (Day et al. 1991). Moreover, it has been reported that the activity of antioxidant enzymes (superoxide dismutase) and glutathione levels decline with cellular aging, while products of oxygen free-radical reactions increase in their levels, potentially altering radiation induced end-points (reviewed in Bernstein and Bernstein 1991).

For experiments, a vial containing 10^6 cells in 1 ml of growth medium (DF supplemented with 13% FCS) at 10% DMSO was taken from liquid nitrogen where frozen cells are routinely stored. The vial was then left on dry ice for about 20 minutes. The cells were quickly thawed by adding growth medium at room temperature to the vial. The cells were finally diluted in 10 ml growth medium and centrifuged (relative centrifugal force: 20g) for 3 to 5 minutes. The medium was decanted and the cells resuspended in 10 ml growth medium and plated in two 80 cm² flasks at a density of 7.5×10^5 and 2.5×10^5 cells/flask respectively. They were then incubated at 37°C in an atmosphere of 2% CO₂ in air. When the cells reached confluency (within 5 days), they were trypsinized and replated according to the following protocol. The medium was decanted, the cell monolayers were rinsed twice with a solution of Earle's balanced salt (EBSS) free of calcium and magnesium ions and containing 10mM NaHCO₃, plus 20mM Hepes (pH 7.4), and once with a 1% trypsin solution (Sigma). A volume of 1.5 ml trypsin solution was

left on the monolayer for 30 s, after which it was aspirated. The monolayer of cells with a film of trypsin remaining was then incubated at 37°C for 3 to 5 minutes. Following the incubation, one firm tap of the flask with the palm of the hand was sufficient to dislodge the cells from the plastic surface. The cells were then immediately resuspended in growth medium, counted on an electronic cell counter (Coulter), and plated at the required cell numbers in 80 cm² flasks containing 25 ml of growth medium.

Unless otherwise noted, for all plateau phase experiments, 5×10^5 cells were seeded in 80 cm² flasks. They were re-fed on day 5 and day 8. On day 10, they were submitted to the various experimental procedures. At that stage, over 90% of the cells were in the G₀/G₁ phase of the cell cycle, as indicated by flow cytometric analysis.

Some antibiotics supplemented to culture media are bacteriostatic. In the event a microbial contaminant is already present in the medium, microbial basal metabolism could potentially release toxic compounds which can adversely affect the growth and response of the cultured cells. To ensure that the cells were free of microbial contamination, the cells were routinely cultured in an antibiotic free medium. Gentamicin sulfate (Gibco, Grand Island, N.Y.) was added at a concentration of 25 µg/ml only to the cells

destined for experimental manipulations.

Treatment With the Protein Synthesis Inhibitor Cycloheximide

Immediately prior to irradiation, the conditioned growth medium, in which the AG1522 cells were growing, was decanted and quickly pooled. A 12.5 ml volume of the pooled conditioned medium was then added to the cells which were then immediately irradiated or control manipulated. Within a minute after the irradiation, a further 12.5 ml volume of the conditioned medium containing 4 $\mu\text{g/ml}$ cycloheximide (Sigma, St. Louis, MO.) was added to the cells which were then incubated at 37°C for up to 8 hours. Following the respective incubation period, the cells were rinsed twice with EBSS and trypsinized as previously described.

Disposable Plastic

The cells were cultured in "Nunc" (Roskilde, Denmark) 80 cm² polystyrene flasks, treated with an electric ion discharge to render them wettable. The flasks were routinely stored in the dark to prevent deterioration of the cell surface attachment properties. Polystyrene plastic is known to be permeable to O₂ and CO₂, hence eliminating growth problems related to gassing.

All media containing DMSO were handled either in glass or polypropylene plasticware to prevent adverse reactions of the chemical with the plastic vessels.

For the micronucleus assay, the cells were grown in tissue culture chamber slides (Nunc), where cellular growth occurs directly on a standard microscope slide.

Flow Cytometry

An Ortho-Diagnostic Cytofluorograf system (50-H) with a dual-laser cytometer, a cell sorter and a dedicated computer was used to analyze cell cycle distributions. The method used to prepare the cells for analysis was a modification of Vindelov's method described in Ortho Protocol 28 (in Szekely et al. 1989). Briefly, a 10^6 cell suspension in pH 7.6 Tris buffer was added to a permeabilizing hypertonic salt solution containing Triton-X-100 (0.1% v/v), RNase A (0.05% w/v) and ethidium bromide (5 $\mu\text{g/ml}$). After an incubation of the suspension at 0°C for 10 min followed by an incubation at room temperature for 10 min, the cells were analyzed by flow cytometry. The fluorochrome, ethidium bromide, intercalated into the DNA was excited by the argon laser (488 nm line). Cells were analyzed at a rate of 200 to 300 cells/s. The fluorescent pulses were passed through a filter and processed. A

two-parameter analysis was performed by examining the red-fluorescent-pulse area versus pulse peak height. A two-dimensional region was then defined to exclude G_0/G_1 doublets, which would otherwise be counted within the G_2/M subpopulation.

Histograms of fluorescence intensity (DNA) per cell were then displayed for the region. The number of cells in each phase of the cell cycle was then determined by the polynomial method using Ortho software.

The cell cycle is divided into two fundamental parts: interphase, which occupies the majority of the cell cycle, and mitosis which lasts about 30 minutes, ending with the division of the cell. During interphase the DNA is decondensed and individual chromosomes can be distinguished by fluorescence *in situ* hybridization. Chromosome replication is restricted to a specific part of interphase called S phase (for DNA synthesis). S phase occurs in the middle of interphase, preceded by a time-gap called G_1 and followed by a time-gap called G_2 . In a typical animal cell cycle, G_1 lasts 12 hours, S phase 6 hours, G_2 6 hours, and mitosis about 30 minutes. The DNA content of G_2 cells is exactly twice that of G_1 cells, and cells in S phase have an intermediate DNA content that increases with the extent of replication.

The radiation sensitivity of cells has been shown to depend of their position in the cell cycle. Survival curves at a number of discrete points in the cell cycle were generated by Sinclair (1968). His results showed that the most sensitive cells are those in M and G₂. At the other extreme, cells at the latter part of S exhibited the most radioresistance. Cells in G₁ and S were intermediate in sensitivity between the two extremes. In addition, analysis of the cell cycle distribution provides information on division delays introduced by various treatments of a cell population.

Irradiation

High-dose-rate irradiations were done using a Siemens Stabilipan-2 X-ray machine operated at 250 kV, and 15 mA with a 1-mm aluminum external filter, giving a half-value layer of 1.29 cm aluminium (effective energy of 87 keV). The culture flasks were placed on a dry, rotating lucite disc on the surface of a temperature controlled water bath used to control the temperature of the irradiated cells. Unless otherwise indicated, the irradiation temperature was room temperature of 25°C. The dose-rate was determined before and after each experiment by means of a Model 550 Victoreen dosimeter (Cleveland, Ohio). In all experiments, the flasks were positioned at a distance of 16 cm from

the X-ray source. The range in acute dose rates used in the experiments was 1.66 Gy to 3 Gy/min. This range reflects changes in the output of the X-ray machine at different times. However the energy spectrum of the X-rays remained constant throughout the course of this project, as the thickness of the half-value layer did not change.

Chronic radiation exposures were from a ^{60}Co γ Beam 150 irradiator (Atomic Energy of Canada Limited). The cells were kept at 37°C and at a distance of 3.55 meters from the source during the chronic exposures. Both the acute and chronic exposures occurred in the absence of light.

Cellular Survival Measured by the Colony Formation Assay

The colony formation assay is a measure of the proportion of cells which remain reproductively viable after an experimental treatment. Following trypsinization of the monolayer, an appropriate number of cells was plated in 80 cm² flasks containing 25 ml of growth medium. Cells were plated in numbers that would yield about 200 colonies/flask. Thirteen days after plating and incubation at 37°C, cellular colonies were visible. The colonies were gently rinsed with a phosphate buffered salt solution (PBS) (pH 7.4), and fixed in absolute ethanol. They were subsequently

stained with a 0.1% Giemsa stain in PBS, rinsed in water and allowed to air dry. It was observed that Giemsa stain resulted in a good contrast staining between the cytoplasm and the nucleus. The established criterion of counting surviving colonies containing >50 cells was followed. A binocular dissecting microscope was used to view the stained colonies.

The plating efficiency of the cells was calculated from the numbers of observed colonies arising from control cells that were not subjected to any treatments. For calculations of cellular survival of treated cells, survival levels were normalized to those of untreated cells. In a particular experiment, cellular survival in 80 cm² flasks was measured in three replicates for the experimental cells, and 4 to 8 replicates for the control cells (plating efficiency). The standard deviation was calculated and is shown on the graphs whenever greater than the datum point symbol.

An average of about 200 surviving colonies in an 80 cm² flask was desired, for it yielded good statistics on a large number of cells at risk from the various treatments. As different cell numbers are usually plated to measure survival following treatments with varying lethal effects, a series of experiments was done to measure the effects of plateau-phase feeder cells (X-irradiated with a 50 Gy dose) on cellular survival and recovery from the

treatments. Neither the feeder cells nor growth medium containing up to 25% conditioned growth medium altered the plating efficiency of the cells or altered the survival level of treated cells under our experimental conditions. However, it should be noted that feeder cells and medium conditioned by specific numbers of cells and at specific stages of their growth can have profound effects on the growth characteristics of cells and hence on their response to treatments (Kabalin et al. 1989, Leith et al. 1991).

Micronucleus Assay

The frequency of micronucleus formation following a specific treatment was measured using the cytokinesis-block technique developed by Fenech and Morley (1986). Immediately after trypsinization, about 3×10^4 AG1522 were plated in a 2 ml volume of growth medium in a chamber flaskette (Nunc), in the presence of 1 $\mu\text{g/ml}$ cytochalasin B, and incubated at 37°C. Forty-eight hours later, the cells were rinsed twice with PBS (pH 7.4), and fixed in acetic acid : methanol (3:1). Following air drying, the cells were stained in a 1 $\mu\text{g/ml}$ acridine orange solution and examined under a fluorescent microscope. Usually a minimum of 1000 cells was examined. Micronuclei were scored in binucleate cells only. Binucleate cells were scored as having 0, 1, 2 or more than 2 micronuclei. Such counts permitted not only the determination of

the frequency of micronucleus formation, but also allowed the measurement of changes in the distribution of the number of micronuclei per binucleate cell following the various treatments. The frequency of micronucleus formation is defined as a ratio of the total number of micronuclei counted in a specific number of binucleate cells.

Cytochalasin B is a toxic metabolite produced by fungi of the subclasses *Ascomycotina* and *Deuteromycotina*. It binds with roughly similar affinity to both the glucose transporter and to the barbed ends of actin filaments (Rampal et al. 1980). Studies on the lack of effect of cytochalasins (there are several members of this class of protein) on cells expressing an actin mutation implicate actin as the major target of cytochalasin (reviewed in Sheterline and Sparrow 1994). Treatment of cells with Cytochalasin B disrupts cytokinesis by enabling nuclear division to take place while preventing cytoplasmic division. Therefore, one can observe which cells in the population were able to undergo at least one cell cycle after exposure to a dose of radiation, or exposure to other agents, by their binucleate appearance under microscopic examination.

Experiments were done to determine the optimum concentration of cytochalasin B to block cytokinesis in the AG1522 cells.

Cytochalsin added at 1 $\mu\text{g}/\mu\text{l}$ for 48 hours was not cytotoxic to the cells and about 50% of control cells were in the binucleate state at that concentration.

Statistical Analysis of Micronucleus Formation Data

Gaussian statistics were applied in the analysis of the data, whereby a certain number of cells were found to be micronucleated in a population of binucleate cells. The frequency of micronucleus formation (r_0) was calculated as: $r_0 = a/b$, where a is the total number of micronuclei scored, and b is the total number of binucleate cells examined. The error associated with r_0 is deduced from Gaussian statistics and is given by the following formula (Hassard 1991):

$$\Delta r_0 = (a/b) (1/a + 1/b)^{1/2}$$

To determine whether the observed and expected results are significantly different, the following statistical considerations from the 'normal distribution' were applied: If the observed values are outside 1.64, 1.96, 2.58, or 3.29 S.D. (standard deviation) of the expected values, then the probability of these values arising by chance alone is $p < 0.1$, 0.05, 0.01 or 0.001 respectively.

If binomial statistics is applied in the analysis of the data , then the error associated with r_0 is given by the following formula (Hassard 1991):

$$\Delta r_0 = [(a/b)(1-a/b)/b]^{1/2}$$

To determine whether the observed frequency of micronucleus formation is significantly different from the expected frequency, it is assumed that this difference follows normal statistics. Therefore, a Z score is defined by the pooled standard deviation according to the following formula:

$$Z = (r_0 - r_e) / [(\Delta r_0)^2/1 + (\Delta r_e)^2/1]^{1/2}$$

where r_e is the expected frequency of micronucleus formation, and Δr_e is the standard error associated with the expected value. The level of significance is then found in a Z score table.

When binomial statistics were used to analyze the results reported in this thesis, the observed values were found to be different from the expected values at a greater level of significance than obtained with gaussian statistics.

RESULTSCHAPTER 1GROWTH CHARACTERISTICS AND RADIATION RESPONSE OF AG1522 CELLS

Figure 5 describes the growth of AG1522 cells as a function of time after plating. Cells (4.6×10^5) from a 6 day old culture in late exponential/early plateau growth phase, which were re-fed 26 hours prior to trypsinization and resuspension in fresh growth medium, were seeded in 80 cm² flasks containing 25 ml of growth medium. The growth of the cells was monitored as a function of cell number relative to time after seeding. At the times indicated in the figure, one or more flasks were trypsinized, the cells were resuspended and counted in an electronic cell counter. It was observed that after about 60 hours of exponential growth, the cells enter a stationary growth phase. From the exponential growth phase, the population doubling time of this cell line is calculated to be about 22 hours. When the lag phase of growth is considered, the effective population doubling time is calculated to be about 26 hours.

Figure 6 shows the effect on growth of refeeding stationary phase AG1522 cells at various times after their entry into stationary growth phase. Refeeding was done by decanting the old

medium and adding fresh growth medium. It is observed that the addition of fresh medium leads to increases in cell numbers, but at a lower rate when compared with that occurring in the exponential growth phase. Refeeding stationary phase cells resulted in a much tighter growth pattern. However, no piling up of cells was ever observed. The increases in cell numbers resulting from refeeding show that the cells of this line do respond to media changes by reaching a higher saturation density, in spite of the fact that they are a non-transformed cell line sensitive to postconfluence inhibition of cell division.

Figure 7 shows the cell cycle distribution of the cells described above, at various times after seeding, and after refeeding plateau-phase cells. The data show that as the cells enter a stationary phase of growth, a substantial percentage of the cells are still in S phase, probably indicating one last round of cell division for the majority of the cells. Also, the increases in cell numbers observed after refeeding stationary phase cells were correlated with redistribution of the cells in the cycle, with greater percentages of cells being observed in S and M phase. Consistent with our present knowledge of progression of cells in the growth cycle, it took longer for stationary phase cells to progress into S phase upon refeeding. Consecutive refeedings have

a stimulatory effect upon the progression of stationary phase AG1522 cells from G₁ to S.

Figure 8 shows the acidification of the growth medium, as revealed by pH measurements, at various times in the growth cycle described in figures 5 and 6. It is of interest to note that while the cells were in an exponential growth/early plateau phase, the pH of the medium was maintained at 7.4. Drastic drops in pH were observed when the cells are in the stationary phase of growth. When stationary phase cells are refed with fresh medium, it took 39 hours for the pH to drop to about 7.25. The effects of pH on the repair of potentially lethal radiation damage (Raaphorst et al. 1988) and on clastogenicity (Morita et al. 1992) have been described. Therefore, the time of refeeding a culture, at a specific population density, prior to experimental treatment is important.

Figure 9 describes the survival of plateau phase AG1522 cells following exposure to X-rays. The cells grown as monolayers in 80 cm² flasks were exposed to X-rays as described in Materials and Methods. Figure 10 is an expanded representation of the initial part of the survival curve. The absence of a shoulder in this curve for exposure to low doses is noteworthy. An increased

low-dose sensitivity relative to an extrapolation from doses greater than 0.75 Gy is observed. The values of D_0 calculated from the portions of the survival curve corresponding to exposures between 0 and 0.1 Gy and 1 and 8 Gy were 0.075 Gy and 1.55 Gy respectively.

Figure 11 also describes the survival of AG1522 cells following exposure to X-rays. However, for the experiment depicted in this figure the protocol used was different. A parental culture of plateau phase cells was trypsinized, resuspended in growth medium and counted. Respective cell numbers expected to yield approximately 50 to 150 colonies following exposure to X-rays were seeded in 80 cm² flasks, 16 hours prior to exposure. With this protocol, all the cells subsequently exposed to radiation originate from one culture; hence the effects on plating efficiency of trypsinization and handling of the cells were eliminated. Trypsinization of cells, if not well controlled can have profound effects on cellular viability (e.g. Raaphorst et al. 1979, Kapiszewska et al. 1991). Therefore, when parental cultures originating from different flasks are subjected to radiation (e.g. as for the data in figure 9) and subsequently trypsinized for the colony survival assay, it is very important to follow a well-controlled regimen for handling the cells. Resulting variations in

the plating efficiencies can modify the apparent survival levels due to the radiation exposures. Moreover, trypsinization of the cells at a well-defined time (1 or 2 min) following exposure could also be critical as mechanisms which modify cell survival can be active during that time. The results described in this figure (which were corrected for cellular multiplicity of 1.12) also indicate a hypersensitivity to very low doses of X-rays followed by reduced rates of cell killing at higher doses.

Figure 12 shows significant repair of potentially lethal radiation damage when AG1522 cells were held in plateau phase following an exposure to a 4.75 Gy dose of X-rays (1.83 Gy/min). A recovery ratio (defined as survival after a holding period divided by survival at time=0) of 4.35 was observed after an 8 hour incubation at 37°C following the radiation exposure. Most of the repair occurred in the initial incubation period following irradiation with a half-time for maximum repair of about 1 hour. However, it should be emphasized that the maximum recovery and recovery ratio can vary from one experiment to the other. Figure 12A shows a 12.6% survival level after a 7 hour incubation at 37°C following a 4.75 Gy dose of X-rays, as compared to 25% observed in figure 12. The maximum recovery ratio was calculated to be 3.7 as compared to 4.35 in the previous figure. The repair of potentially

lethal radiation damage (PLDR) was routinely measured in experiments designed to test the adaptive response to ionizing radiation in human fibroblasts. It is of interest to note that whenever a survival AR was not detected, an absence of such repair was also noted. Figure 12B shows that the recovery from potentially lethal radiation damage as noted in figures 12 and 12A can correlate with a decrease in the frequency of micronucleus formation.

Figure 13 shows that the repair of potentially lethal radiation damage is dependent on translational activities that are not present in non-irradiated cells. The increases in cellular survival observed following holding of X-irradiated cells in plateau phase were decreased when non-toxic concentrations ($2\mu\text{g/ml}$) of cycloheximide were added 4 minutes after X-irradiation and remained present for various holding periods.

Figure 14 shows the survival of plateau phase AG1522 cells following exposure to γ -rays, from ^{60}Co , delivered at 0.0025 Gy/min . A comparison of these data with the survival data following exposure to high dose-rate irradiation (Figures 10 and 11) reveals a substantial dose-rate sparing effect on cell killing.

Figure 15 shows minimal repair of potentially lethal damage when cells are held in plateau phase for a period up to 10 hours following exposure to a 9 Gy dose of γ -rays delivered at 0.0025 Gy/min. A maximum recovery ratio of 1.2 is observed after a 10-hour holding period.

While figure 12 shows that plateau phase AG1522 cells are able to repair potentially lethal radiation damage, figure 16 shows an absence of repair of potentially lethal radiation damage when actively growing AG1522 cells, re-fed 60 min earlier are exposed to 3.25 Gy and held at 37°C for 3 hours. Absence of such repair is also observed when the cells are pre-exposed to a fractionated regimen consisting of 3 consecutive doses of 0.39 Gy each, separated by various time intervals as indicated on the figure, prior to the 3.25 Gy dose. A complete lack of increase in cellular survival is observed during the holding period in every case tested. A lack of repair of potentially lethal damage was also noted following exposure of actively growing AG1522 cells to 4.75 Gy (data not shown). As a result of these data, plateau-phase cells are more suitable for studying radiation-induced radioresistance in AG1522 cells, as their repair of radiation damage is easily detected, and also because they consist of cells

mostly at the same stage in the cell cycle. Actively growing populations consist of cells at different stages of the cell cycle with different radiosensitivities (Sinclair 1968).

Figure 17 is a cell cycle analysis of the cells prior to the treatments described in figure 16. The abscissa depicts channel number, which is related to DNA content per cell. The first peak in the profile represents cells in G_0/G_1 phase, and the second peak, the cells in G_2 and M phases, while the cells in between the peaks are in S-phase. The results indicate that the culture of cells was actively growing with about 35% of the cells being in S phase, and 26% in G_2/M .

Figure 5. Growth curve of AG1522 cells, The increase in numbers of cells was monitored as a function of time after plating.

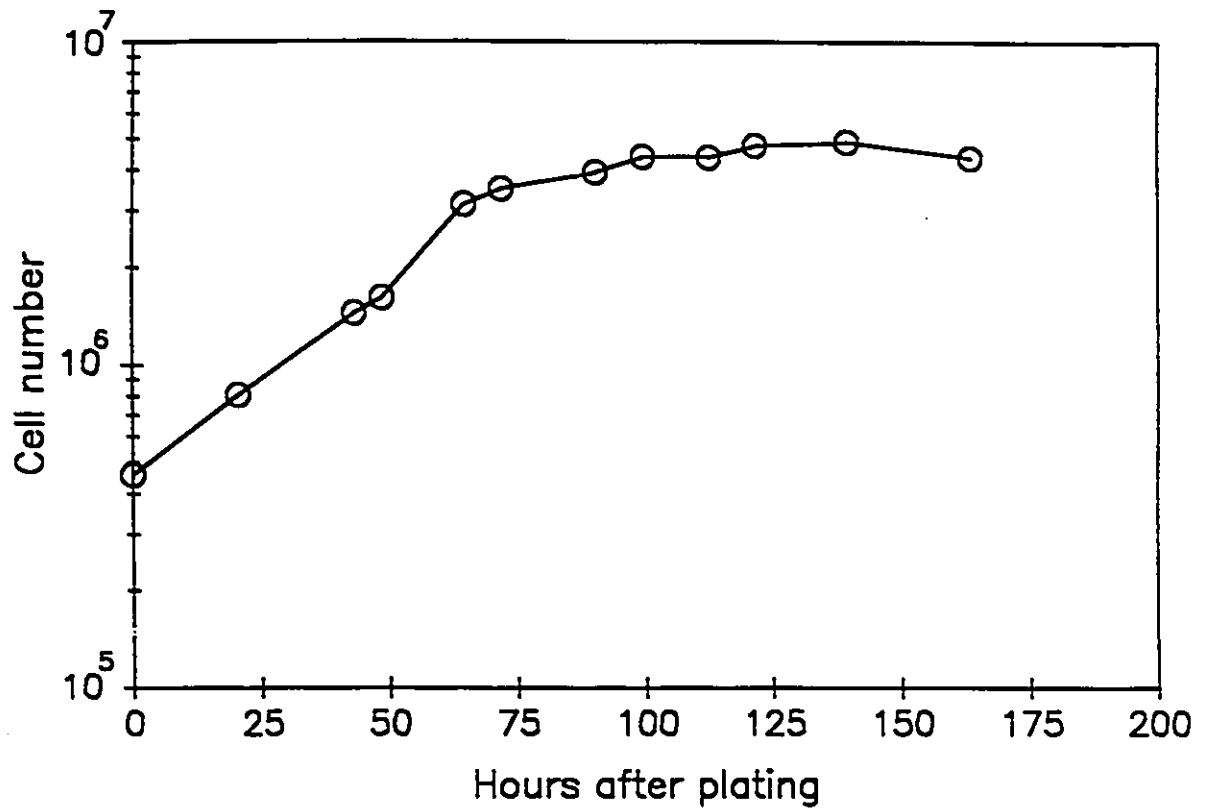


Figure 6. Growth curve of AG1522 cells: the effect of refeeding. The increase in cell numbers was monitored as a function of time after plating. Open circles: cells are not refed. Closed circles: cells were refed. (): time in hours between refeeding and cell count.

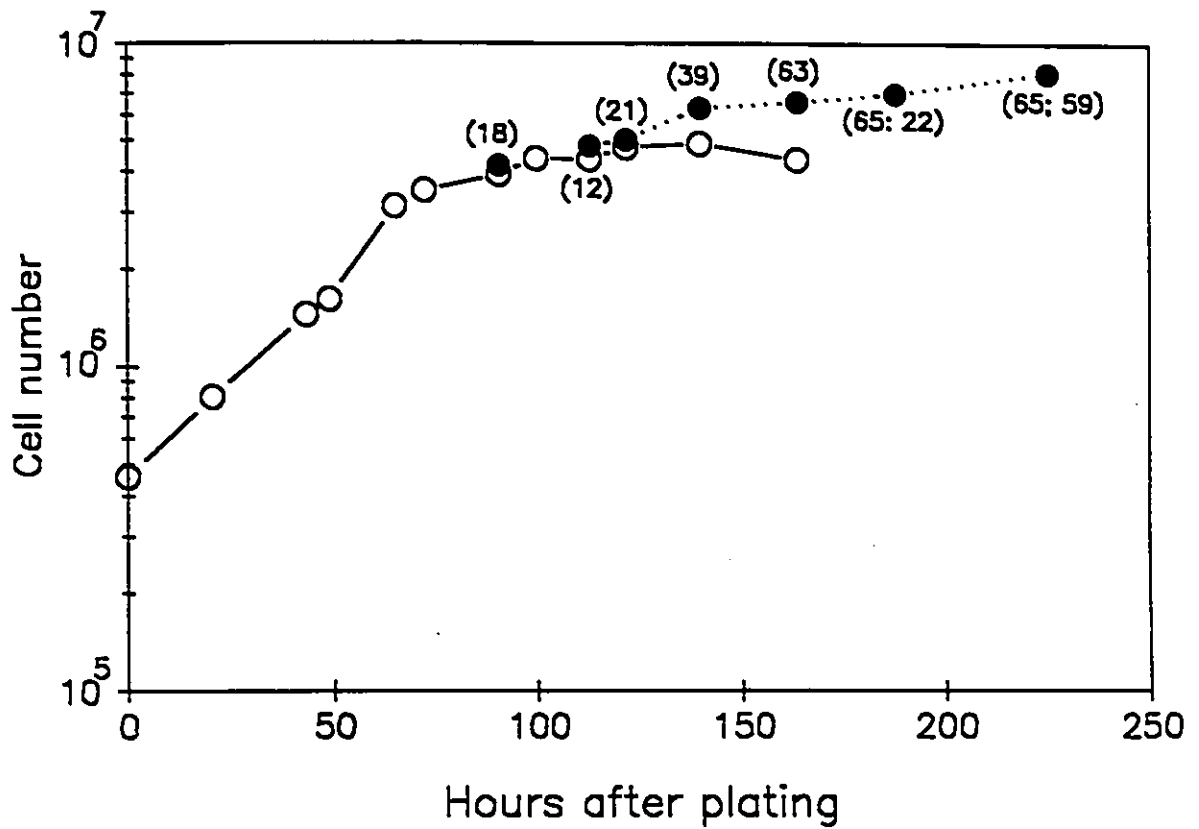
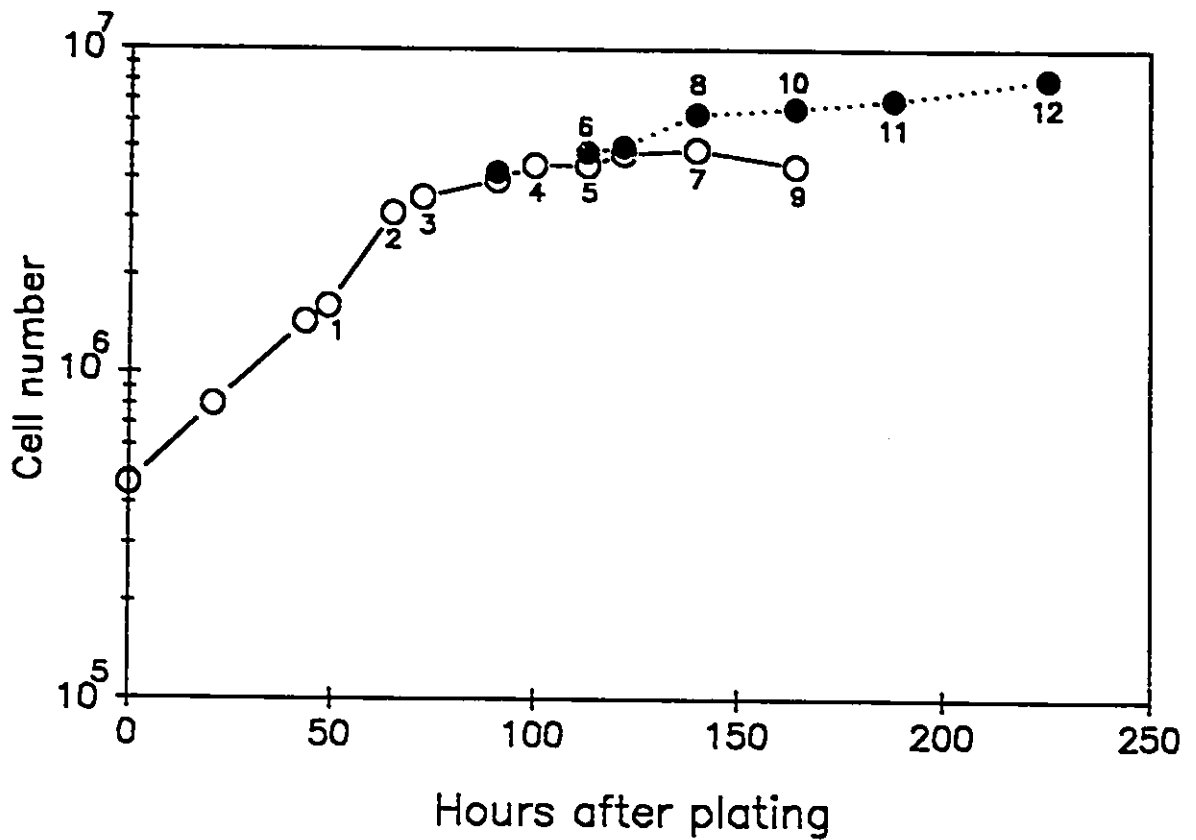


Figure 7. Growth curve of AG1522 cells: the cell cycle distribution as a function of time after plating. Open circles: cells were not refed. Closed circles: cells were refed.



	1	2	3	4	5	6	7	8	9	10	11	12
G_0 / G_1	46.4	69.1	68.7	90.4	93.1	92.3	92.4	85.8	91.9	91.3	83.6	89.2
S	43.1	10.8	27.1	2.8	1.4	3.1	1.0	4.2	1.6	1.1	9.1	4.9
G_2 / M	10.5	19.0	4.2	6.7	5.0	4.5	6.5	10.0	6.5	7.6	7.3	5.9
Time in hrs of refeeding prior to cell count and cell cycle analysis						12		39		63	65.5 21.8	65.5 59

Figure 8. Acidification of the growth medium of AG1522 cells as a function of time after plating. Open circles: cells were not refed. Closed circles: cells were refed. (): time in hours between refeeding and measurement of pH. pH measurements were made at 25.5°C using the cells depicted in the growth curves in figure 6.

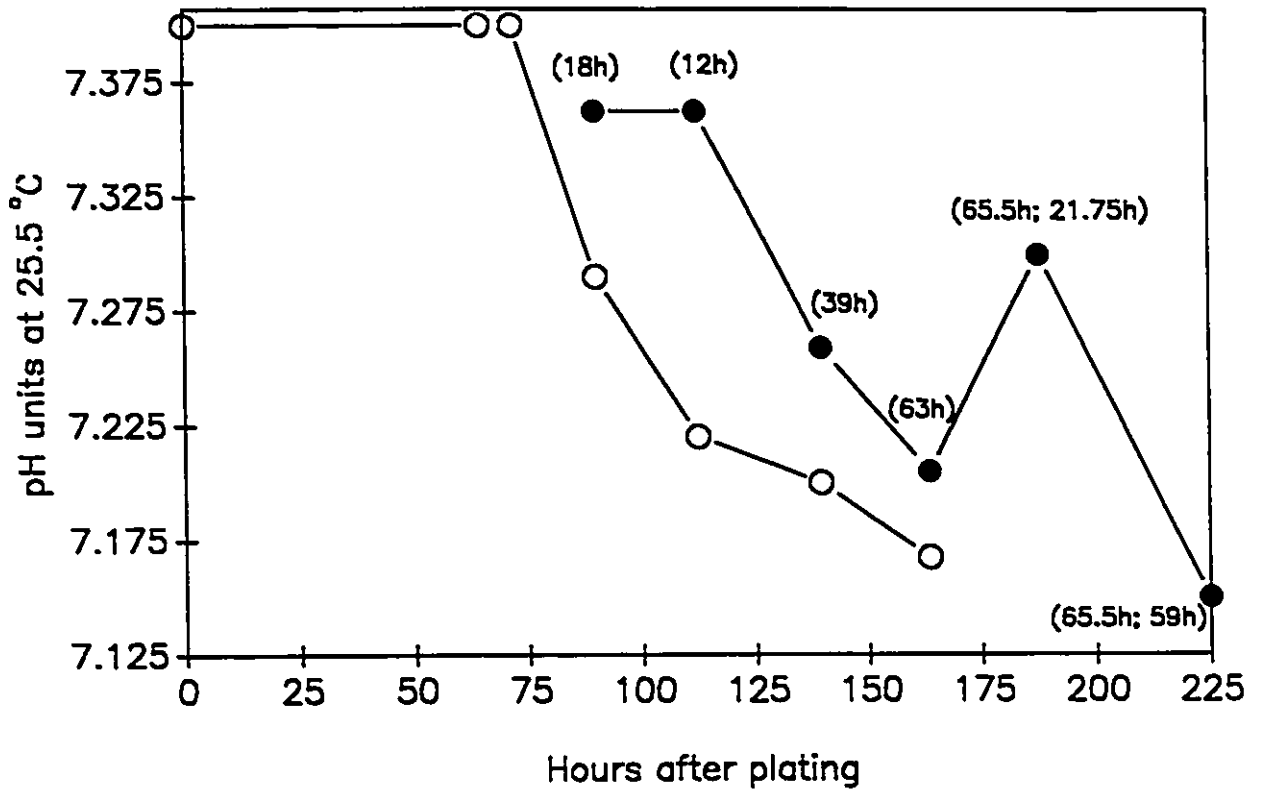


Figure 9. Clonogenic survival of plateau phase AG1522 cells exposed to X-rays (1.66 Gy/min).

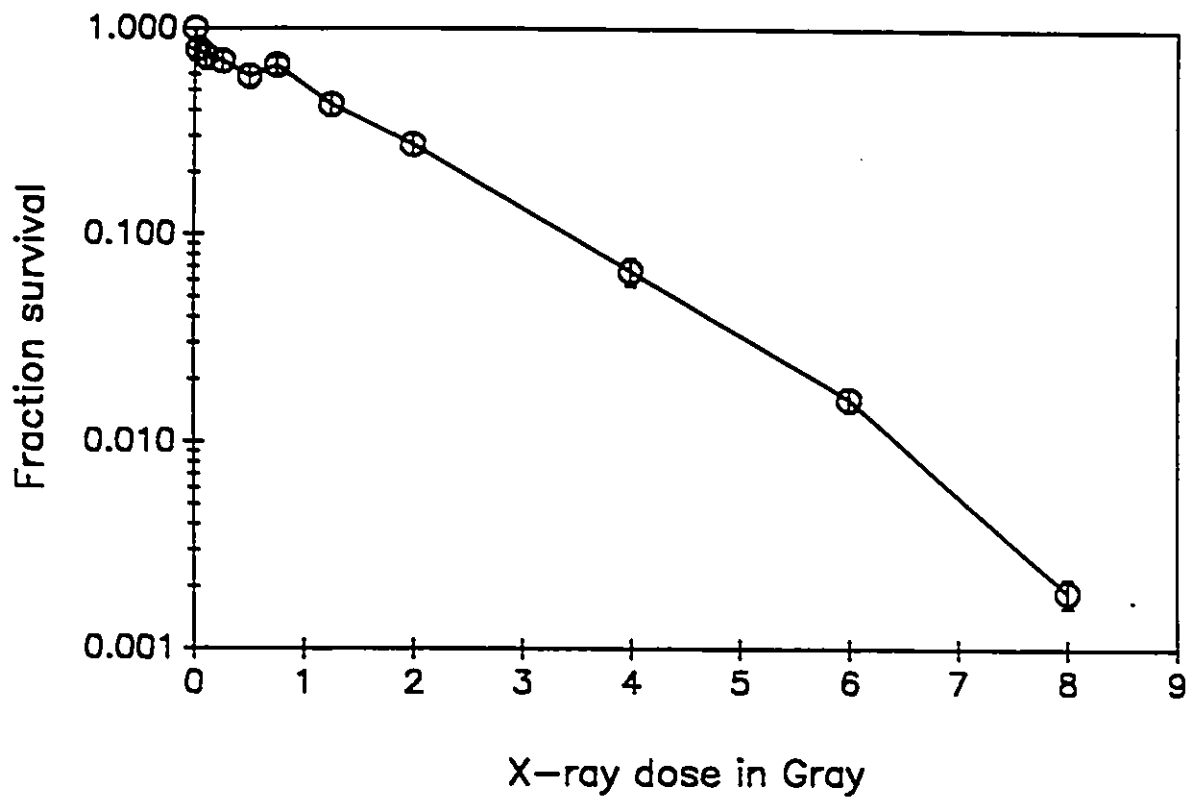


Figure 10. Expanded representation of the low-dose region of the survival curve described in Figure 9.

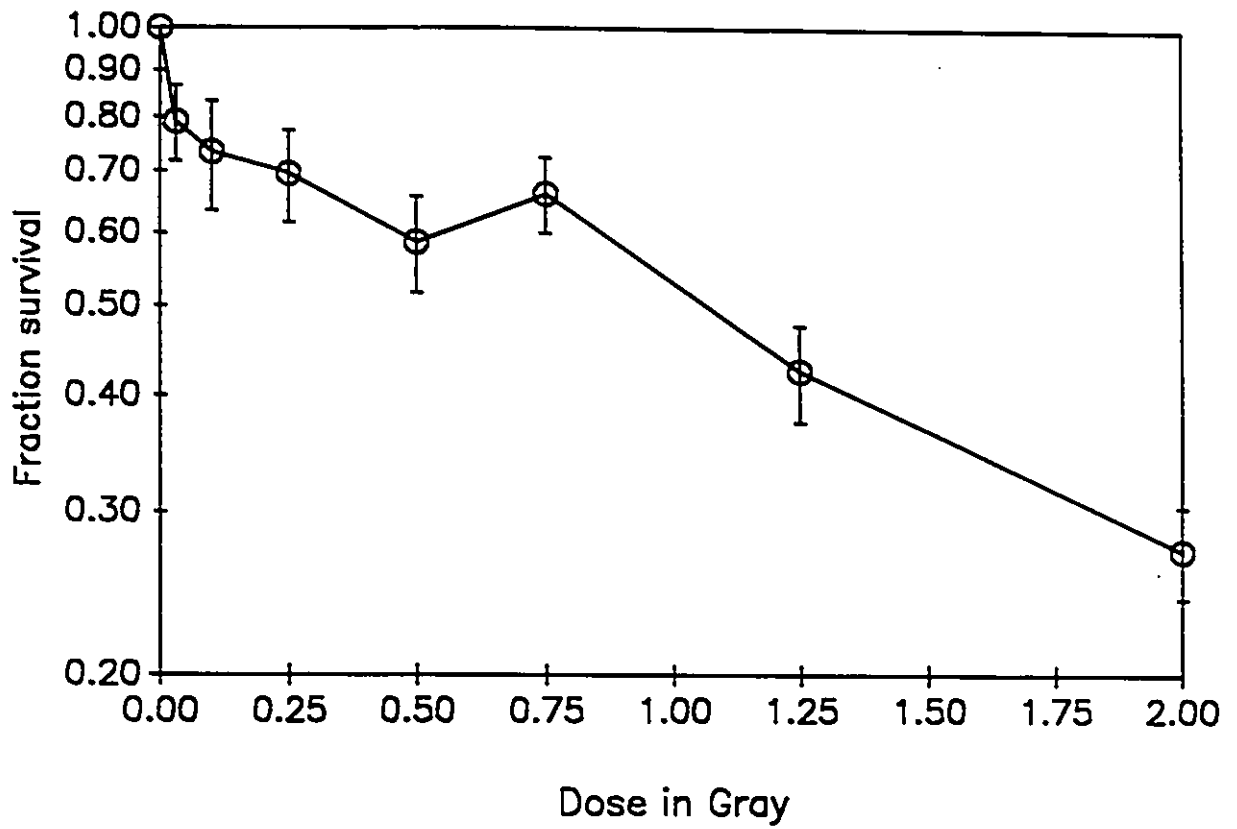


Figure 11. Survival of AG1522 cells plated 16 hours prior to X-irradiation (3 Gy/min).

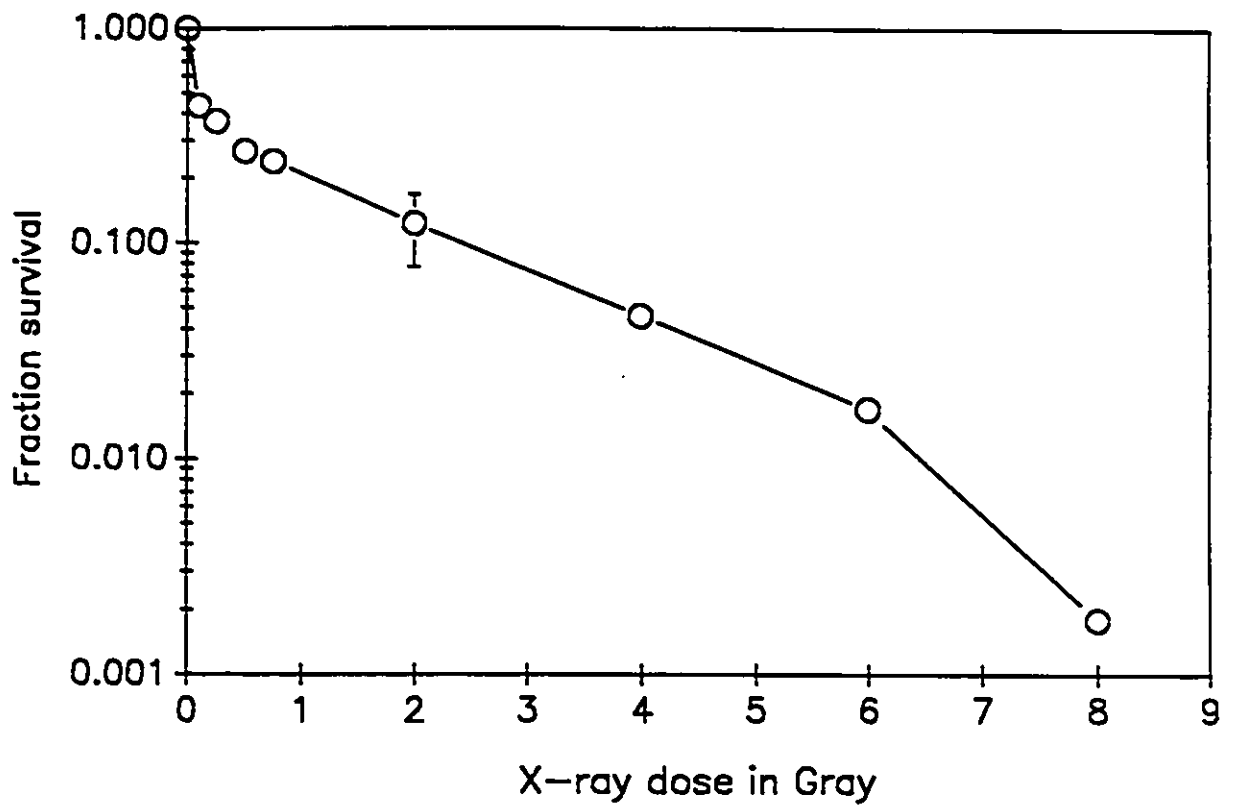


Figure 12. Clonogenic survival of plateau phase AG1522 cells exposed to X-rays (4.75 Gy at 1.83 Gy/min) and incubated at 37°C for various times prior to trypsinization and plating.

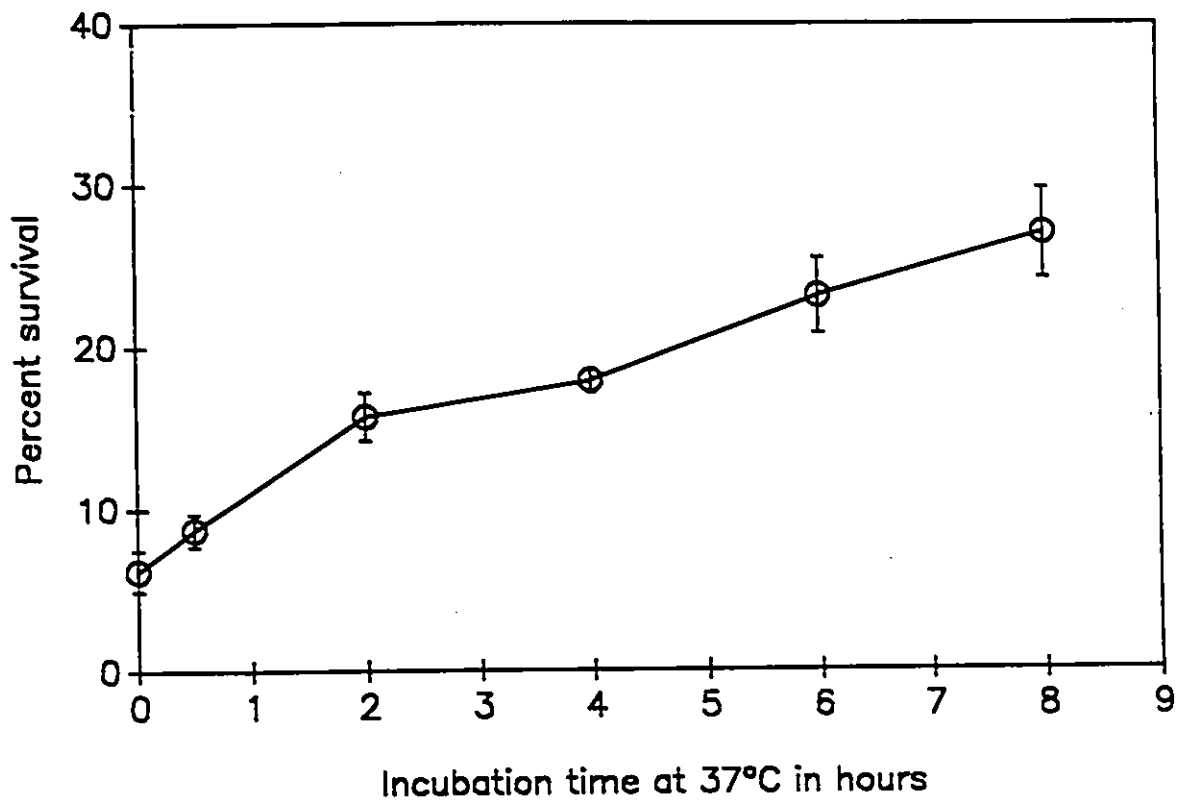


Figure 12A. Clonogenic survival of plateau phase AG1522 cells exposed to X-rays (4.75 Gy at 1.72 Gy/min) and incubated at 37°C for various periods prior to trypsinization and plating.

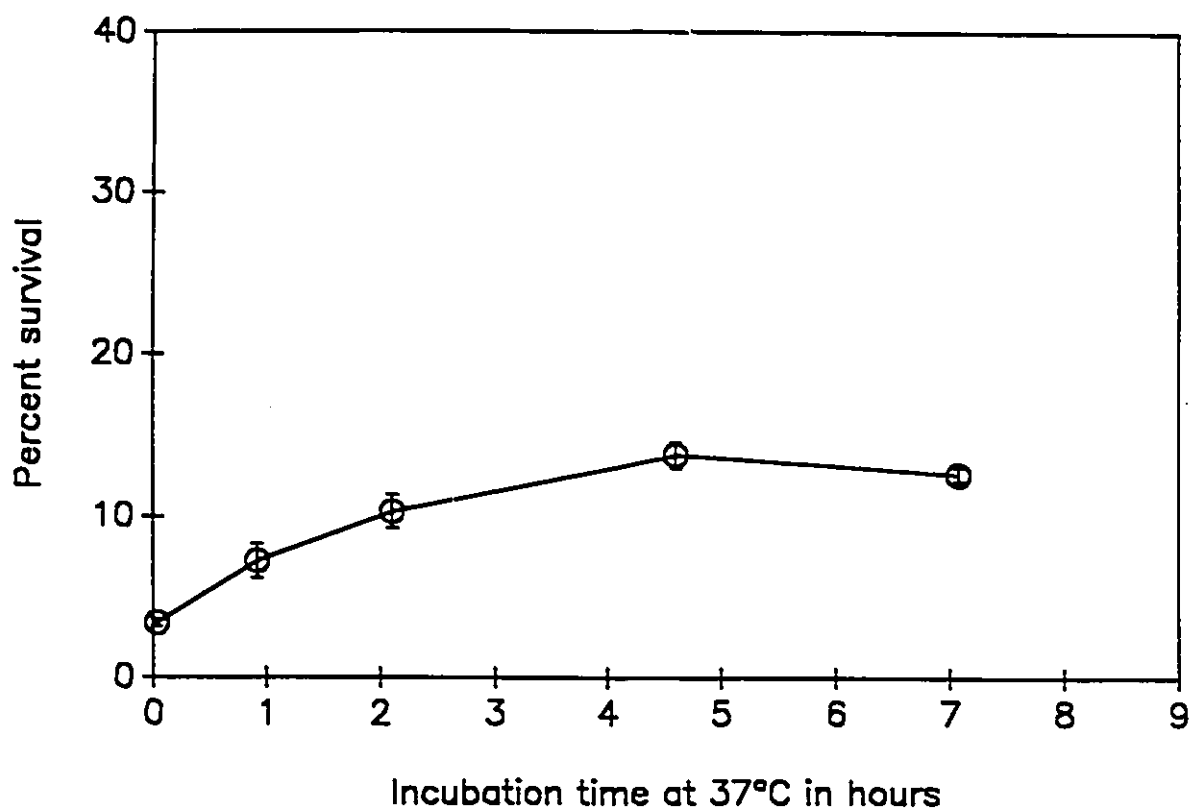


Figure 12B. Frequency of micronucleus formation in plateau phase AG1522 cells exposed to 4 Gy (2 Gy/min) and subsequently incubated at 37°C for various times.

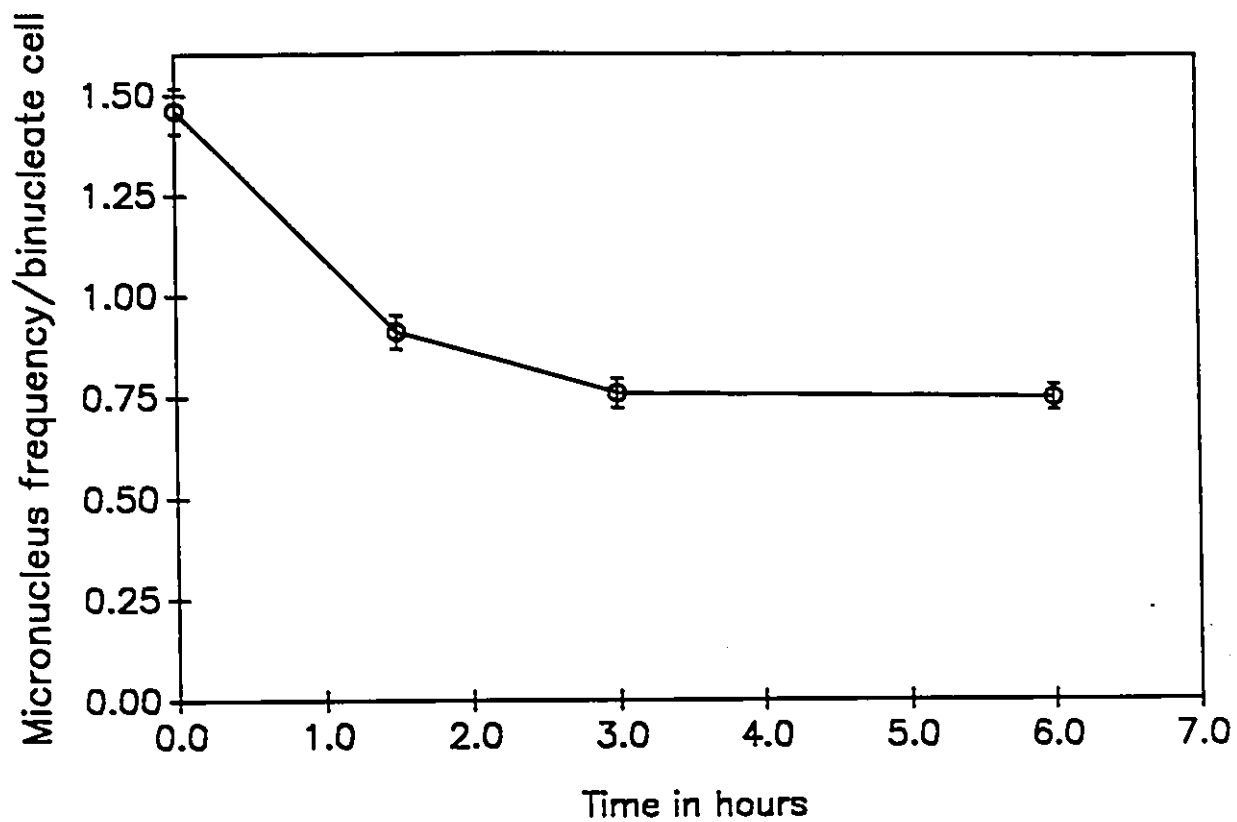


Figure 13. Effect of cycloheximide ($2\mu\text{g/mL}$) on cellular survival during incubation at 37°C following an X-ray dose of 3.23 Gy (2.58 Gy/min). Filled circles: with cycloheximide; open circles: no cycloheximide.

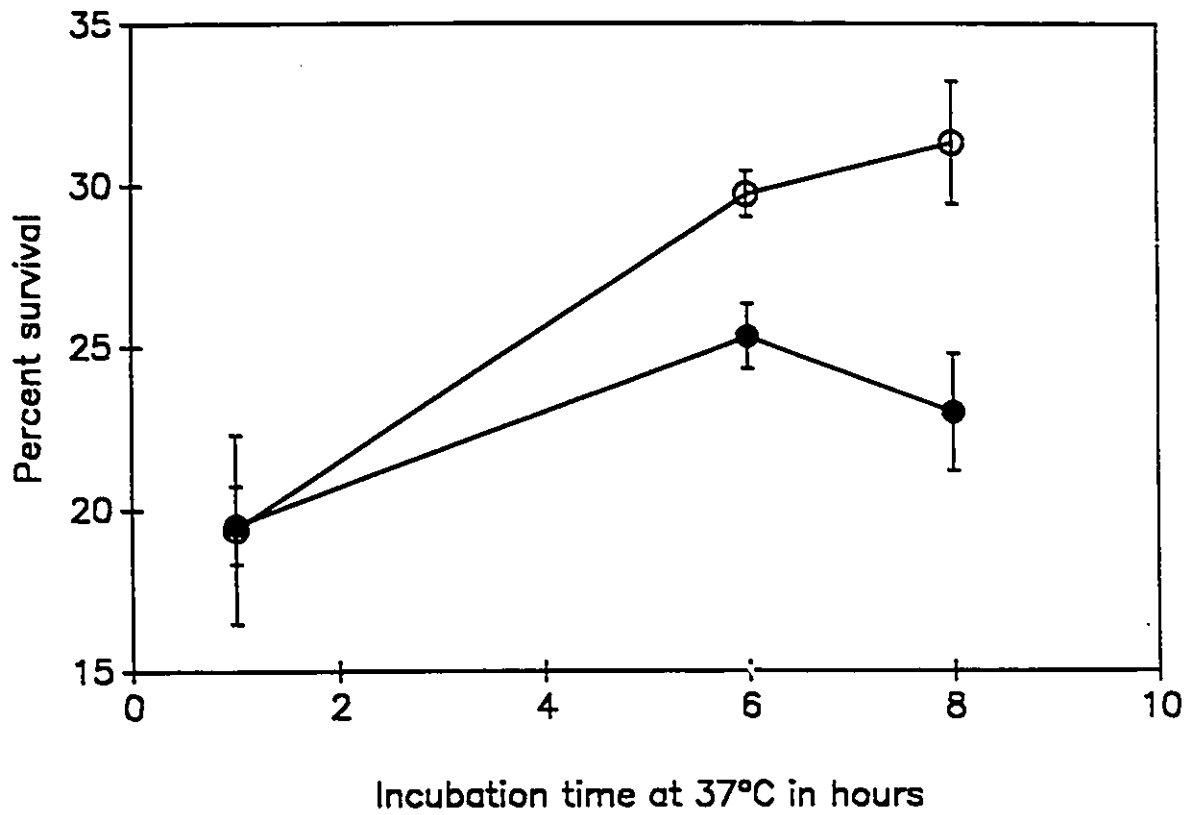


Figure 14. Survival of plateau-phase AG1522 cells following exposure to γ -rays from ^{60}Co (0.0025 Gy/min).

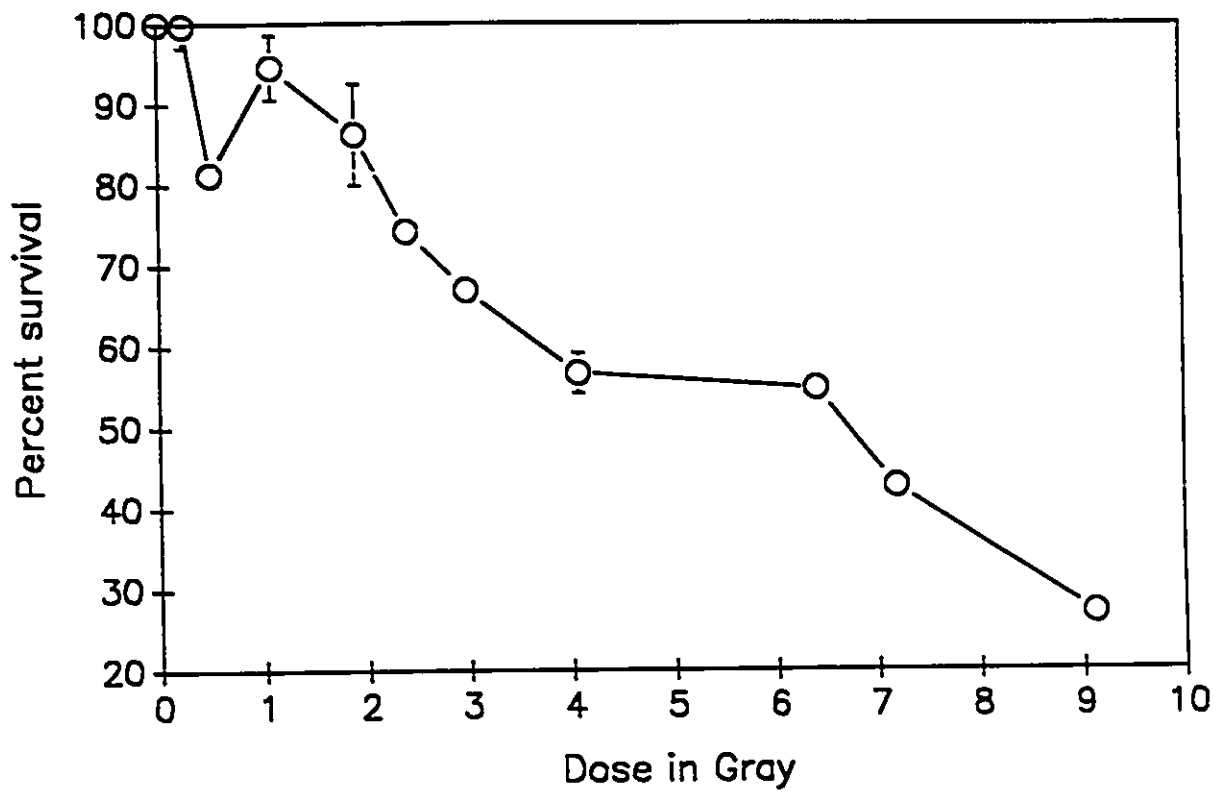


Figure 15. Clonogenic survival of plateau-phase AG1522 cells held at 37°C for various incubation periods following exposure to γ -rays (9 Gy at 0.0024 Gy/min).

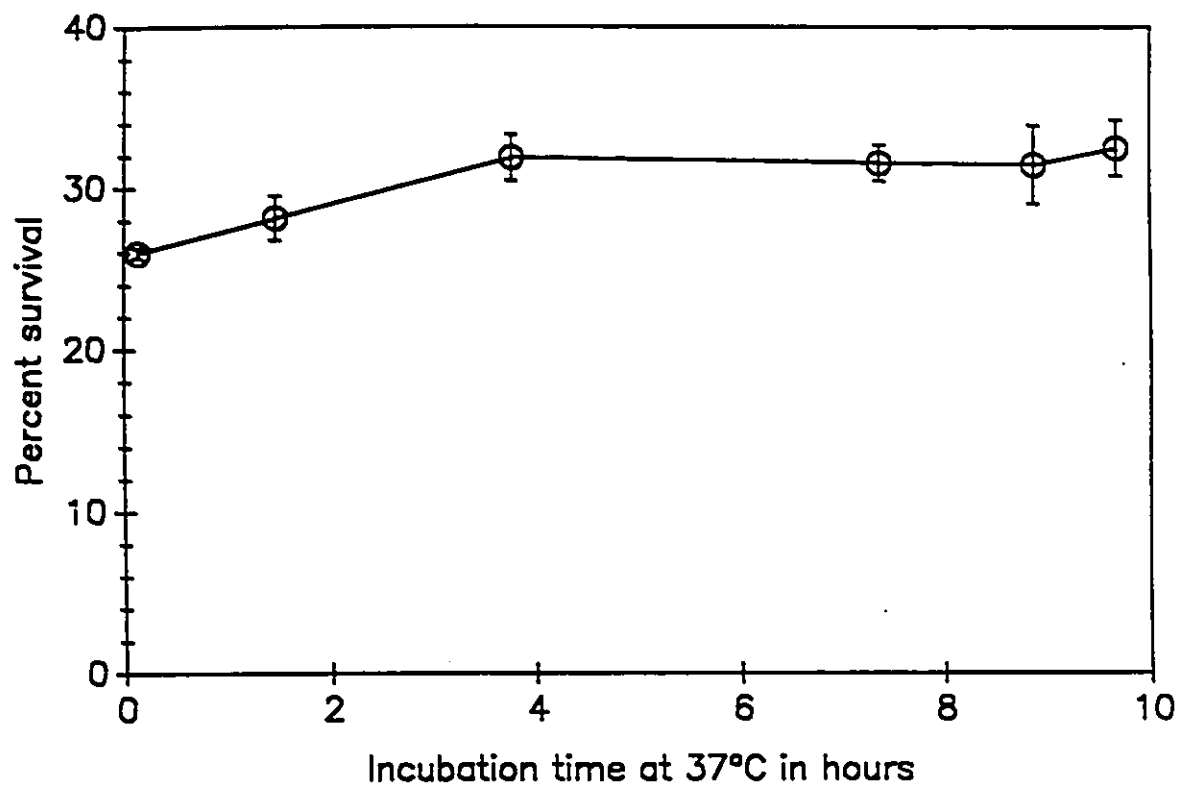
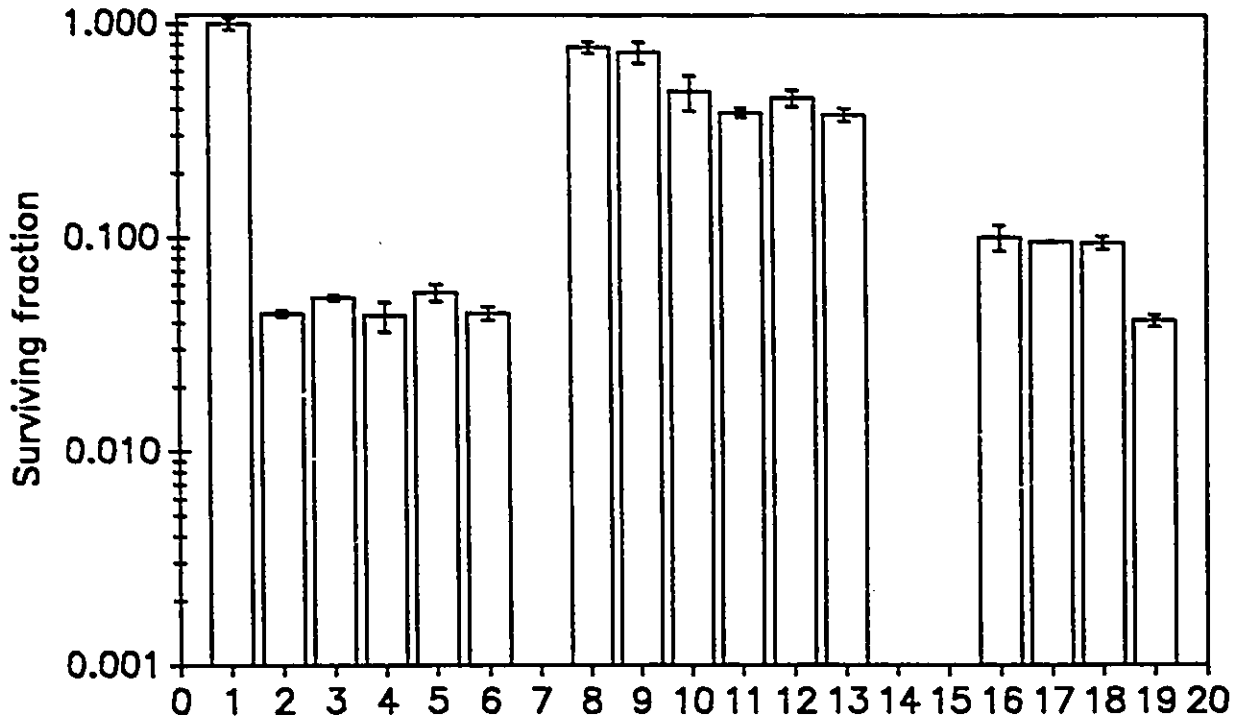


Figure 16. Clonogenic survival of exponentially growing AG1522 cells. The cells were pre-exposed to multiple dose-fractions of X-rays prior to a challenge X-ray dose of 3.25 Gy (2.5 Gy/min). The cells were then incubated at 37°C for various times prior to trypsinization and plating for the survival assay.



x: 0.39 Gy

X: 3.25 Gy

→ : incubation at 37°C

1. Control

2. $X \xrightarrow{1h} X \xrightarrow{1h} X \xrightarrow{1h} X \xrightarrow{5min}$

3. $X \xrightarrow{1h} X \xrightarrow{1h} X \xrightarrow{3h} X \xrightarrow{5min}$

4. $X \xrightarrow{1h} X \xrightarrow{1h} X \xrightarrow{6h} X \xrightarrow{5min}$

5. $X \xrightarrow{1h} X \xrightarrow{1h} X \xrightarrow{1h} X \xrightarrow{3h}$

6. $X \xrightarrow{1h} X \xrightarrow{1h} X \xrightarrow{3h} X \xrightarrow{3h}$

8. $X \xrightarrow{5min}$

9. $X \xrightarrow{1h}$

10. $X \xrightarrow{1h} X \xrightarrow{1h}$

11. $X \xrightarrow{1h} X \xrightarrow{1h} X \xrightarrow{1h}$

12. $X \xrightarrow{1h} X \xrightarrow{1h} X \xrightarrow{3h}$

13. $X \xrightarrow{1h} X \xrightarrow{1h} X \xrightarrow{6h}$

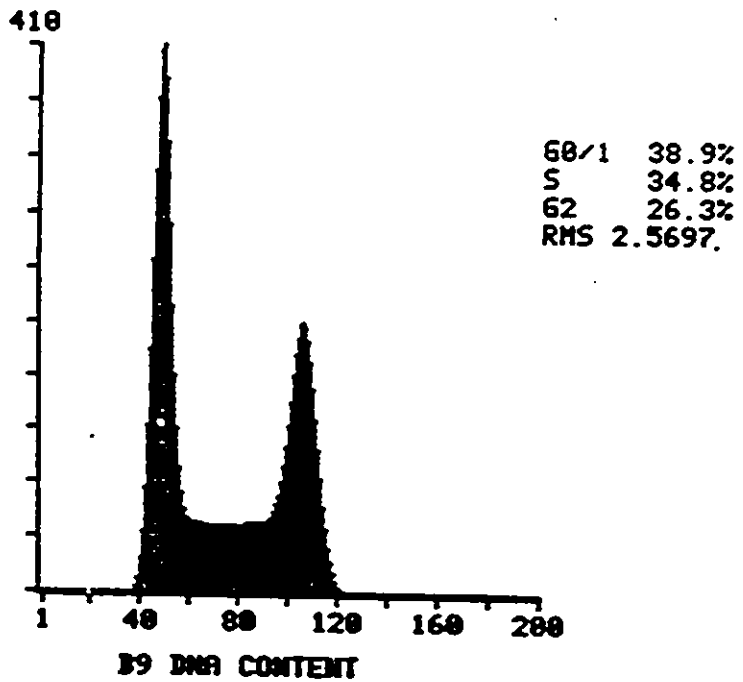
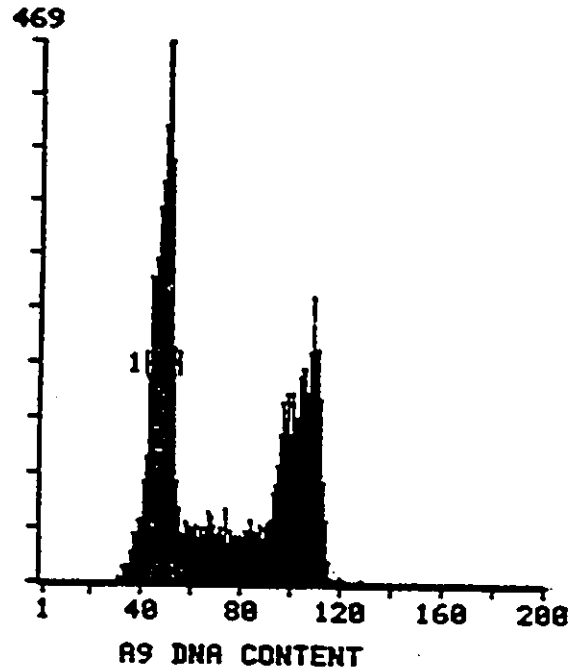
16. $X \xrightarrow{5min}$

17. $X \xrightarrow{1h}$

18. $X \xrightarrow{3h}$

19. $4.46 \text{ Gy} \xrightarrow{5min}$

Figure 17. Flow cytometric profile of exponentially growing AG1522 cells submitted to the experimental treatments described in Figure 16. Top panels represent acquired data; bottom panel represents computer fitted data and the percentage of cells at the various cell cycle stages.



CHAPTER 2EXISTENCE OF AN ADAPTIVE RESPONSE TO IONIZING RADIATION IN AG1522 CELLS

Figure 18 indicates that a pre-exposure of plateau phase cells to γ -rays (4.25 Gy), delivered at 0.003 Gy/min protects the cells against a challenge dose of X-rays (4.25 Gy at 2.5 Gy/min) given immediately after the chronic exposure. Up to a two-fold improvement in cellular survival is observed, as compared to the results expected for individual exposures.

The data (Figure 19) suggest the existence of threshold and optimum doses for induction of an adaptive response which protects against the lethal effects of ionizing radiation in plateau-phase AG1522 cells. A threshold dose greater than 1.5 Gy (0.003 Gy/min) appears necessary to trigger a survival adaptive response in this cell line, and a dose of about 4.25 Gy appears to be optimum for inducing such a response when the challenge dose is delivered immediately after the adapting dose.

Furthermore, we have consistently observed that at seven days after plating cells for survival, the colony size (number of

cells/colony; from cells surviving the sequential low dose-rate and acute exposures, described in figure 18, is about 4 times larger than the size of colonies from cells given an acute exposure only (Figure 20). These data indicate that colony size can be significantly altered, even when effects on clonogenic survival are modest (Figure 18).

Figure 21 shows that the flow cytometric analysis done following the various radiation exposures described in figure 18 did not show any alteration in the distribution of the cells in the cell cycle during the treatments, with over 90% of the cells being in G_0/G_1 .

To investigate whether the AR to IR can be measured by a biological end-point other than cellular survival, the formation of micronuclei following the various treatments described in figure 18 was evaluated in cytochalasin-B-treated cells. This analysis allows us to investigate whether the increased levels of survival (Figure 18) over what was expected were due to a reduction in the residual level of DNA damage from the combined low dose-rate pre-exposure and acute challenge exposure. Figure 22 shows a significant reduction (2-fold) in the frequency of micronucleus formation for the cells receiving a combined treatment of a 4.25 Gy

low dose-rate pre-exposure (0.003 Gy/min), followed immediately by 4.25 Gy of acute X-rays (2.5 Gy/min), when compared to the results expected for individual exposures (the sum of the micronucleus frequency from the individual exposures).

Figure 23 is a photograph of AG1522 cells viewed under a fluorescence microscope (400X magnification) following fixation and staining of the cells with acridine orange. Only binucleate cells were counted to assay for micronucleus formation following the various treatments.

Figure 24 shows that not only did we observe a reduction in the frequency of micronucleus formation in cells adapted with a large low dose-rate pre-exposure and challenged with a 4.25 Gy acute exposure (Figure 21), but a shift in the distribution of micronuclei was also observed. The data shown in this figure indicate a reduction ($p < 0.05$) in the percentage of micronucleated cells with 2 micronuclei in cells receiving the combined low dose-rate and acute exposures (23 ± 1.8), as compared to cells receiving the acute exposure only (31.7 ± 2.2). This reduction in micronucleated cells with 2 micronuclei was accompanied by an increase in the percentage of cells with 1 micronucleus (55.7 ± 3.1 and 47.3 ± 2.8 for cells receiving the combined or acute treatment

respectively).

The following figures show that the adaptive response for micronucleus formation can be observed for lower adapting doses and at different dose rates. Figure 25 shows a decrease in micronucleus frequency for plateau phase AG1522 cells pre-exposed to 3.7 Gy (0.0025 Gy/min) prior to a 4.25 Gy challenge (1.8 Gy/min). A 21% reduction ($p < 0.01$) in the observed frequency of micronucleus formation was noted for cells receiving the combined low- and high-dose-rate treatment as compared to the cells receiving the challenge dose only. When the expected micronucleus frequency was considered, the observed result for the combined treatment shows a 35% reduction in the frequency of micronucleus formation.

Figure 26 shows a decrease in micronucleus frequency for plateau phase AG1522 cells exposed to a 0.5 Gy adapting dose (0.0025 Gy/min) prior to being challenged with a 4 Gy acute dose (1.8 Gy/min), as compared with the micronucleus frequency observed after the 4 Gy challenge exposure only. Greater reductions were observed when a 5 hour incubation period separated the 2 treatments.

Figure 27 shows reductions in micronucleus formation in AG1522 plateau phase cells pre-exposed to 0.5 Gy (0.0005 Gy/min) and challenged immediately after the low dose-rate exposure with either a 3 or 4 Gy acute exposures (2 Gy/min). These results show that the AR to IR exists for adapting doses delivered at yet lower dose-rates.

Figure 28 shows the micronucleus frequency of AG1522 plateau phase cells pre-exposed to a 0.5 Gy dose delivered at high dose-rate (2 Gy/min), and challenged with a 4.25 Gy dose (2 Gy/min) either immediately after the pre-exposure or after various incubation periods at 37°C. When the challenge dose immediately followed the pre-exposure, the resulting micronucleus frequency was an additive effect of the two exposures. However, as the incubation period separating the two exposures increased, the micronucleus frequency decreased, indicating that time is needed for expression of the radioprotective effect due to the adapting dose. The observed micronucleus frequencies when 6.5, 12 or 15 hours separated the two exposures were 6.3, 16.3 or 25.5% lower than the expected micronucleus frequencies. Figure 29 shows that among the micronucleated binucleate cells, the percentage of cells with >2 micronuclei decreased as the incubation period separating the treatments increased as described in figure 28.

Distribution of the cells in the cell cycle was analyzed in all experiments. There was no redistribution of the cells in the cycle during the various treatments, with over 90% of the cells remaining in G₀ and 1% in S phase. Also, the number of cells per 80 cm² flask did not vary as a result of the various treatments, indicating a lack of cell division activity as evidenced by the flow cytometric data. This result also demonstrates the absence of any loss of cells which may have selected for a specific radioresistant population of pre-exposed cells receiving the challenge X-ray exposure.

Figure 18. Survival of plateau-phase AG1522 cells chronically (0.003 Gy/min) or acutely (2.5 Gy/min) exposed to 4.25 Gy, or both with the acute dose delivered immediately after the chronic exposure.

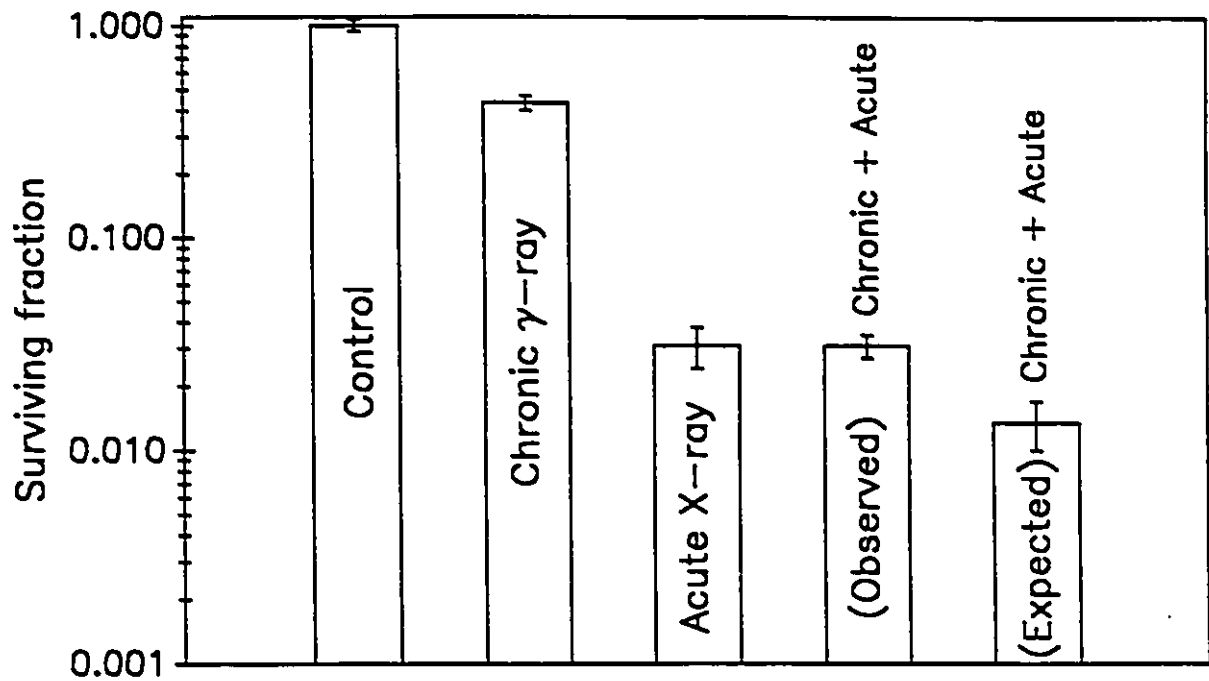


Figure 19. Enhancement of clonogenic survival. Survival was measured after an acute (2.5 Gy/min) 4.25 Gy dose in cells pre-exposed to various doses of low dose-rate (0.003 Gy/min) γ radiation.

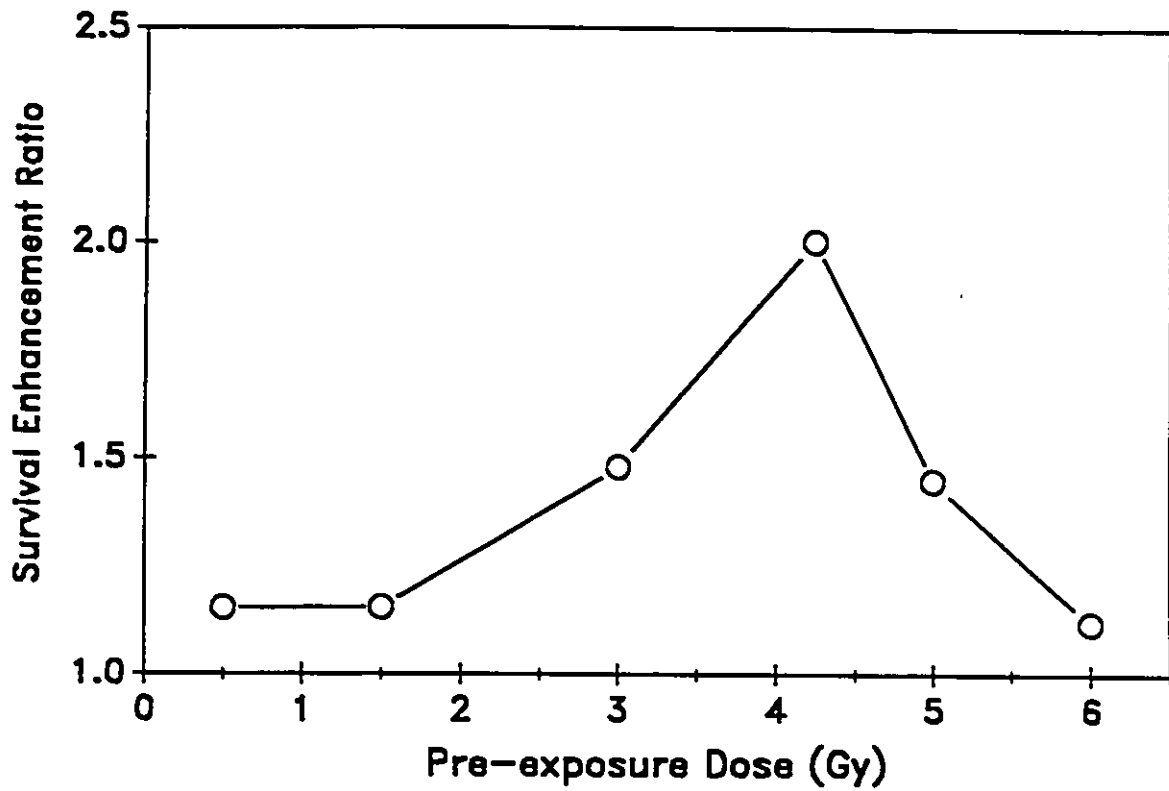


Figure 20. Average number of cells per colony. Cells were counted 7 days after plating following a 4.25 Gy chronic dose (0.003 Gy/min), a 4.25 Gy acute dose (2.5 Gy/min) or the chronic dose followed immediately by the acute dose. Control cells were unexposed to radiation.

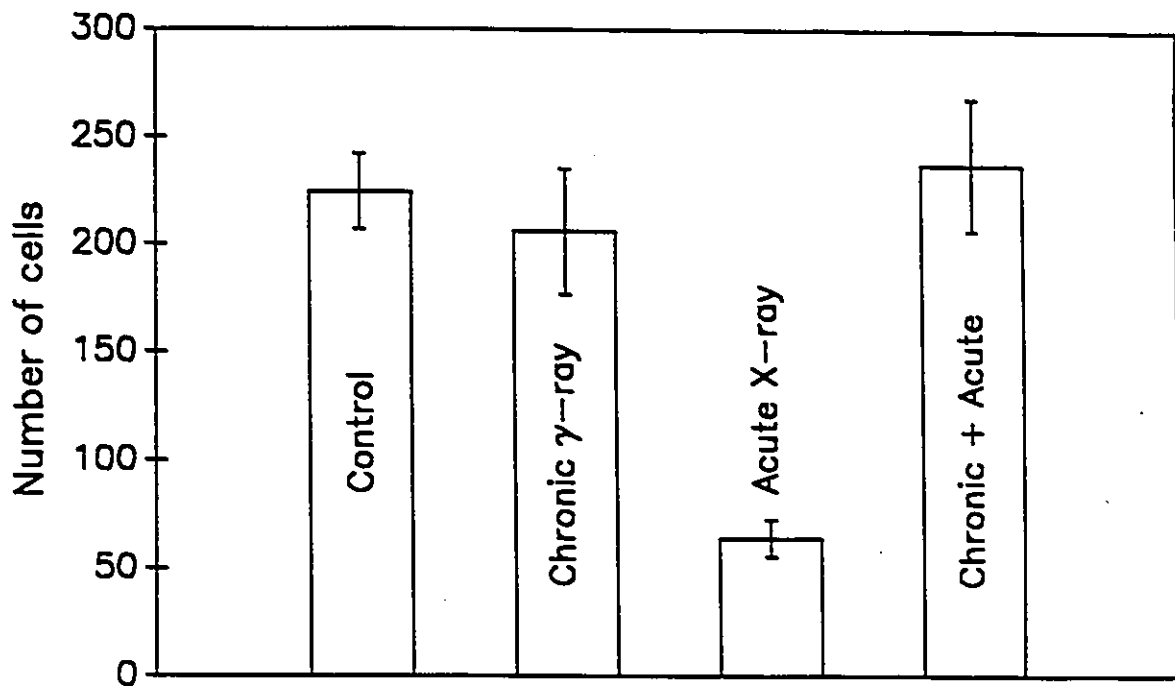


Figure 21. Flow cytometric analysis of control cells and cells exposed to 4.25 Gy (0.003 Gy/min). Top panels represent acquired data; bottom panels represent computer fitted data and the percentage of cells at the various cell cycle stages.

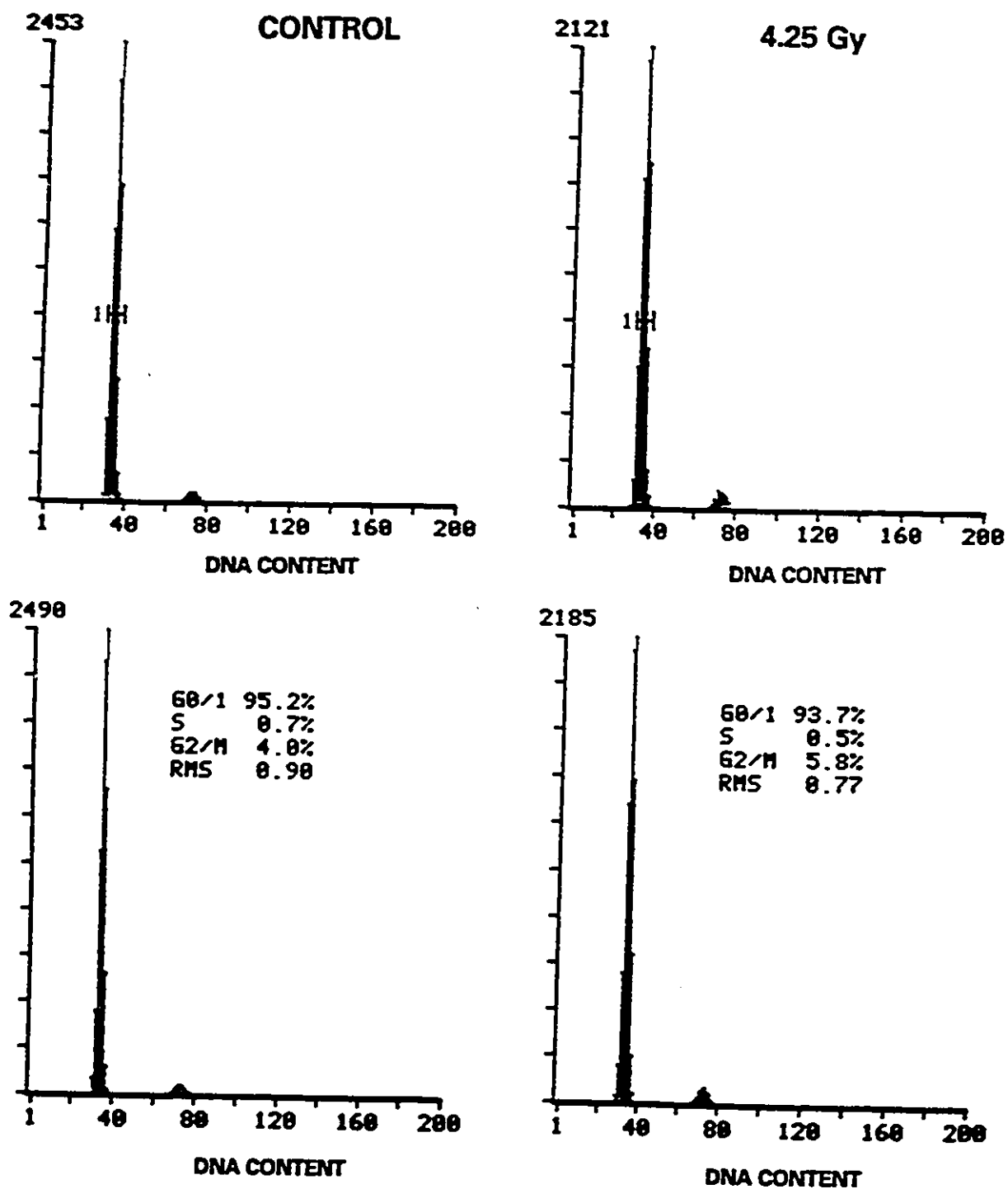


Figure 22. Reduction in the frequency of micronucleus formation in adapted cells. Plateau phase AG1522 cells were exposed to a chronic 4.25 Gy dose (0.003 Gy/min) and/or an acute 4.25 Gy dose (2.5 Gy/min) of radiation. When the two exposures were combined, the acute exposure began immediately after the chronic exposure.

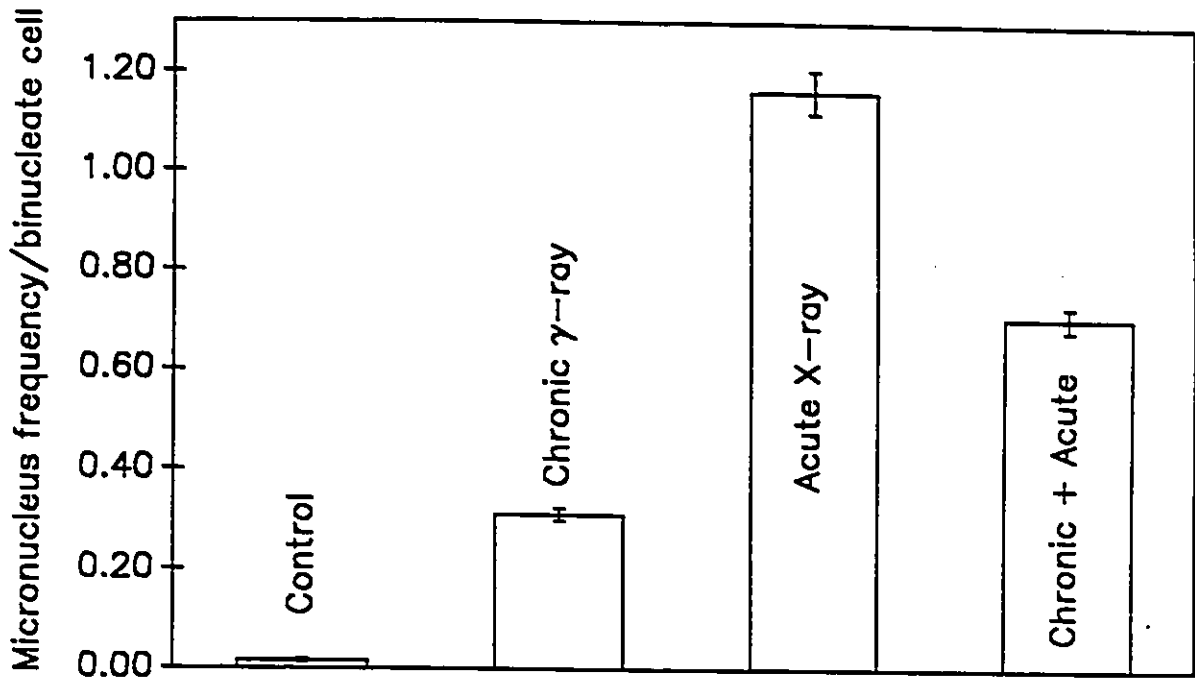


Figure 23. Photograph of AG1522 cells after cytochalasin B treatment, fixation and staining as described in Materials and Methods (400X magnification). Mononucleate cells and a micronucleated binucleate cell are observed. N: nucleus; MN: micronucleus.

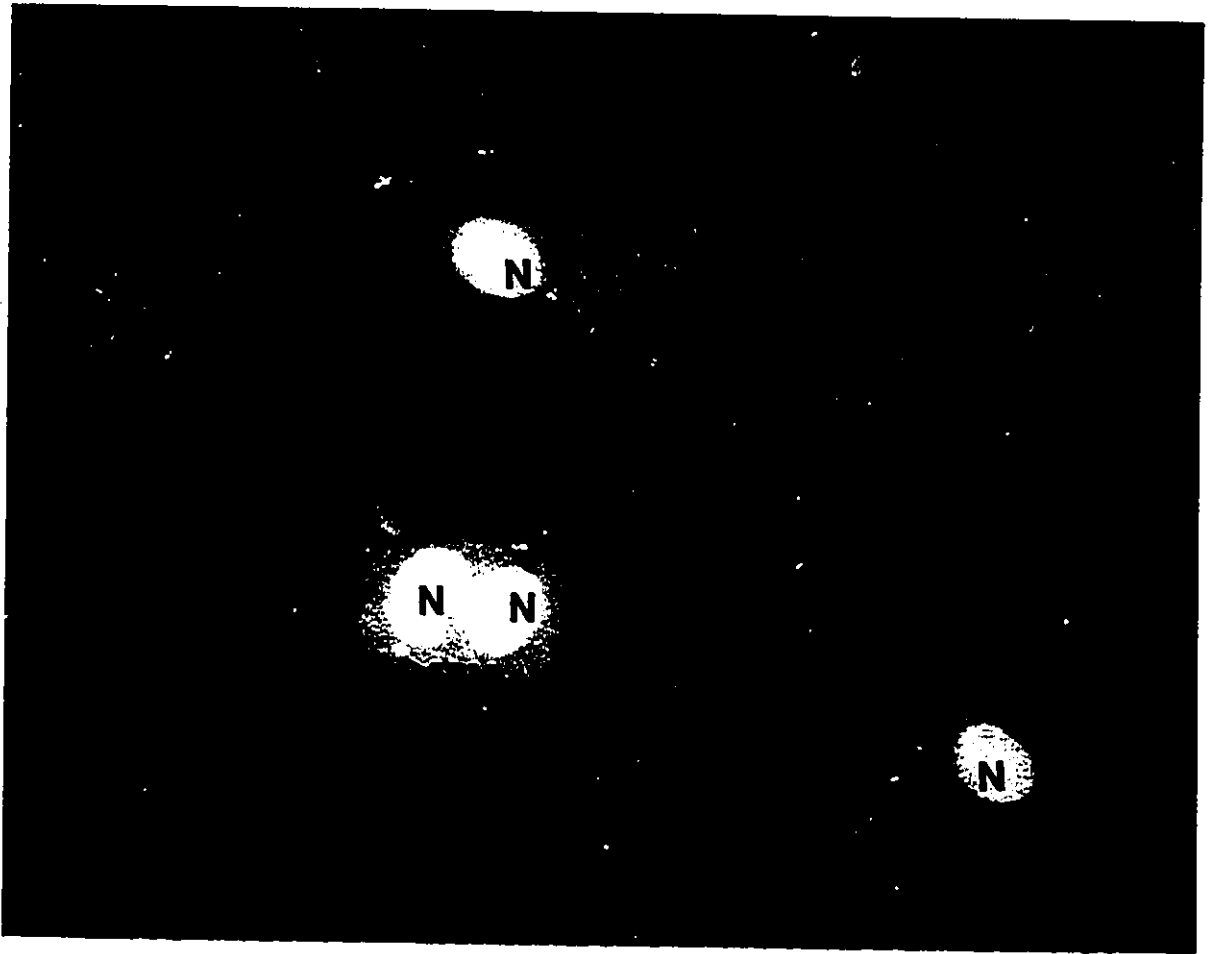


Figure 24. Shift in distribution of micronuclei in radiation adapted human cells. The cells were exposed to an acute 4.25 Gy dose (2.5 Gy/min) or a chronic 4.25 Gy dose (0.003 Gy/min) followed immediately by a 4.25 Gy acute dose.

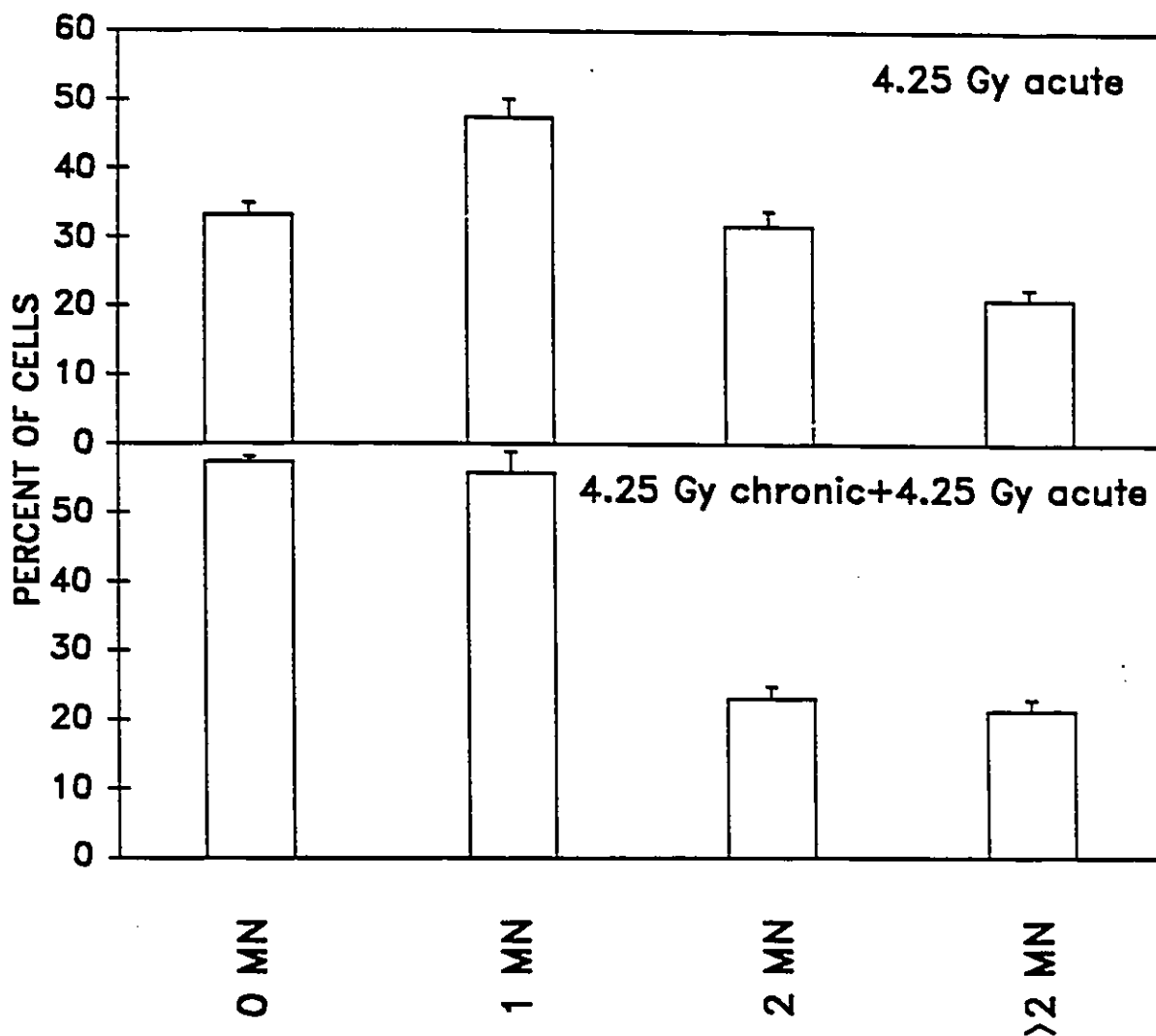


Figure 25. Frequency of micronucleus formation in plateau-phase AG1522 cells exposed to γ -rays (3.7 Gy at 0.0025 Gy/min) and/or X-rays (4.25 Gy at 1.8 Gy/min).

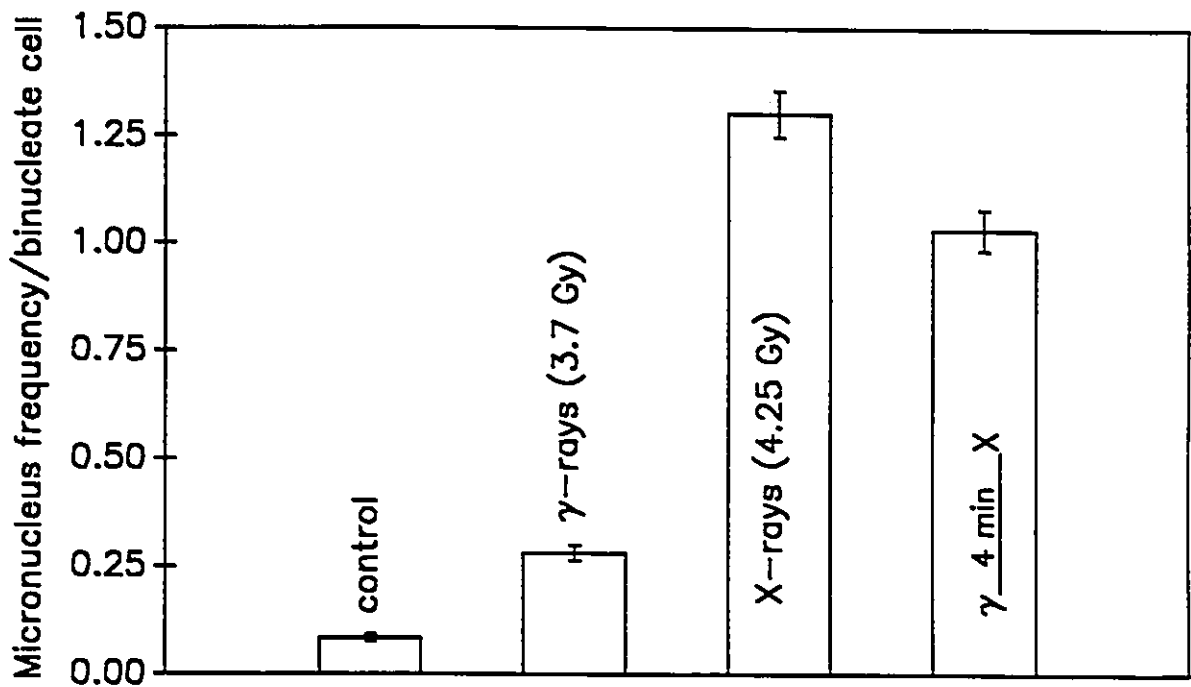


Figure 26. Frequency of micronucleus formation in plateau-phase AG1522 cells exposed to γ -rays (0.5 Gy at 0.0025 Gy/min) and/or X-rays (4 Gy at 1.8 Gy/min).

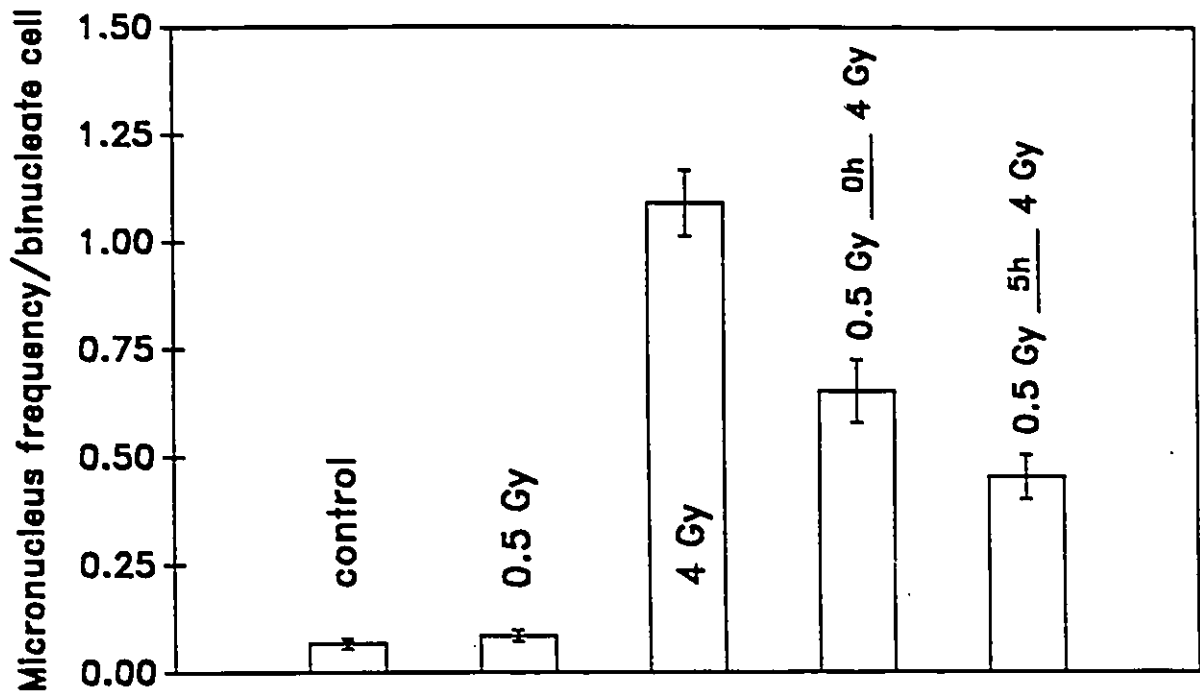


Figure 27. Frequency of micronucleus formation in plateau-phase AG1522 cells exposed to γ -rays (0.5 Gy at 0.0005 Gy/min) immediately prior to 3 or 4 Gy doses of X-rays (2 Gy/min).

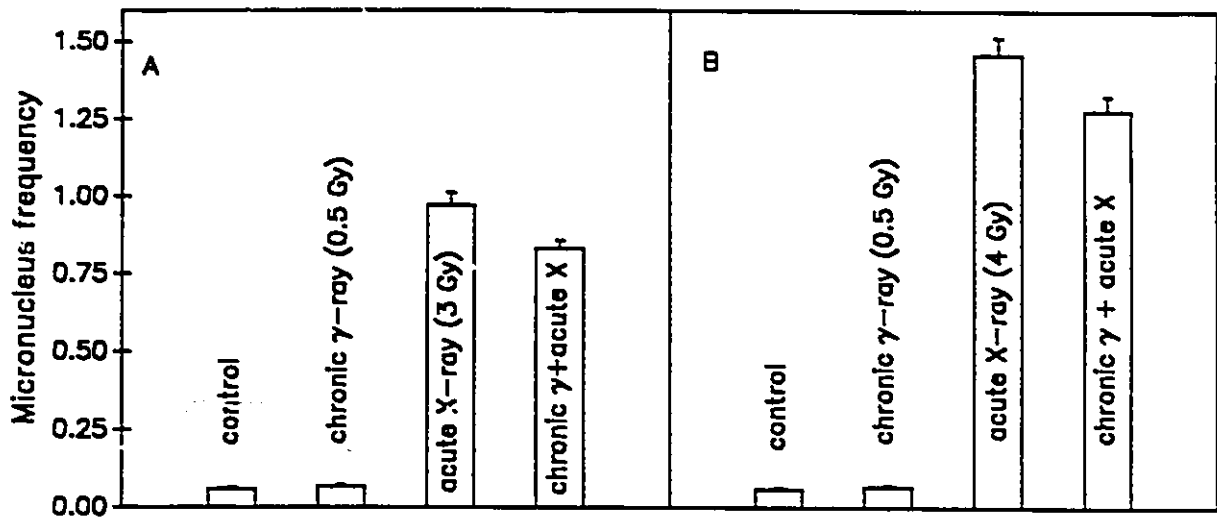


Figure 28. Change in frequency of micronucleus formation in plateau-phase AG1522 cells pre-exposed to an acute 0.56 Gy X-ray dose (2 Gy/min) at various times prior to a second acute 4.25 Gy X-ray dose (2 Gy/min).

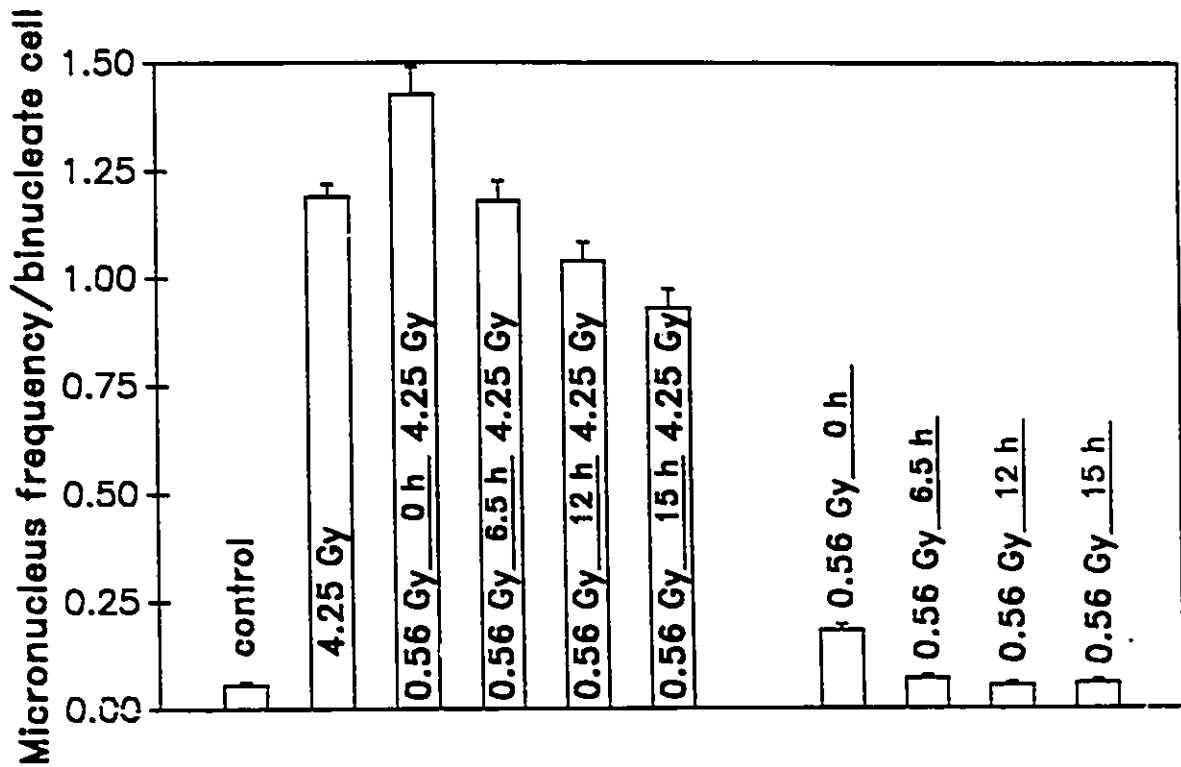
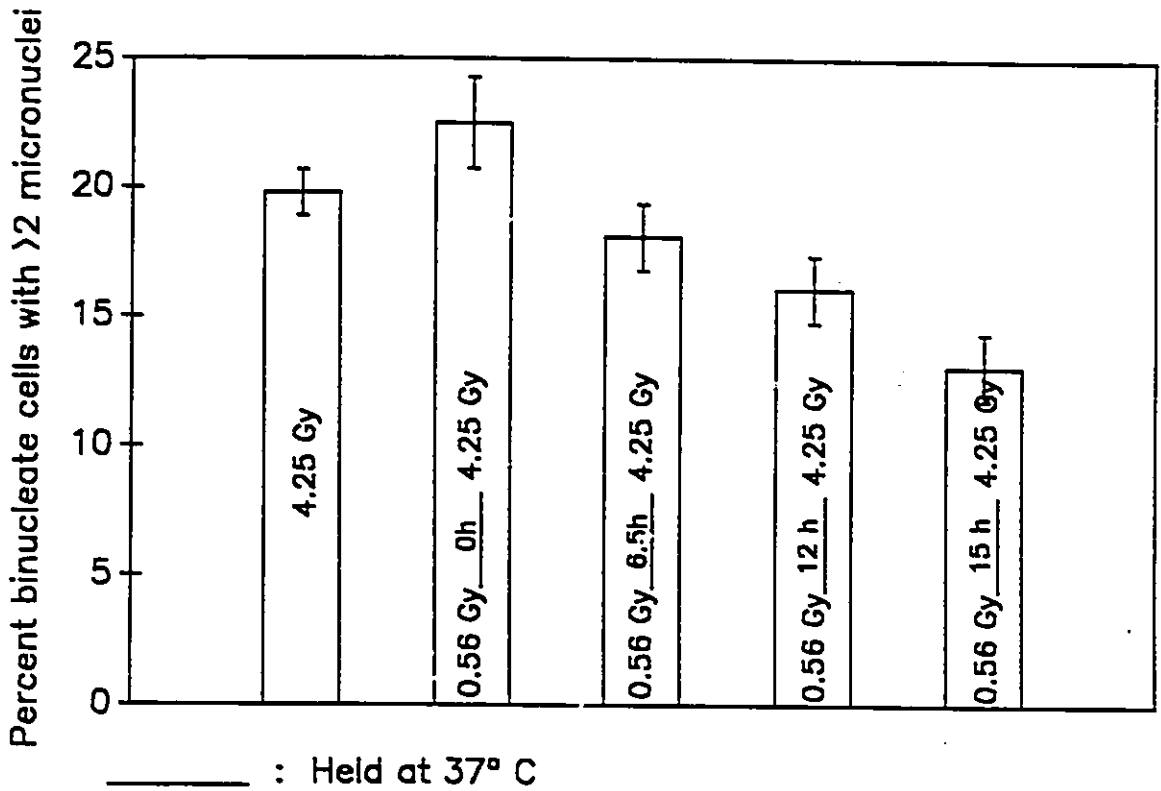


Figure 29. Shift in the percentage of binucleate cells with more than 2 micronuclei. Plateau-phase AG1522 cells were pre-exposed to an acute 0.56 Gy dose (2 Gy/min) at various times prior to a second acute 4.25 Gy dose (2 Gy/min) challenge exposure, as in figure 28.



CHAPTER 3MECHANISMS LEADING TO THE EXPRESSION OF AN ADAPTIVE RESPONSE TO IONIZING RADIATION IN PLATEAU PHASE AG1522 CELLS

The data in table 3 show an enhancement of the rate of repair of DNA damage due to an acute challenge dose of radiation, when the cells have been pre-exposed to a low dose-rate adapting dose. The data in the table indicate that without pre-exposure, holding of plateau phase AG1522 cells at 37°C for two hours following a 3 Gy (2 Gy/min) challenge exposure, resulted in a 29.9% reduction in the frequency of micronucleus formation due to the challenge dose (treatments C and E compared). This reduction was further enhanced when the cells were pre-exposed to a 0.5 Gy dose delivered at 0.0005 Gy/min (Treatments D and F and E and F compared), which triggered an adaptive response to the 3 Gy challenge dose of X-rays (treatments C and D compared). The greater reductions in micronucleus frequency during the 2 hour holding period constitute an increase in the rate of repair of DNA during the holding period.

The data in table 4 show the development of repair of potentially lethal damage for cells exposed to a 4 Gy dose and subsequently held in plateau phase for 2 or 6 hours. The recovery

ratios were 2.2 and 3.3 for 2- and 6-hour holding periods respectively. While a 0.5 Gy adapting dose did not detectably alter the lethal effects of a 4 Gy challenge dose, it significantly enhanced the recovery of the cells during the 2 hour holding period after the 4 Gy challenge exposure, whether the adapting dose was terminated immediately or 2 hours prior to the challenge exposure. These increases in survival levels correlated with respective reductions in the frequency of micronucleus formation.

The data in table 5 also show an adaptive response to ionizing radiation, as measured by the frequency of micronucleus formation, when cells pre-exposed to 0.5 Gy (0.0005 Gy/min) were challenged with a 4 Gy dose (2 Gy/min). Without pre-exposure, holding of the cells at 37°C following the challenge exposure resulted in a reduction of the frequency of micronucleus formation. These reductions appear to level off at about 3 hours of holding in plateau phase. Pre-exposure to a low dose-rate adapting dose of 0.5 Gy enhanced slightly (statistically non-significant) the rate of repair of DNA damage during the initial 1.5 hour holding period at 37°C. No further reductions were observed for longer holding periods, indicating that normally unreparable lesions remained unrepaired in adapted cells.

The data presented in table 6 show that plateau phase cells

exposed to a dose of 4.75 Gy (1.9 Gy/min) and held at 37°C for 0.5 or 2 h prior to trypsinization, exhibit recovery ratios of 1.4 and 2.53 respectively when assayed for cellular survival. A pre-exposure to 0.5 Gy (0.0025 Gy/min) 2 or 7 h prior to the challenge dose which was followed by a 0.5 h holding period resulted in recovery ratios of 1.56 and 2.04 respectively. Pre-exposure to the adapting dose, 7 hours prior to the challenge dose, resulted in a 46% increase over the expected survival level ($p < 0.001$) when the cells were held in plateau phase for 30 min following the challenge dose. However, these pre-exposure conditions did not produce any enhancement of the recovery ratio when the cells were held for 2 hours following the challenge exposure. As above, these results indicate an enhancement of the rate of repair of normally repairable lesions leading to increases in cellular survival. The data in figure 12A indicate that the repair of potentially lethal radiation damage following a 4.75 Gy can be complete within a 2 h holding period at 37°C following the exposure. Therefore, these data also suggest that normally unreparable lesions remained unrepaired in adapted cells.

It is well established that cells in culture respond to DNA damage with transient delays in both G_1 and G_2 phases of the cell cycle. Therefore, we investigated whether pre-exposure of plateau phase human fibroblasts to an adapting dose of radiation further

alters the progression of the cells in the cell cycle following trypsinization and plating after the second exposure. The data presented in figure 30 represent the percentage of binucleate cells in the total population of cells treated with cytochalasin B. The data indicate that 48 hours after plating, 48% and 43% of control cells and cells exposed to 0.5 Gy (0.0005 Gy/min) respectively were in the binucleate state. Following exposure to a 3 or a 4 Gy dose, about 38.5% of the cells were in the binucleate state. This latter percentage dropped to 30% when the cells were pre-exposed to the 0.5 Gy chronic exposure which resulted in an adaptive response to the challenge exposures of 3 and 4 Gy as measured by micronucleus formation.

The results shown in figure 31 indicate that holding of cells in plateau phase following a large challenge dose of radiation correlated with a decrease in the percentage of cells in the binucleate state as expected. However this decrease was enhanced when the cells were pre-exposed to an adapting dose of radiation which had been shown to enhance the rate of repair of DNA damage (Table 3).

Table 3. Frequency of micronucleus formation in plateau-phase AG1522 cells exposed to 0.5 Gy (0.0005 Gy/min) prior to a 3 Gy challenge dose (2 Gy/min), and subsequent incubation of the cells at 37°C for 2 hours

TREATMENT	MN FREQUENCY \pm S.E	TREATMENTS COMPARED	PERCENT DECREASE IN FREQUENCY	STATISTICAL SIGNIFICANCE
(A) Control	0.052 \pm 0.0068			
(B) 0.5 Gy (0.0005 Gy/min)	0.065 \pm 0.0077			
(C) 3 Gy (2 Gy/min)	0.97 \pm 0.04			
(D) 0.5 Gy $\xrightarrow{0h}$ 3 Gy	0.83 \pm 0.027	(C) and (D)	14.4	p < 0.05
(E) 3 Gy $\xrightarrow{2h}$	0.68 \pm 0.026	(C) and (E)	29.9	p < 0.001
(F) 0.5 Gy $\xrightarrow{0h}$ 3 Gy $\xrightarrow{2h}$	0.49 \pm 0.02	(D) and (F) (E) and (F)	40.9 27.9	p < 0.001 p < 0.001

\rightarrow : Incubation at 37°C

Table 4. Clonogenic survival and frequency of micronucleus formation of plateau phase AG 1522 cells pre-exposed to 0.5 Gy (0.0025 Gy/min) and challenged with 4 Gy (1.8 Gy/min).

Treatment	Observed % Survival Level \pm S.D.	Expected % Survival Level \pm S.D.	% Increase in Survival Over Expected Level	Statistical Significance	Micronucleus Frequency per Binucleate Cell \pm S.D.
(A) Control	100 \pm 2.4				0.067 \pm 0.01
(B) 0.5 Gy	75.7 \pm 7.6				0.085 \pm 0.01
(C) 4 Gy	7.5 \pm 1.1				1.1 \pm 0.08
(D) 0.5 Gy $\xrightarrow{6h}$ 4 Gy	6.7 \pm 0.3	5.7 \pm 1.0	15.2	N.S.	
(E) 4 Gy $\xrightarrow{7h}$	16.7 \pm 1.5				0.9 \pm 0.06
(F) 0.5 Gy $\xrightarrow{6h}$ 4 Gy $\xrightarrow{2h}$	20.8 \pm 2.3	12.6 \pm 1.7	39.2	p < 0.05	
(G) 0.5 Gy $\xrightarrow{2h}$ 4 Gy $\xrightarrow{2h}$	21.6 \pm 1.7	12.6 \pm 1.7	41.5	p < 0.01	0.72 \pm 0.06
(H) 4 Gy $\xrightarrow{6h}$	24.7 \pm 0.7				0.7 \pm 0.05

\rightarrow : Incubation at 37°C

Table 5. Frequency of micronucleus formation in plateau phase AG 1522 cells exposed to 0.5 Gy (0.0005 Gy/min) prior to a challenge dose of 4 Gy (2 Gy/min) and subsequent incubation of the cells at 37°C.

Treatment	MN Frequency ± S.E.	Treatments Compared	% Decrease in MN Frequency	Statistical Significance
(A) Control	0.058 ± 0.0068			
(B) 0.5 Gy	0.066 ± 0.0077			
(C) 4 Gy	1.46 ± 0.057			
(D) 0.5 Gy $\xrightarrow{0h}$ 4 Gy	1.28 ± 0.048	(C) and (D)	12.3	p < 0.1
(E) 4 Gy $\xrightarrow{1.5h}$	0.91 ± 0.041	(C) and (E)	37.7	p < 0.001
(F) 0.5 Gy $\xrightarrow{0h}$ 4 Gy $\xrightarrow{1.5h}$	0.77 ± 0.032	(D) and (F)	39.8	p < 0.01
		(E) and (F)	15.4	p < 0.1
(G) 4 Gy $\xrightarrow{3h}$	0.76 ± 0.036	(C) and (G)	47.9	p < 0.001
(H) 0.5 Gy $\xrightarrow{0h}$ 4 Gy $\xrightarrow{3h}$	0.73 ± 0.035	(D) and (H)	42.9	p < 0.001
		(G) and (H)	3.9	NS
(I) 4 Gy $\xrightarrow{6h}$	0.75 ± 0.031	(C) and (I)	48.6	p < 0.001
(J) 0.5 Gy $\xrightarrow{0h}$ 4 Gy $\xrightarrow{6h}$	0.74 ± 0.035	(D) and (J)	42.2	p < 0.001
		(I) and (J)	1.3	NS

→ : Incubation at 37°C

Table 6. Clonogenic survival of plateau-phase AG1522 cells pre-exposed to 0.5 Gy (0.0025 Gy/min) and challenged with 4.75 Gy (1.8 Gy/min) and subsequently held in plateau phase at 37°C for 0.5 or 2 hours.

Treatment	Observed % Survival ± S.D.	Expected % Survival	% Increase in Survival Over Expected Level	Recovery Ratio	Statistical Significance
(A) Control	100 ± 4				
(B) 0.5 Gy $\xrightarrow{0h}$	105 ± 6				
(C) 0.5 Gy $\xrightarrow{2h}$	108 ± 2			1	
(D) 0.5 Gy $\xrightarrow{7h}$	100 ± 0			1	
(E) 0.5 Gy $\xrightarrow{10h}$	101 ± 5			1	
(F) 4.75 Gy $\xrightarrow{0h}$	6.2 ± 1.3				
(G) 4.75 Gy $\xrightarrow{30min}$	8.7 ± 0.1			1.4	
(H) 0.5 Gy $\xrightarrow{2h}$ → 4.75 Gy $\xrightarrow{30min}$	9.7 ± 0.8	9.4 ± 1.07	3.0	1.56	NS
(I) 0.5 Gy $\xrightarrow{7h}$ → 4.75 Gy $\xrightarrow{30min}$	12.7 ± 0.1	8.7 ± 0.07	46.0	2.04	p < 0.01
(J) 4.75 Gy $\xrightarrow{2h}$	15.7 ± 1.5			2.53	
(K) 0.5 Gy $\xrightarrow{2h}$ → 4.75 Gy $\xrightarrow{2h}$	16.3 ± .03	17 ± 1.6	--	2.63	--
(L) 0.5 Gy $\xrightarrow{7h}$ → 4.75 Gy $\xrightarrow{2h}$	15.1 ± 1.4	15.7 ± 1.5	--	2.43	--

→ : Incubation at 37°C

Figure 30. Induced division delay. The figure shows the percentage of AG1522 cells reaching the binucleate stage 48 h after plating. Plateau phase cells were exposed to 0.5 Gy (0.0005 Gy/min) immediately prior to a 4 or a 3 Gy (1.8 Gy/min) challenge dose.

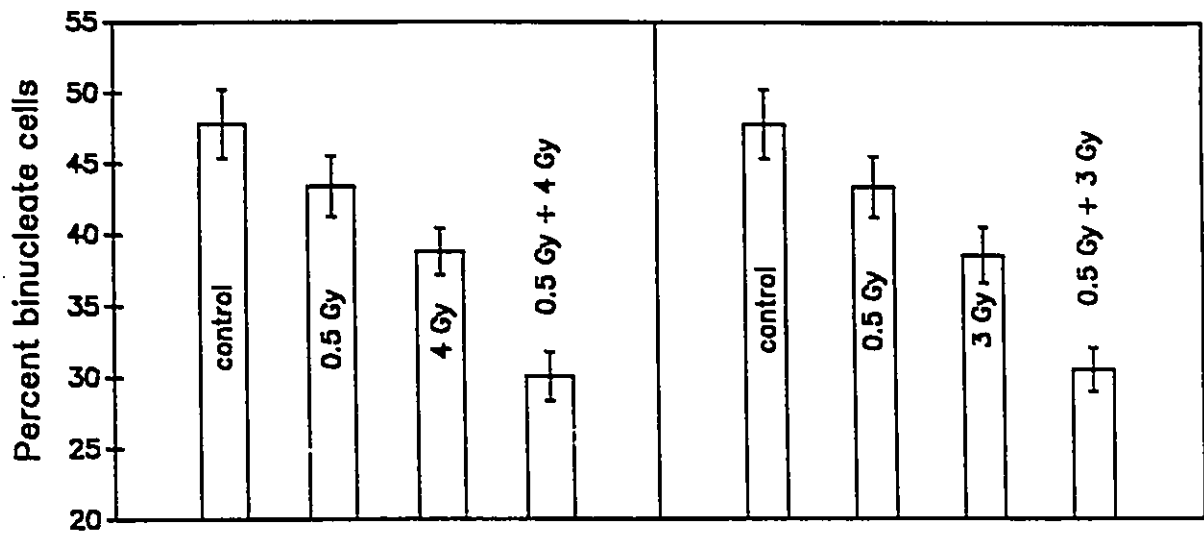
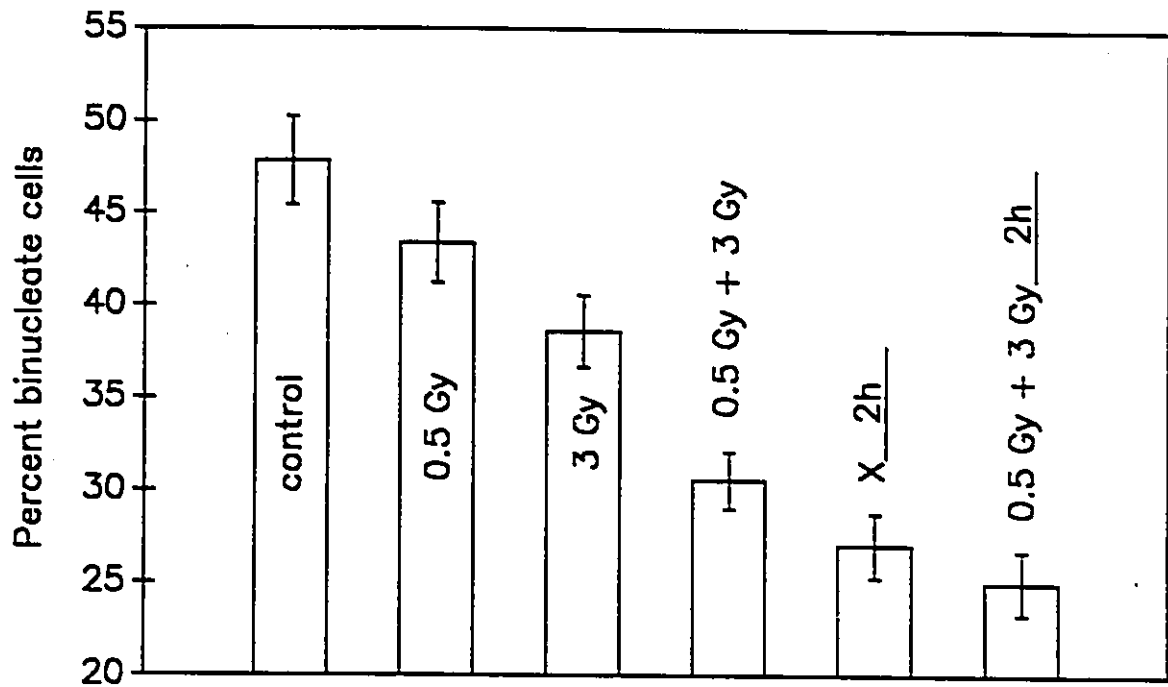


Figure 31. Induced division delay. Plateau phase AG1522 cells exposed to 0.5 Gy (0.0005 Gy/min) immediately prior to 3 Gy (1.8 Gy/min) followed by incubation at 37°C for 2 hours.



DISCUSSION**EXISTENCE OF AN ADAPTIVE RESPONSE TO IONIZING RADIATION IN NORMAL HUMAN SKIN FIBROBLASTS AS INDICATED BY MULTIPLE BIOLOGICAL PARAMETERS**

The experimental results describing cellular survival, colony size, micronucleus formation and cellular distribution of micronuclei in plateau phase AG1522 cells, pre-exposed to a dose of radiation and subsequently challenged with a second dose of radiation, indicate that adaptive responses to ionizing radiation exist in normal human fibroblasts (Azzam et al. 1992a; 1992b). These results extend the previous knowledge of the induction of the AR to IR in human lymphocytes (Olivieri et al. 1984), and constitute the first proof of an AR to IR in a human fibroblast cell line (Wolff 1992a). The survival results showed that adaptation can also reduce the amount of radiation killing. A threshold and an optimum adapting dose were observed for the survival AR when the challenge dose was delivered immediately after termination of a chronic pre-exposure. The survival AR was still observed 72 hours after the adapting dose (data not shown). Moreover, colonies from adapted cells which survived a subsequent challenge exposure were larger in size than colonies arising from cells that were exposed to the challenge dose only (Figure 18). The larger colony size indicates that the adapted cells surviving a challenge acute dose of radiation may be: 1- fitter cells, that have normal doubling time, as compared to cells which received the

acute dose only, or 2- in the case of cells receiving the acute dose only, the damaging events may have expressed themselves at a later cell generation, thus resulting in aborted or smaller colonies. While the effect on clonogenic survival of pre-exposure to a 4.25 Gy low dose-rate irradiation was relatively modest (2-fold increase in survival of pre-exposed and acutely challenged cells (4.25 Gy) as compared to challenged cells only), the colony size itself was altered significantly (4-fold more cells in the adapted and challenged cells as compared to the challenged cells only). Measurement of colony growth as described in these experiments can, therefore, be used as another indicator of expression of the survival AR to IR. Unfortunately, this approach is prohibitively time-consuming and only a few studies (Spadinger et al. 1994) have ever made use of it to study radiation effects, and none of these have focussed on effects at low doses or low dose rates. Recently, with the assistance of an automated image cytometry apparatus (Palcic and Jaggi 1986), colony size measurements have revealed important features of the low-dose radiation response (Walter et al. 1993). These measurements are revealing aspects of the radiation response that would not be discerned or would be obscured by the usual clonogenic scoring. The use of an image analysis system to quantify the number of abortive colonies (<50 cells) in the adapted and challenged cell population and comparison with the occurrence of such colonies in

the challenged population may reveal whether cells that would normally lyse following the challenge dose are stimulated to divide and become clonogenic.

Our report on the existence of a survival and cytogenetic AR in plateau phase human fibroblasts coincided with the report of Shadley and Dai (1992) on an association between increases in survival and increases in non-aberrant cells in G₁ phase lymphocytes. These reports have been considered significant (Wolff 1992a), for they have shown that the reductions in chromosomal aberrations and mutations that various researchers have observed, in adapted cells exposed to a challenge dose of radiation, can correlate with increases in cellular survival. However, the increases in survival, when observed and detected by current scoring techniques are not as large as the decreases in chromosomal damage observed following similar exposure protocols. A correlation between survival and a cytogenetic end-point has not always been observed. Sanderson and Morley (1986) have previously measured survival and mutation in G₀ exposed human lymphocytes from a number of donors. They found reductions in X-ray induced 6-thioguanine resistance, but no effect on survival. Recently, Rigaud et al. (1993) and Rigaud and Moustacchi (1994) also found significant reductions (up to 70%) in the mutant frequency at the HPRT gene locus in human lymphoblastoid cells pre-exposed to a low

dose of 0.02 Gy γ rays and then challenged with a high dose of 4 Gy. However, cell survival remained unchanged, suggesting that different mechanisms dealing with lethal or mutagenic lesions, respectively, can be induced by radiation. Published data exist (Thacker and Stretch 1983) which indicate that mutation induction and cell killing are not necessarily related. Also, Shadley and Dai's (1992) work with human lymphocytes showed that a cytogenetic adaptive response does not necessarily result in a survival adaptive response. Their data showed that reductions in multiply-aberrant cells can result in a cytogenetic AR, but, if nonaberrant cells are not increased, then a survival AR is not detected by the colony formation assay. Mutant bacteria and lower eukaryotic cells altered in their mutagenic response to genotoxic agents without changes in their survival are well known (Walker 1984, Larimer et al. 1989). Recently, Noodt et al. (1994) did not find a correlation between DNA strand breaks and HPRT mutation induced by photochemical treatments in V79 cells. Petrovecki et al. (1994) reported a lack of relationship between DNA double-strand breaks and the survival of murine bone marrow cells irradiated *in situ*. In the experiments described in this thesis, reductions in micronucleus formation were consistently greater than the increases in cellular survival observed as a result of pre-exposure of AG1522 cells to an adapting dose of radiation. These results suggest that survival is probably additionally dependent on mechanisms different

from those leading to the adaptive response which reduced micronucleus formation. An understanding of the relationship between the various parameters of survival, DNA damage and mutations is important for the understanding of the processes leading to carcinogenesis. Moreover, in the course of these studies, we observed that culture conditions, serum lot and age of serum at 4°C have an important effect on cellular survival (data not shown). The influence of growth medium on the yield of X-ray-induced chromatid exchanges has recently been investigated by Moore et al. (1993). The effect of the physiological state of the cell at the time of the adapting exposure was highlighted by Olivieri et al. (1990). The role of lymphokines on the radio-response of human lymphocytes is being studied by Shadley and Dai (1993a).

Following our initial report (Azzam et al. 1992a, 1992b), Shadley and Dai (1994) and Meyers et al. (1994) have confirmed the existence of a survival AR in normal human fibroblasts. In the studies of Shadley and Dai (1994), AG1522 cells were cultured and exposed to radiation doses similar to those in our studies. The consistent observation of a survival AR to IR in the AG1522 cells, under defined parameters, by various investigators in different laboratories, highlights the usefulness of experimenting with this established normal human cell line to characterize the mechanisms underlying the AR to IR.

The increase in cellular survival of pre-exposed and challenged cells over the expected level may indicate that: a) possibly the damage due to the challenge dose was better repaired as a result of induced DNA repair activity, more time for repair, increased access of the repair enzymes, or possibly due to the induction of factors controlling the fidelity of repair. b) Less damage occurred from the challenge dose as a result of conformational change, whereas a consequence of the chronic pre-exposure, the DNA was less accessible to the damaging events. A recent report by Belayev et al. (1993) demonstrated that the chromatin conformational state (CCS) of human leukocytes is highly sensitive to low dose X-rays. Their method of study based on anomalous viscosity time-dependences to identify changes in CCS can detect changes in chromatin conformation at doses that by themselves do not produce breaks in the genome; apparently the method is independent of experimental conditions and donor traits. c) Radio-adaptation could be due to an up-regulation of defence mechanisms against oxidative damage. An increased synthesis or altered activity of antioxidant enzymes would minimize the indirect damaging effects of IR, i.e., the reaction of free radicals produced by water radiolysis with DNA, the membrane and other cellular organelles. This could occur via activation of enzymes such as superoxide dismutases, peroxidases (e.g. glutathione peroxidase) and catalase. As an example of such induction, cells

pretreated with xanthine-xanthine oxidase became less susceptible to the mutagenic and killing effects of a subsequent dose of radiation, and this was associated with a 2-fold increase in the activity of superoxide dismutase but an unchanged catalase activity (Laval 1988). As will be reported in appendix 1, pre-exposure of AG1522 cells to low dose-rate γ radiation, where a cytogenetic and survival AR was observed, did not alter the mRNA levels of Cu-Zn or Mn-superoxide dismutases, catalase or glutathione peroxidase. However, post-translational regulation could result in increased activity of these enzymes. A radioprotective role of antioxidant compounds has been extensively described (Petkau 1987), and an extension of life-span by overexpression of Cu-Zn superoxide dismutase and catalase in *Drosophila melanogaster* has recently been reported (Orr and Sohal 1994).

In the studies described in this thesis, multiple biological end-points were measured simultaneously to elucidate the number of mechanisms involved in the AR to IR and their relationship to each other. Prior to our first report (Azzam et al. 1992a) on the presence of an AR to IR in normal human fibroblasts, only Sanderson and Morley (1986) measured simultaneously the end-points of survival and mutations in G_0 exposed lymphocytes. Following observation of the survival AR in AG1522 cells, it was reasonable to hypothesize that the increased levels of survival over what was

expected are due to less DNA damage resulting from the combined treatments. In a set of following experiments, DNA damage was evaluated through the measurement of micronucleus formation along with measurements of clonogenic survival. An AR response to IR was observed using both end-points (Figures 18, 22).

Micronuclei are structures in the cytoplasm which contain the same type of chromosomal material as the main nucleus. They are predominantly derived from acentric fragments and result mainly from unrejoined DNA double-strand breaks. The significant decrease in the formation of micronuclei due to a challenge dose of radiation, in cells pre-exposed to an adapting dose of radiation, may be due to any of the mechanisms just described, which possibly led, at least in part, to the expression of the survival AR. The net result of expression of the radio-protective mechanism(s) is the restitution of DNA double-strand breaks or the reduced formation of such breaks.

Upon characterization of the AR to IR in human lymphocytes, Olivieri et al. (1984) hypothesized that the adapting dose of radiation induced a chromosomal (or DNA) repair mechanism that allowed the high number of initial breaks induced by the subsequent high dose of radiation to become repaired efficiently, thus resulting in less total damage being left in the cell. This

hypothesis is still being adopted by researchers in this field (Wolff 1992b, Shadley 1994). Supportive evidence came from the initial experiments of Olivieri et al. (1984) which have been confirmed by others (Ikushima 1989, Vijayalaxmi and Burkart 1989), whereby the AR to IR was inhibited by 3-aminobenzamide (3AB), an inhibitor of poly(ADP)-ribosyl-transferase. The latter enzyme has been shown to be induced in response to the production of strand breaks within the cell (Gradwohl et al. 1990), and is thought to be involved in the repair of such breaks (Sato and Lindahl 1992). Poly(ADP-ribose)-depleted cells are deficient in nucleosomal unfolding, and they fail to excise bulky adducts from DNA (Malanga and Althaus 1994), and their ability to rejoin DNA strand breaks is delayed (Ding et al. 1992). Abolition of the induced protective effect was also observed if the protein synthesis inhibitor, cycloheximide, was added within the 4- to 6-h interval after the adapting dose (Youngblom et al. 1989). More recently, Boothman et al. (1989) have detected several radiation-inducible proteins in radioresistant human melanoma cells exposed to high doses of X-rays. Their identification and their role is being investigated. Preliminary experiments by Wolff (1992b) showed that an exposure of human lymphocytes to 1 cGy of X-rays induces or stimulates the production of proteins that bind to damaged DNA. These proteins are being considered as good candidates for being the induced DNA-protecting or DNA-repair proteins (Wolff 1992a).

In our studies, not only did pre-exposure to radiation reduce the frequency of micronucleus formation due to a challenge dose of radiation, but the distribution of micronuclei among the micronucleated binucleate cells was also altered. A reduction in the frequency of binucleate cells with 2 or >2 micronuclei was observed in adapted and challenged cells as compared to the challenged cells only (Figures 24, 29). This shift in distribution of micronuclei is another proof that adaptive responses to IR exist in human fibroblasts, whereby induced radioprotective mechanisms resulted in less residual chromosomal damage. Previously Shadley and Dai (1992) found that a cytogenetic AR could lead to increases in survival of lymphocytes if there was a sufficient increase in non-aberrant cells. They suggested (1992) and showed evidence (1993c) that the use of challenge doses that gave mainly singly aberrant cells improved the detection of a survival response. The same could be hypothesized for human fibroblasts, and the observed survival AR could possibly be greater if the cells were exposed to smaller challenge doses producing a reduced frequency of cells with 2 or >2 micronuclei. In those studies of Shadley and Dai (1993c), where a challenge dose (1 Gy) resulting in singly aberrant cells was used, the increase in non-aberrant cells was not sufficient to account for the increase in survival. They suggested that a large fraction of the increase in survival was due to a decrease in lethal damage in cytologically non-aberrant cells. Such damage

could range from sub-microscopic lesions, to alterations not visible in Giemsa-stained cells. An enhanced clonogenicity above "100% survival" of colony-forming ability had been reported by Calkins et al. (1989) and confirmed by others (Smith et al. 1992) for mammalian cell lines after exposure to low doses of γ -rays. It was suggested that low-dose exposure recruited non-clonogenic cells into colony forming cells. In an attempt to explain this "Lazarus effect", Calkins had proposed that a small increment of radiation over a threshold dose can induce a repair mechanism which produces a net improvement in the status of the irradiated cells. He argued that repairable damage to cells arises not only from radiation, but also seems to develop in the natural course of handling and subculturing of cells, thus leading to the capacity of small, 'repair-inducing' doses of irradiation to improve the plating efficiency of irradiated cultures.

MECHANISMS UNDERLYING THE ADAPTIVE RESPONSE TO IONIZING RADIATION

Our intention throughout these studies was to elucidate some of the mechanisms underlying the AR to IR, and an analysis of the reported results may reveal some of the processes leading to the observed radioprotective effects. The data in figures 18 and 22 show that a fairly large dose of radiation (4.25 Gy) delivered at

a low dose-rate (0.003 Gy/min) triggers a survival and a cytogenetic adaptive response respectively. The dependence of the AR both on the total dose of the pre-treatment and on the rate at which the dose is given has been previously studied (Shadley and Wiencke 1989). It appears from these studies that a high X-ray dose must be given at a low dose-rate to induce the AR, whereas a low dose must be given at a high dose-rate. In their study of the AR to IR in lymphocytes of rabbits exposed *in vivo* to very low dose-rate γ -radiation, Liu et al. (1992) also found that a large dose of radiation (1.5 Gy) can induce a significant protection against the action of an acute challenge dose of 1.5 Gy. Overall, these studies indicate that the inducing signal for the AR must be a certain number of lesions produced in a certain time. The survival data in figures 9 and 11 indicate that the D_{10} value (dose required to reduce survival to 10%) for AG1522 cells is among the highest for normal human fibroblasts (Gentner et al. 1988, Little et al. 1988). This inherent radioresistance may explain the induction of an AR with fairly large doses of IR.

As well, in our experiments, lower adapting doses delivered at 0.0025 Gy/min were also able to induce, in AG1522 cells, an AR against chromosomal damage by a challenge dose of radiation (Figures 25, 26). However, when a 0.5 Gy adapting dose was delivered at that dose rate, greater reductions in micronucleus

formation were observed when an incubation period, at 37°C, separated the adapting and challenge exposures (Figure 26). These results indicate that the AR can be observed at lower adapting doses, and that time is needed for expression of the response. For the data shown in figure 22, the adapting dose of 4.25 Gy was delivered over 24 hours, a sufficient time period which allowed for expression of the processes leading to the AR. An adaptive response induced by 0.5 Gy was also observed when the adapting dose was delivered at yet a lower dose-rate (0.0005 Gy/min or 0.5 Gy/15 h) (Figure 27). In this latter case, the challenge dose was delivered within minutes following the adapting exposure. The AR was also induced when the cells were adapted with an acute (2 Gy/min) dose of 0.5 Gy (Figure 28). The data shown in the figure indicate that the longer the incubation period separating the two treatments, the greater is the magnitude of the AR. When the adapting dose was followed immediately by the challenge dose, the effect on micronucleus formation was additive, and a minimum period of 6 hours appears to be needed for significant reductions in micronucleus formation to be observed. This time-interval is consistent with the data from studies of lymphocytes (Shadley et al. 1987) and V79 cells (Ikushima 1989), where a similar time was required for expression of the AR.

Data from the literature (e.g Shadley and Wiencke 1989),

from this work (not presented) and from many others clearly indicate that high adapting doses delivered at high dose rate do not induce an AR to IR. The data presented here showed that a low adapting dose (0.5 Gy), whether delivered at high or low dose-rate, induces an AR to an acute challenge dose provided that time is allowed for expression of the radioprotective activity. Our data is, therefore, consistent with the concept that there is a certain amount of damage per unit time with which the cell can cope and which induces a protective mechanism rendering the cell resistant to further damage. The fact that a 0.5 Gy priming dose, delivered chronically, induced an AR to a challenge dose given immediately after the pre-exposure suggests that an AR can be induced, in plateau phase AG1522 cells, with lower doses delivered acutely, as long as an incubation period at 37°C is provided to allow for expression of the radioprotective activity [preliminary data (not presented) was obtained confirming this suggestion].

The data in figures 26 and 28 describe an AR to IR in AG1522 cells pre-exposed to 0.5 Gy delivered either chronically (over 3 hours) or acutely (30 seconds) and challenged with a 4 Gy dose at various times following the pre-exposure. The rate of increase with time in resistance to a second challenge dose is observed to be lower for the acute than for the chronic adapting dose, indicating that the ability of these cells to adapt to radiation

increases with decreasing rates of damage. If valid *in vivo*, these results would have important implications in radiation therapy and the field of radiation protection. These data shed light on the cellular response at low doses and low dose-rates that has been extrapolated from data obtained at high doses and high dose-rates of radiation.

In earlier studies with human lymphocytes, Shadley and Wiencke (1989) suggested that the repair activity does not occur during the low dose-rate irradiation but later, after the challenge dose has been given. Also, experiments with the poly(ADP)ribosyltransferase inhibitor, 3AB, showed that when the chemical is present during the pre-exposure only, an AR was observed (Wiencke et al. 1986). However, when the chemical was present only during the holding period after the challenge dose had been delivered, the AR was not observed (Shadley and Wolff 1987, Vijayalaxmi and Burkart 1989), indicating that the radio-protective effect is expressed following the challenge exposure. In those studies of the AR reported in the literature, the induced radioprotective effect was measured after a holding period following the challenge dose. In our studies, however, the AR was measured both immediately following the challenge dose, and after various incubation periods subsequent to the challenge exposure. Our data clearly indicate that the process(es) responsible for the

radioprotective effect can be expressed during the low dose-rate pretreatment, or during an incubation period following the adapting dose, provided that the time-interval from the initiation of the adapting exposure until the time the challenge exposure is delivered is not less than 3 hours. During this time-period, some induced changes leading to protection against a subsequent dose of radiation seem to occur. Such changes could include induction of repair mechanisms, changes in chromatin conformation or up-regulation of antioxidant defence mechanisms. Our studies show that an AR to a challenge dose is observed, in pre-exposed cells, when trypsinization and plating of the cells for clonogenic survival and micronucleus formation assays are done immediately after the challenge dose. Our data is consistent with the report of Feinendegen *et al.* (1987), Petkau (1987), Laval (1988), Boreham *et al.* (1990), Belayev *et al.* (1993), and others indicating that changes at the molecular level occur following low dose or low dose-rate radiation exposures (Woloschak *et al.* 1990; de Toledo *et al.* 1994a, 1994b). These changes can contribute to expression of an adaptive response to radiation. As well, as shown by others, our data, as will be discussed later, show that the adapting dose is able to prime or induce DNA repair mechanism(s) expressed during an incubation period following the challenge dose. Poly-ADP-ribosylation enzymes modulate these DNA repair mechanisms (Sato and Lindahl 1992, Malanga and Althaus 1994), and the data on the

inhibition of the AR to IR by 3AB results from an effect on these mechanisms, which might be involved in the AR along with other possible protective processes.

While characterizing the radioresponse of AG1522 cells, we observed that plateau phase cells repair potentially lethal damage (Figure 12). Also, as demonstrated by Koval in lepidopteran insect cells (1986), treatment of AG1522 cells by cycloheximide following a challenge dose of radiation (Figure 13) resulted in an inhibition of the repair of potentially lethal damage, suggesting the induction of a process responsible for the observed increased survival, which was dependent on de-novo protein synthesis. The increases in survival as a result of PLDR correlated with decreases in micronucleus formation (Figure 12B). The data presented in table 3 indicate an enhanced decrease in the rate of formation of micronuclei when the cells were initially exposed to a priming dose of 0.5 Gy delivered over a 15 h period, and subsequently exposed to a 3 Gy challenge exposure followed by a 2 hour incubation at 37°C. The greater reductions in micronucleus formation observed after the holding period, in cells that had been pre-exposed to the adapting dose, indicate a faster DNA repair rate as a result of that adapting dose. Such an enhancement could result from an increase in the cell's enzymatic capacity to repair DNA, or from a better accessibility of repair enzymes to the chromatin (enzymes involved

in poly-ADP-ribosylation can shuttle histone proteins off and back onto the DNA template, making it accessible to other proteins involved in DNA damage processing (Althaus and Kleczkowska 1992). The stimulation of additional cellular mechanisms (e.g. antioxidant defence) intervening in the formation of DNA breaks cannot be excluded.

The data in table 5 indicate that the further reductions in micronucleus formation, as a result of pre-exposure of AG1522 cells to an adapting dose of radiation and holding of the cells at 37°C following the challenge exposure, occur during the early part of the holding period. The lack of further reductions observed for longer incubation periods suggests that normally unreparable lesions remained unrepaired in adapted cells. It is possible, therefore, to conclude that the observed radioprotection must result at least in part from the stimulation of a DNA repair mechanism which does not result in more extensive repair than that achievable by the constitutively available DNA repair mechanisms, if those mechanisms are given sufficient repair time.

The data in table 4 indicate that the greater reductions in micronucleus formation occurring during the holding period, when the cells are pre-exposed to an adapting dose, are translated into increases in cellular survival. This could represent either

increased fidelity of repair or simply more repair accomplished before cells are committed to divide. Evidence from Schwartz' laboratory (Schwartz 1994) indicate that the rejoining of DNA double-strand breaks is faster in radioresistant cell lines, as measured by neutral filter elution techniques and pulsed field gel electrophoresis. His data indicate that the faster rejoining of DNA double-strand breaks was associated with a greater fidelity of repair as X-rays induced fewer chromosome exchange-type aberrations in resistant cell lines.

Youngblom et al. (1989) presented evidence that the adaptive response is attributed to the induction of proteins 4-6 h after the adapting dose. These proteins possibly play a role in modulating the response of the cell to a challenge dose of radiation, and protect against the damaging effects by either rendering the DNA less accessible to the damaging events, competing with DNA for the radiation-produced damaging species or by repairing the damage during a subsequent holding period. Also, the role of post-translational regulation of constitutively existing proteins cannot be excluded. Such regulation may result in enhanced enzymatic activity.

The synthesis of proteins which modulate the repair of DNA damage result from radiation-induced alterations in gene activity

which could also affect apoptosis and cell cycle progression. As replication or segregation of damaged DNA is likely to have deleterious consequences for the survival of the cell, the cell has evolved mechanisms to cope with the damage by transient delays in G_1 or G_2 phase. A delay in G_1 would allow more time for repair prior to DNA replication. A G_2 delay permits repair prior to mitosis. If replicative DNA synthesis or mitosis occurred before repair of the damage, then mutagenic lesions could be 'fixed' and propagated (Cohen and Ellwein 1990) and could contribute to the progressive increase in genomic changes which may lead to neoplastic transformation. As will be discussed in appendix 1, progression through the various stages of the cell cycle is regulated by cyclin proteins whose levels vary throughout the cell cycle. Ionizing radiation has been shown to cause delays in the G_1 and G_2 phases (reviewed in Maity et al. 1994), and decreased levels of the cyclin A protein (Dulic et al. 1994) and cyclin B protein and mRNA (Muschel et al. 1991) have been observed in G_1 and in G_2 arrested cells respectively. Lack of exhibition of G_1 arrest following exposure to IR was recently shown in cells lacking *p53* genes and cells with mutant *p53* genes (Kastan and Kuerbitz 1993). However, cells with mutant *p53* genes continued to arrest in G_2 after radiation treatments. Induction of the wild type *p53* has also been shown to regulate apoptosis (Clarke et al. 1993). Figure 30 shows that exposure to 3 or 4 Gy of radiation resulted in a 19% decrease

in the percentage of binucleate cells. This decrease suggests a delay in progression through the cell cycle leading to the binucleate state. These delays were enhanced when the cells were exposed to a low dose-rate exposure which resulted in an AR indicating that division delay is a possible mechanism of adaptation (Azzam et al. 1994c). Analysis of gene expression of cells exposed to an adapting dose of radiation (appendix 1) showed a decrease in the expression level of cyclin A and cyclin B which is consistent with a mechanism leading to a delay in the progression through the cell cycle. Of further interest is the novelty of the finding that the reductions in micronucleus formation, as a result of holding of the cells at 37°C, correlate with delays in progression in the cell cycle. Reductions in micronucleus formation with holding of the cells in plateau phase was shown to correlate with increases in cell survival (PLDR) (Figure 12 and 12B). The saturation of PLDR, as described in figure 12A, correlates with lack of further delays in the cell cycle. Elucidation of the molecular mechanism regulating progression in the cell cycle should contribute to our understanding of the AR to IR and the stress response in general.

The enhanced delay in progression of the cells in the cell cycle in pre-exposed and challenged cells seems contradictory to the previously reported increase in colony size of cells surviving

this treatment, when compared to the challenged cells only. However, it might be reasonable that an initial delay in progression of the cells may have permitted optimal constitutive and induced repair of the damage due to the challenge dose, following which the propagated cells resumed a fitter growth pattern.

While the adapting dose of radiation caused delays in progression of the cells in the cycle, flow cytometric measurements showed that the distribution in the cell cycle, of the plateau phase cells used, was unaltered during the chronic pre-treatment, or after the various incubation periods that followed the adapting or acute exposures. Hence, the observed AR cannot be attributed to selection of cells at a radioresistant stage of the cell cycle. Work by Sinclair (1968) has shown that the position of the cell in the cell cycle at the time of irradiation can alter the cellular radiation response. Moreover, throughout this thesis work, cell counts were performed following each treatment (data not shown). The cell counts for control and irradiated cells were constant such that the observed AR could not be attributed to selective loss of radiosensitive cells.

Of particular importance, this thesis research has clearly demonstrated that the adaptive response to ionizing radiation can

be triggered in cells which are predominantly in G_0/G_1 . The induction of an AR to IR in G_1 , S, or G_2 phases of the cell cycle has been demonstrated in human lymphocytes (reviewed in Shadley 1994, Rigaud and Moustacchi 1994). However, there is conflicting evidence for induction in G_0 phase for lymphocytes. Induction of an AR in resting cells is important, as most cells in the human body are relatively quiescent, entering an active cell division cycle infrequently or in some cases not at all during the life span of an individual. Moreover, in these studies, it was observed that radiation induced a potentially lethal damage repair system(s) (PLDR), in quiescent AG1522 cells, that was readily observed and quantified (Figure 12). This PLDR system was not observed, by using clonogenic survival as an end-point, in cells with high levels of DNA replicative synthesis and cell division (Figure 16). In these studies, the assignment of cells in the cell cycle has been done according to flow cytometric measurements. Caution has to be exercised in assigning cells to a particular stage of the cell cycle based on time relative to mitogenic stimulation as it is done with lymphocyte studies. A promising but time-consuming method based on BrdU replication banding has been developed by Aghamohammadi and Savage (1992) to obtain a fine analysis of the cell cycle and to permit cohort analysis.

EVIDENCE FOR INDUCED RADIORESISTANCE IN NORMAL HUMAN SKIN FIBROBLASTS FROM SUBSTRUCTURES IN SURVIVAL CURVE; RELATIONSHIP OF THIS END-POINT TO THE ADAPTIVE RESPONSE

The AR studies as described here and by others (reviewed in Wolff 1992a, 1992b; Shadley 1994, Rigaud and Moustacchi 1994) indicate that cells respond to ionizing radiation by inducing processes leading to repair of DNA injury, which remain available to protect against further damage. Induction of radioprotective processes has also been suggested by the study of the substructures of survival curves obtained by exposing cells to a range of single doses of radiation, generated in several laboratories using various cell lines over many years (reviewed in Joiner 1994). The study of these sub-structures is currently an area of intensive study (Skov 1994). The published reports (e.g. Lambin et al. 1993) indicate a hypersensitivity to low radiation doses followed by an increased radioresistance to higher doses. The concept of a threshold dose above which cells would acquire radioresistance was first put forward by Calkins in 1967 in his induced repair model ('T-N') to explain the non-monotonic dose-response relation he observed in irradiated protozoa (Calkins 1967). He proposed that in addition to constitutive ("Non-triggered" = N) repair which is always present, there is damage-induced ("Triggered" = T) repair which becomes functional upon exceeding a threshold level of damage. The radiation exposure protocols adopted in either Calkins' repair

model or the induced repair mechanisms proposed by Olivieri et al. (1984) are obviously different; however the models are not mutually exclusive. Calkins triggered repair assumes that DNA damage diverts the cell into a pathway quite distinct from the response after subthreshold doses. The AR suggests that forewarning of damage renders the exposed cell more able to cope with future lesions. In his early papers, Calkins (1967) predicted that T repair stimuli will fade or disappear with time, and to reach the threshold inducing dose will require higher total doses when given as split doses or at low dose-rates than as a single acute dose. Evidently, much experimental proof is still required to elucidate the processes induced in these two systems. Koval's studies (1984) strongly support inducible recovery as basis for the multiphasic survival curves of cultured eukaryotic cells. Koval (1988) has shown that the maximum amount of PLDR or SLDR is the maximal repair which can be induced in lepidopteran cells.

As previously described, our data indicate that an AR to IR exists in AG1522 cells. Also, the survival curves shown in figures 9, 10 and 11 are noteworthy for absence of a shoulder observed at low doses of radiation. In these curves, which represent the first clonogenic survival response of a normal human cell line exposed to very small doses of radiation, an increased low-dose sensitivity relative to extrapolation from doses greater than 0.75 Gy is

observed. The initial sensitive response converts into a more resistant phase through a plateau. As proposed by Calkins these observations could indicate that: 1. At low doses induced (T) repair is not triggered and the incremental rate of killing is maximal. 2. In the plateau, the induced repair threshold is exceeded, and the rate of killing is reduced. 3. At the end of the plateau, due to the summation of the constitutive and triggered repair capacity, the incremental response is lower than the incremental response below the threshold for the triggered repair where only the constitutive repair is operational. Hence, both types of repair mechanisms as proposed by Calkins (1967) and Olivieri et al. (1984) may occur in plateau phase AG1522 cells exposed to ionizing radiation. It is of interest to note that the acute radiation dose which induced an adaptive response to a subsequent dose (Figure 28) falls in the plateau region of figure 10 where the rate of killing is observed to be diminished.

While evidence for induced processes in irradiated cells is accumulating, definitive proof of the nature of the signalling lesion is still lacking. In their study of the survival response of Chinese hamster cells to low doses of ionizing radiation, Marples and Joiner (1993) had previously observed a multiphasic survival response to X-rays. Similar to the report of Calkins et

al. (1991), a low-dose hypersensitive region followed by increased radioresistance was observed. These substructures in the survival curves were not observed with neutrons (Marples and Joiner 1993), and exponential survival curves were obtained. In studies of the AR to IR in lymphocytes, neither protons (Khandogina et al. 1991) nor fast neutrons (Wiencke et al. 1987) induced a response. Also, while studying radiation-induced radioresistance in yeast, Boreham and Mitchel (1991) found that low LET radiation induced higher levels of resistance to a second dose than high LET radiations. As previously noted, low LET radiations produce mainly single-strand breaks in irradiated cells, while high LET radiations produce mainly double-strand breaks. However, clear evidence that a specific DNA lesion signals the induction of the adaptive process is lacking. As reviewed, the adaptive response to ionizing radiation was induced by a variety of agents. One common effect of many of these agents (e.g. bleomycin, hydrogen peroxide) is the generation of free radicals. Free radicals can interact with other structures in the cell, such as membranes, which play a role in signal transduction (Halliwell and Gutteridge 1989, Hockenbery et al. 1993). The effect of free radical scavengers or signal transduction inhibitors on induction of the AR has not been attempted.

Clearly, accumulating experimental evidence shows that an AR to IR exists for multiple biological end-points including cell survival, chromosomal aberrations and mutations. The effect of adaptive responses on the carcinogenic risk of radiation exposure has not been addressed, and the studies in the following chapter will attempt to show whether radiation-induced adaptive responses against neoplastic transformation exist. Small repeated radiation exposures, permitted under current radiation safety programmes are delivered to a large number of people, and data regarding the effects of such exposures is lacking. Cellular transformation studies based on the AR protocol as reported in the following section might be a promising model to study the carcinogenic effects of multiple exposures to ionizing radiation.

CHAPTER 4CONSEQUENCES OF THE ADAPTIVE RESPONSE TO IONIZING RADIATION

The data just described in our human fibroblast cell system and the results of other studies in the lymphocyte system indicate that the adaptive response to ionizing radiation induces radioprotective mechanisms which result in reduced residual DNA damage from a subsequent challenge dose of radiation. Enhancement of both the rate and time available for repair of DNA damage caused by the challenge dose may contribute, at least in part, to the induced effects. What remains unclear, however, is the effect, if any, that the radiation-induced radioprotective mechanisms observed in mammalian cells have on the carcinogenic risk of a subsequent radiation exposure.

A model system suitable for the study of the carcinogenic effects of a radiation-induced adaptive response is the C3H 10T½ "transformation assay" (Reznikoff et al. 1973b), where non-transformed cells in tissue culture can be transformed into demonstrably malignant cells by exposure to radiation. Using this system, we report here the effects of chronically delivered adapting doses on killing, micronucleus formation and neoplastic transformation by an acute challenge dose. For low LET radiations, protraction in delivery of a dose of radiation, either by low dose-

rate or fractionation, has been shown to result in a decreased biological effect (Han et al. 1980, Hill et al. 1984, Balcer-Kubiczek et al. 1987). Adaptive responses to low dose-rate irradiation which have any effect on the carcinogenic risk of a subsequent radiation exposure would impact on the field of radiation protection where the risk of sequential doses is assumed to be additive. The linear "no threshold" theory of radiation action assumes that the effect is always additively deleterious (UNSCEAR 1988). Results of the following experiments will prove that, under the conditions used here, this assumption does not hold. Moreover, these results add to the accumulating evidence that low-dose effects cannot be predicted from high-dose experiments.

As proliferating cells exposed to ionizing radiation could give rise to redistribution of cells in the cell cycle and alter radiation sensitivity, especially when low dose-rate experiments are performed (Sinclair 1968, Redpath and Sun 1990, Brenner et al. 1993), the following study used plateau phase C3H 10T½ cells which have been shown to be highly sensitive to post-confluent inhibition of cell division (Reznikoff et al. 1973a). Since we observed that the adaptive response to ionizing radiation occurred in G₀/G₁ cells, we extended our studies to C3H 10T½ cells at a similar stage of the cell cycle.

MATERIALS AND METHODS

Cell culture. The C3H 10T½ clone 8 cells were obtained from the American Type Tissue Culture Collection (ATCC, Rockville, MD). Cells in passage 8 to 10 were seeded at a density of 2×10^5 cells per 80 cm² flask (Nunc) containing 25 ml of DF culture growth medium (Gibco) supplemented with 15 mM NaHCO₃ and 10% fetal calf serum (Sigma, lot 11H-0915). Cells were incubated at 37°C in 2% CO₂. Under these conditions, the pH of the medium was maintained at 7.4. The doubling time of an exponentially growing cell population was about 18 hours. The cells were plated 7 or 8 days prior to irradiation for these studies using confluent cultures (89% in G₀/G₁ phase as determined by flow cytometry). Immediately after irradiation (or in some cases after irradiation followed by a defined holding period at 37°C), cells were trypsinized and replated to yield approximately 300 clonogenic cells per flask. Cell survival was determined by colony formation 8 to 10 days after replating. Plating efficiencies ranged from 15 to 30%. Flasks for the neoplastic transformation assay were re-fed on day 10, and then at 7-day intervals, with DF medium supplemented with 5% heat-inactivated (56°C, 30 min) fetal calf serum and 25 µg/ml gentamicin sulfate (Gibco). The pH of the medium was stable between 7.3 and 7.4 throughout the experiment. After 45 days of incubation, the monolayers were washed with PBS, fixed in ethanol and stained with

a 2% aqueous Giemsa (Fisher). Neoplastic transformation frequency was estimated using morphological criteria (Reznikoff et al. 1973b), with only type II and III foci being counted.

Prior to the start of the experiments, an exhaustive preselection of bovine fetal calf serum was done. The supplementation of serum from the selected batch (Sigma lot 11H-0915) to DF medium supported good growth of the cells, resulted in acceptable plating efficiency of the cells (about 20%), and above all it maintained the contact-inhibited monolayer. When control monolayers were re-fed with medium supplemented with this specific lot, the background transformation frequency was very low (Figure 32B). The survival level and transformation frequency following a 4 Gy dose as described in table 7 are consistent with published data (Miller et al. 1979, Terasima et al. 1983, Raaphorst et al. 1990). Studies by Kennedy et al. (1980) had demonstrated that, after a 4-Gy dose of X-rays, neoplastic transformation frequency was independent of the number of cells irradiated.

The dependence of cellular transformation, either spontaneously developed or induced by exogenous agents including radiation, upon serum has been described by many investigators (Carbone et al. 1974, Terasima et al. 1981). However, the reasons for this phenomenon remain unclear. Studies by Terasima et al.

(1983) have shown that the transformation frequency of C3H 10T½ cells depended not only on the serum used for pre-irradiation culture, but also on the serum used post-irradiation. Different batches of serum had significantly different quantitative effects on the X-ray-induced transformation yield. Studies by the same authors have also shown that the culture serum affected, in a batch-dependent manner, the removal of transformation damage which occurred during the first 6h of the post-irradiation period. The half-time required for the development of insensitivity to serum factors in radiation-initiated cells was determined to be about 3 days.

Criteria for type II and type III foci. Foci were classified in conformity with published data for the transformation assay (IARC/NCI/EPA Working Group 1985). Type III were described as having the following properties: dense; multilayered; basophilic; random orientation at focus edge; invasion into the monolayer and being predominantly composed of spindle-shaped cells. Type II foci are distinguished from type III foci primarily by their more ordered and defined edge. They are dense, multilayered and less basophilic than type III foci. According to the IARC/NCI/EPA Working Group, foci of less than 1 to 2 mm should not be scored.

Irradiation. All cellular adapting doses were from ⁶⁰Co γ-rays

(Atomic Energy of Canada Limited) at a chronic dose-rate of 0.0024 Gy/min, at 37°C. Challenge doses were delivered acutely (2 Gy/min) from a Siemens Stabilipan II X-ray machine operated at 250 kV and 15 mA with a 1 mm aluminum filter. For the challenge dose, flasks were removed from the 37°C incubator for the time required to deliver the dose at room temperature (2 to 3 min). Incubations between or after the radiation treatments were at 37°C.

Analysis of transformation data. Two different methods were used for data analysis. The experimental mean of the neoplastic transformation frequency and the standard error of the mean were calculated directly from the number of replicate flasks for each data point. However, since neoplastically transformed cells are not contact-inhibited, cells may be dislodged during refeeding and form new colonies. To test for errors arising from this potential problem of reseeding, the mean neoplastic transformation frequency and standard error were also calculated from Poisson analysis of the number of flasks with no transformants [$P(0) = e^{-\lambda}$], where $P(0)$ represents the probability that a flask will receive zero transformants when the average value per flask is λ (Han and Elkind 1979). The tables and graphs show neoplastic transformation frequency per viable cell (i.e. surviving cell as determined by colony formation) and per cell at risk (cells at risk are the total number of cells submitted to the various treatments).

RESULTS

The growth and radiation response of C3H 10T½ cells used in the following studies have been previously characterized (Rezinkoff et al. 1973a, Borsa et al. 1984, Raaphorst and Azzam 1992).

Table 7 shows the results of three independent experiments measuring the effects of an adapting dose of 0.1 to 1.5 Gy low dose-rate radiation on the survival and neoplastic transformation frequency of plateau-phase cells subsequently given a 4-Gy acute challenge dose of X-rays. The data indicate that none of the adapting doses detectably improved clonogenic survival, whether the cells were plated immediately after the acute exposure or held in plateau phase for 3.5 h. However, the results of all three experiments indicate that a prior chronic adapting dose resulted in about a two-fold reduction in the neoplastic transformation frequency per viable cell due to the challenge dose of 4 Gy. Increasing adapting doses (0.1, 0.65 or 1.5 Gy) did not show a clear dose response for decreasing neoplastic transformation frequency per viable cell, all resulting in about the same two-fold reduction. By themselves, the 0.1 and 0.65 Gy adapting doses resulted in neoplastic transformation frequencies which were four- and two-fold higher, respectively, than background, while the 1.5

Gy adapting dose produced a transformation frequency similar to background. Significantly, the combined chronic and acute treatments were consistently lower than those due to the acute treatment alone.

Calculation of the Poisson mean from the number of flasks containing no transformants indicated that secondary foci formation due to reseeded was not significant. The two-fold decrease in the neoplastic frequency per viable cell of adapted cells was also observed after Poisson calculations.

Consistent with published data (Raaphorst et al. 1990), when the cells were not pre-exposed, holding them in plateau phase for 3.5 h after the challenge dose also resulted in about a two-fold reduction in the neoplastic transformation per viable cell. This holding effect produced a similar or greater additive reduction when the cells were pre-exposed to the adapting doses prior to the challenge dose (Table 7). An exception was observed in experiment III, where the 1.5 Gy adapting dose resulted in an increase in the neoplastic transformation frequency per viable cell expressed during the recovery period after the test dose. However, the Poisson calculation shows that this increase might have been due to reseeded. The results of experiment III show that, unlike the

effect of holding after the acute challenge dose, holding of the cells for 3.5 h after the 0.1 Gy chronic adapting dose did not alter the neoplastic transformation frequency per viable cell.

Neoplastic transformation frequency expressed in terms of cells at risk also showed a reduction resulting from pre-exposure prior to the challenge dose. When calculated this way, the 0.1 and 0.65 Gy pre-exposure doses further reduced the neoplastic transformation beyond that achieved by holding the cells at 37°C for 3.5 h after the challenge dose.

A total of 7 transformed foci of type III were observed, following a challenge dose of 4 Gy, in the three experiments described in table 7. Not a single focus of this type was observed when the cells were pre-exposed chronically to 0.1, 0.65 or 1.5 Gy prior to the challenge dose. The foci observed and scored were of type II.

Figure 32A is a photograph of a type III, giemsa-stained, focus composed of multilayered criss-crossed arrays of densely stained cells. Figure 32B is a photograph of giemsa-stained tissue culture flasks showing monolayers of C3H 10T½ cells, 6 weeks after control and X-ray (4 Gy at 2 Gy/min) treatments. Figure 33 is a

photograph of a binucleate C3H 10T½ cell (400X magnification) used to assay for micronucleus formation following the various treatments described in the previous tables. The integrity of the cells was not disrupted following the fixation and staining of the cells.

The data in table 8 show the effect of pre-exposure to low-dose-rate radiation on micronucleus formation induced by the 4 Gy challenge dose. A reduction in the frequency of micronucleus formation in binucleate cells was observed in adapted cells in all the treatments in the three experiments, as reported above for human fibroblasts. Furthermore, it is observed that in all the treatments, the percentage of cells with more than 2 micronuclei is reduced in the cells pre-exposed to an adapting dose and challenged with 4 Gy as compared to the challenged cells only.

Figure 34 further illustrates the reductions in the frequency of binucleate cells with multiple micronuclei in the cells pre-exposed to 1.5 Gy (0.0024 Gy/min) and challenged with 4 Gy (2 Gy/min) 3.5 hours later, compared to the cells that were exposed to the challenge dose only. A 24% reduction (27.7 ± 2.2 versus 36.5 ± 2.4) in the percentage of cells with >2 micronuclei was observed, while the percentage of cells with 1 micronucleus was

increased by 31% ($44.4\% \pm 2.9$ versus 31.9 ± 2.2). Figure 35 also illustrates the spectrum of shift in the distribution of micronucleus frequency in cells that were pre-exposed to 0.65 Gy (0.0024 Gy/min), challenged with a 4 Gy dose (2 Gy/min) 3.5 hours later and then held in plateau phase for 3.5 hours, as compared to cells exposed to 4 Gy (2 Gy/min) and held in plateau phase at 37°C for 3.5 Hours. A 33% decrease (16.8 ± 1.2 versus 25.2 ± 1.7) and a 21% increase (57.2 ± 2.6 versus 47.4 ± 2.4) in the frequency of cells with >2 micronuclei and 1 micronucleus, respectively, were observed in adapted versus non-adapted cells.

As described in figure 28 for AG1522 cells, the data in table 9 also possibly indicate an induced division delay in C3H 10T½ cells, as a result of pre-exposure to an adapting dose of radiation prior to the 4 Gy challenge dose. At 48 hours after plating, 21.3 ± 1.5 % of the cells reached the binucleate state following a 4 Gy dose. However, pre-exposure to a 1.5 Gy chronic dose prior to the 4 Gy challenge dose resulted in 11.0 ± 1.1 % of the cells being binucleate. Similarly, reductions in the percentage of binucleate cells were observed in populations of cells pre-exposed to the 1.5 Gy chronic dose, challenged with a 4 Gy dose and held in plateau phase for 3.5 hours, as compared to cells submitted to the same treatments without the pre-exposure (14.2 ± 1.3 versus

24.0±1.7).

The histograms in figure 36 show the transformation frequency per viable cell (TF/VC), following exposure of plateau-phase C3H 10T½ cells to γ -rays from ^{60}Co delivered at 0.0024 Gy/min. The data indicate that for the range of doses from 0.1 to 5 Gy, the TF/VC decreases as the dose increases to 3.5 Gy. The transformation frequency per viable cell (10^3) being 0.37, 1.6, 0.85, 0.38, and 0.34 for cells exposed to 0, 0.1, 0.65, 1.5 and 3.5 Gy respectively. For cells exposed to 5 Gy, the TF/VC sharply increased to 2.4 (10^3). It is noteworthy that the TF/VC following 1.5 and 3.5 Gy delivered at low dose-rate were similar to the background TF/VC in this cell line.

Table 7. The effect of adapting doses on radiation-induced transformation

Treatment	Percent Survival (±S.D.)	No. of flasks	No. of foci	No. of flasks without foci	Transformation frequency x 10 ³		
					Per viable cell	Poisson calculation	Per cell at risk
Experiment I							
PE	17.3 (0.4)	12	1	11	0.27 (.27)	0.28 (.27)	.05
X (4Gy)	26.6 (1.8)	12	15	0	3.2 (.33)	>6.3 (1.1)	.15
X →	41.3 (2.4)	12	6	6	1.5 (.45)	2.0 (.69)	.11
γ (0.67Gy)	102.4 (4.2)	12	2	10	0.5 (.32)	0.5 (.35)	.08
γ → X	23.9 (1.2)	12	10	4	1.7 (.43)	2.3 (6.3)	.07
γ → X →	35.1 (3.1)	12	0	12	<0.15	<0.16 (.15)	<.0092
Experiment II							
PE	30.1 (0.10)	12	1	11	0.29 (.29)	0.30 (.29)	.09
X (4Gy)	23.1 (1.04)	13	22	0	3.7 (.38)	>5.6 (.96)	.25
X →	30.0 (3.2)	13	11	4	2.0 (.45)	2.8 (.72)	.18
γ ₁ (0.1Gy)	91.9 (9.7)	12	5	7	1.5 (.54)	2.0 (.77)	.42
γ ₁ → X	27.6 (0.98)	12	10	3	1.7 (.35)	2.9 (.71)	.14
γ ₁ → X →	31.0 (3.47)	13	9	5	1.6 (.41)	2.2 (.63)	.15
γ ₂ (0.65Gy)	87.9 (4.79)	12	4	8	0.86 (.37)	1.1 (.48)	.23
γ ₂ → X	20.8 (0.51)	12	13	2	2.5 (.44)	4.0 (.87)	.15
γ ₂ → X →	26.4 (3.53)	15	13	3	2.2 (.33)	4.0 (.82)	.17
γ ₃ (1.5Gy)	75.3 (7.81)	12	2	9	0.37 (.25)	.65 (.35)	.09
γ ₃ → X	19.6 (1.22)	12	9	3	1.7 (.29)	3.1 (.76)	.10
γ ₃ → X →	25.9 (0.74)	12	5	7	0.89 (.32)	1.2 (.45)	.07

Table 7. (continued)

Experiment	Treatment	Percent Survival (\pm S.D.)	No. of flasks	No. of foci	No. of flasks without foci	Transformation frequency $\times 10^{-3}$		
						Per viable cell	Poisson calculation	Per cell at risk
PE		15.6 (1.1)	12	1	11	0.56 (.56)	0.59 (0.09)	.08
X	(4Gy)	27.2 (1.2)	15	22	2	5.4 (.95)	7.4 (1.30)	.23
X \rightarrow		46.8 (1.9)	12	8	6	1.9 (0.72)	2.0 (0.68)	.14
γ_1	(0.1Gy)	93.4 (1.4)	12	3	9	1.7 (0.88)	1.9 (1.00)	.25
$\gamma_1 \rightarrow$		56.7 (2.4)	12	2	10	1.5 (1.00)	1.7 (1.10)	.14
$\gamma_1 \rightarrow$ X		29.5 (0.66)	12	9	5	2.7 (0.79)	3.2 (0.99)	.12
$\gamma_1 \rightarrow$ X \rightarrow		42.5 (2.3)	12	3	10	0.95 (0.68)	0.69 (0.47)	.06
γ_2	(0.65Gy)	99.4 (4.1)	12	3	9	1.2 (0.64)	0.76 (0.41)	.19
$\gamma_2 \rightarrow$ X		24.3 (1.9)	14	12	4	3.5 (0.84)	5.1 (1.22)	.13
$\gamma_2 \rightarrow$ X \rightarrow		37.2 (1.0)	12	6	8	1.8 (0.88)	1.4 (0.65)	.10
γ_3	(1.5Gy)	105.7 (8.9)	12	1	11	0.4 (0.40)	0.42 (0.41)	.07
$\gamma_3 \rightarrow$ X		24.2 (1.0)	12	6	7	2.1 (0.82)	2.3 (0.89)	.08
$\gamma_3 \rightarrow$ X \rightarrow		42.6 (1.1)	13	14	7	3.5 (1.39)	2.0 (0.7)	.23

PE: ^{60}Co γ -irradiation at 0.0024 Gy/min

\rightarrow : incubation at 37°C for 3.5h
X: X-irradiation at 2 Gy/min

Figure 32A. Photograph of a Giemsa stained type III transformed focus of C3H 10T1/2 cells.

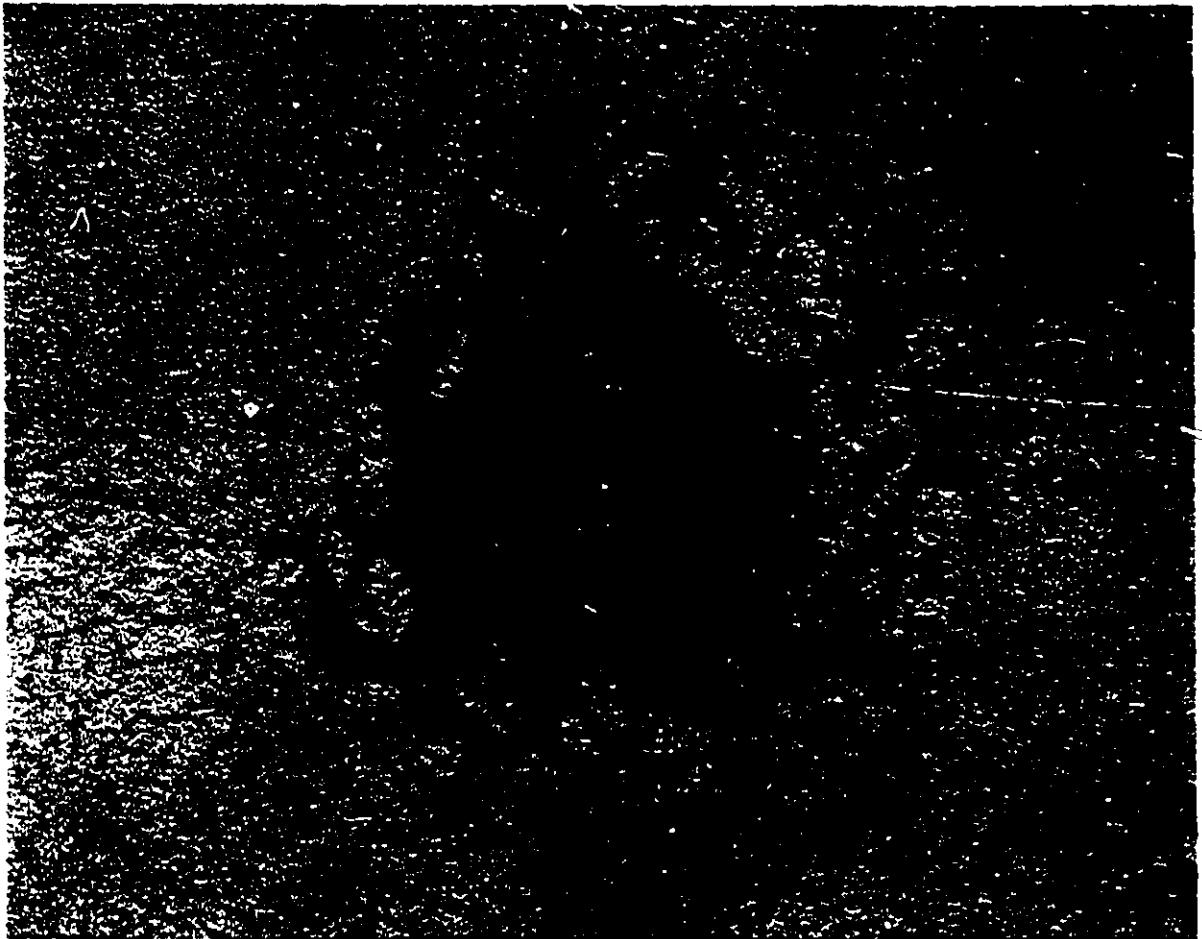


Figure 32B. Photograph of Giemsa stained tissue culture flasks showing monolayers of C3H 10T1/2 cells, six weeks after control and x-ray (4 Gy) at 2 Gy/min treatments

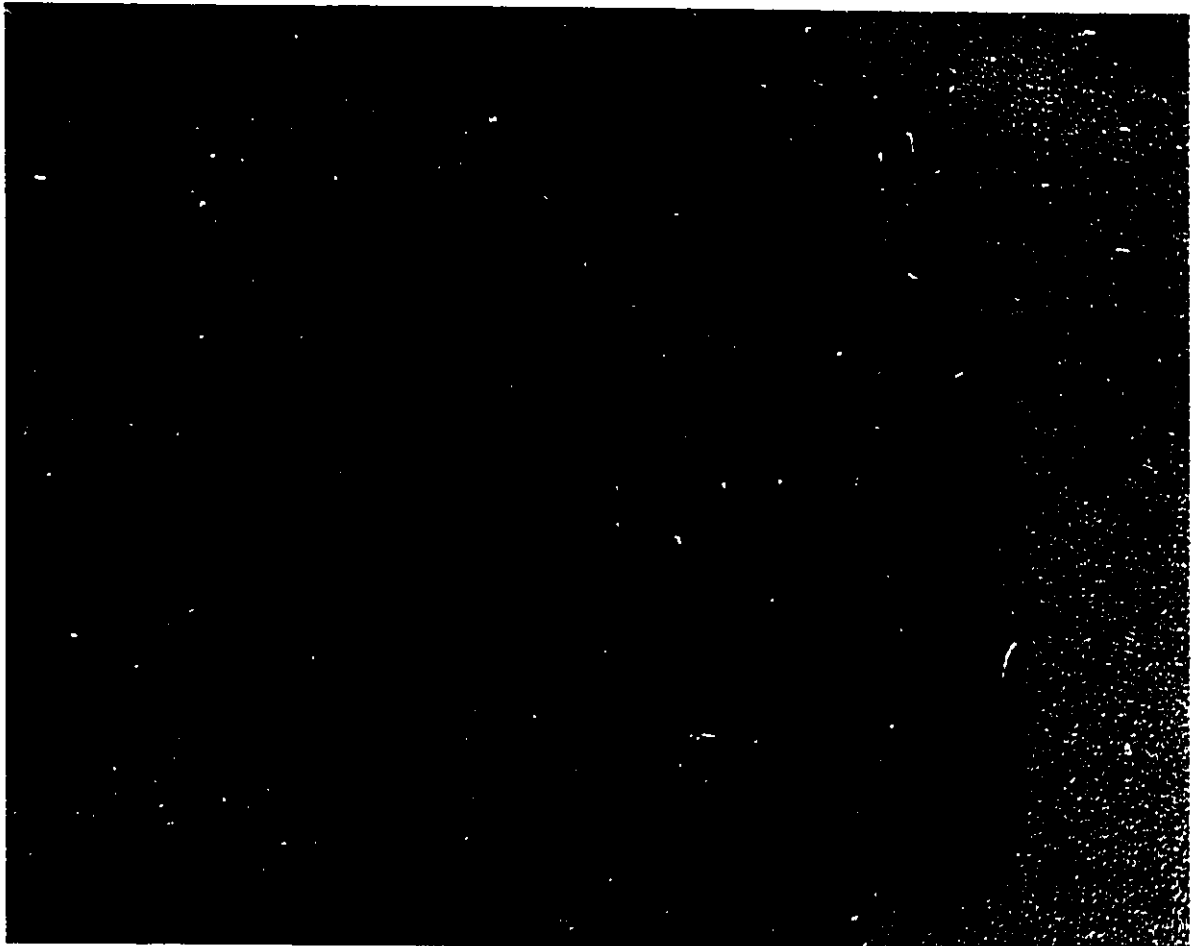


Figure 33. Photograph of mono- and binucleate C3H 10T1/2 cells after cytochalasin B treatment, fixation and staining as described in Materials and Methods (400 X magnification)

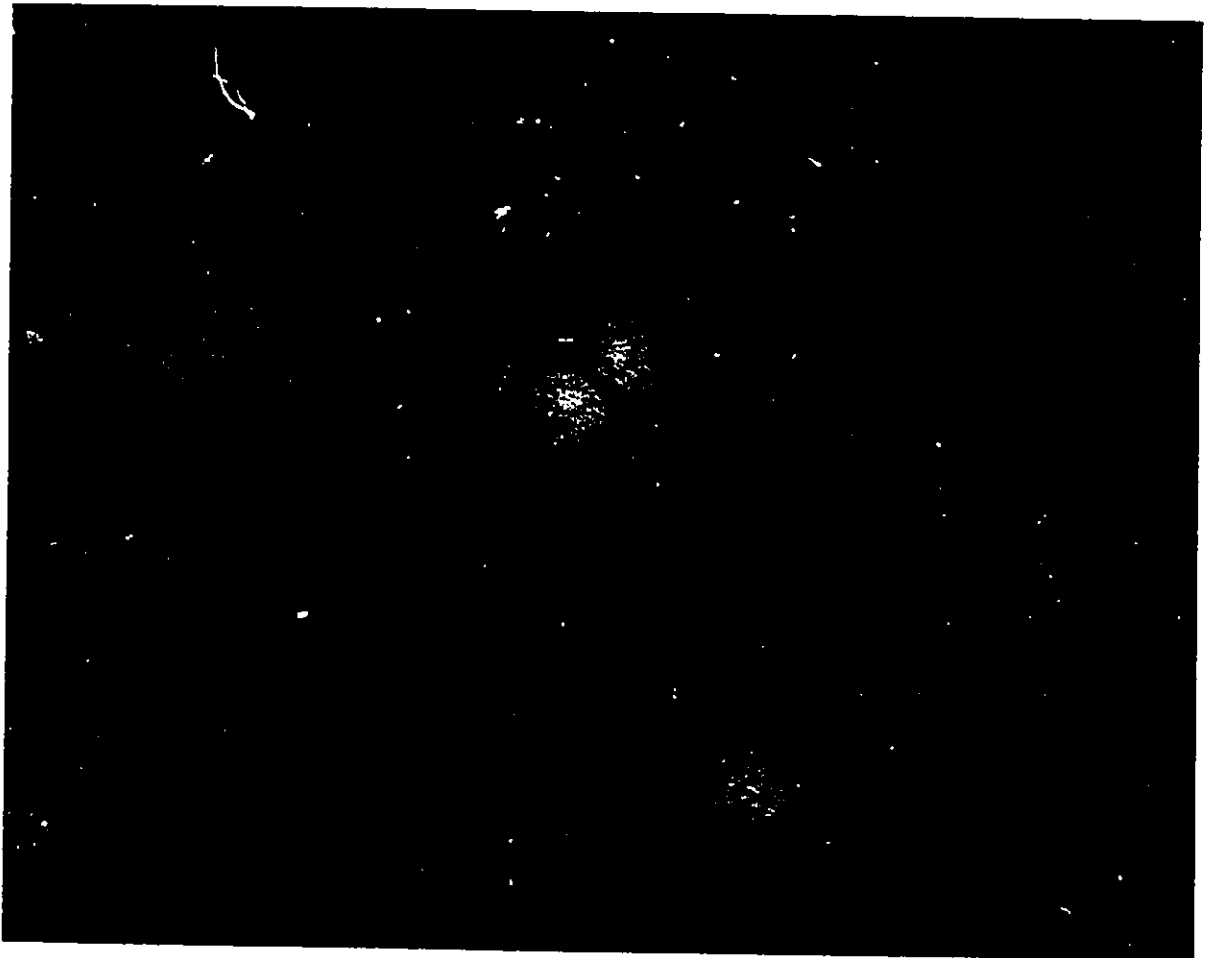


Table 8. Effect of an adapting dose on radiation-induced micronucleus formation in plateau-phase C3H 10T1/2 cells

Treatment	% of binucleated cells with micronuclei			% of Binucleate Cells with > 2 Micronuclei Observed \pm S.D.
	Observed \pm S.D.	Expected	Significance	
	Experiment I			
Control	10.2 (1.3)			16.4 \pm 5.1
X (4Gy)	84.7 (3.4)			37.4 \pm 2.1
X \rightarrow	73.2 (2.9)			25.2 \pm 1.7
γ (0.65Gy)	17.0 (1.1)			10.3 \pm 1.9
$\gamma \rightarrow X$	81.4 (3.2)	91.6 \pm 3.8	NS	36.2 \pm 2
$\gamma \rightarrow X \rightarrow$	63.8 (2.3)	80.0 \pm 3.4	p < 0.01	16.8 \pm 1.2
	Experiment II			
Control	11.5 (0.75)			7.3 \pm 1.2
X (4Gy)	85.3 (2.3)			45.2 \pm 1.7
X \rightarrow	72.4 (2.2)			33.9 \pm 1.6
γ_1 (0.1Gy)	16.2 (0.73)			6.7 \pm 1.5
$\gamma_1 \rightarrow X$	81.5 (2.0)	90.0 \pm 2.5	p < 0.1	39.4 \pm 1.4
$\gamma_1 \rightarrow X \rightarrow$	70.2 (1.5)	77.2 \pm 2.4	p < 0.1	27.0 \pm 1.0
γ_2 (0.65Gy)	19.1 (0.80)			11.4 \pm 1.5
$\gamma_2 \rightarrow X$	81.9 (2.1)	92.9 \pm 2.5	p < 0.05	37.8 \pm 1.4
$\gamma_2 \rightarrow X \rightarrow$	72.8 (2.1)	80.0 \pm 2.4	p < 0.1	30.0 \pm 1.4
γ_3 (1.5Gy)	24.0 (1.0)			9.8 \pm 1.2
$\gamma_3 \rightarrow X$	84.9 (2.3)	97.8 \pm 2.6	p < 0.01	47.0 \pm 1.6
$\gamma_3 \rightarrow X \rightarrow$	70.9 (1.3)	85.0 \pm 2.5	p < 0.001	29.9 \pm 0.8
	Experiment III			
Control	5.49 (0.54)			16.5 \pm 4.2
X (4Gy)	85.1 (4.0)			36.5 \pm 2.4
X \rightarrow	62.4 (2.9)			17.8 \pm 1.7
γ (1.5Gy)	14.1 (1.2)			5.5 \pm 1.9
$\gamma \rightarrow X$	69.3 (3.3)	93.7 \pm 4.2	p < 0.01	27.7 \pm 2.2
$\gamma \rightarrow X \rightarrow$	60.0 (2.8)	70.9 \pm 3.2	p < 0.1	14.9 \pm 1.5

X: X irradiation at 2 Gy/min
 \rightarrow : incubation at 37°C for 3.5 h
 γ : ^{60}Co irradiation at 0.0024 Gy/min

Table 9. Induced division delay: Percentage of C3H 10T1/2 cells reaching the binucleate state 48 hours following exposure to 1.5 Gy (0.0024 Gy/min) and/or 4 Gy (2 Gy/min).

Treatment	% of Binucleate Cells
Control	46.2 ± 2.5
X _{4Gy} (acute)	21.3 ± 1.5
X →	24.0 ± 1.7
γ 0.15Gy (chronic)	45.3 ± 1.8
γ → X	11.0 ± 1.1
γ → X →	14.0 ± 1.3

→: Incubation at 37°C for 3.5 h

Figure 34. Shift in distribution of micronuclei in radiation-adapted C3H 10T1/2 cells. Plateau-phase cells were exposed to an acute 4 Gy dose (2 Gy/min), or to a chronic 1.5 Gy dose (0.0024 Gy/min) followed 3.5 h later by a 4 Gy acute dose.

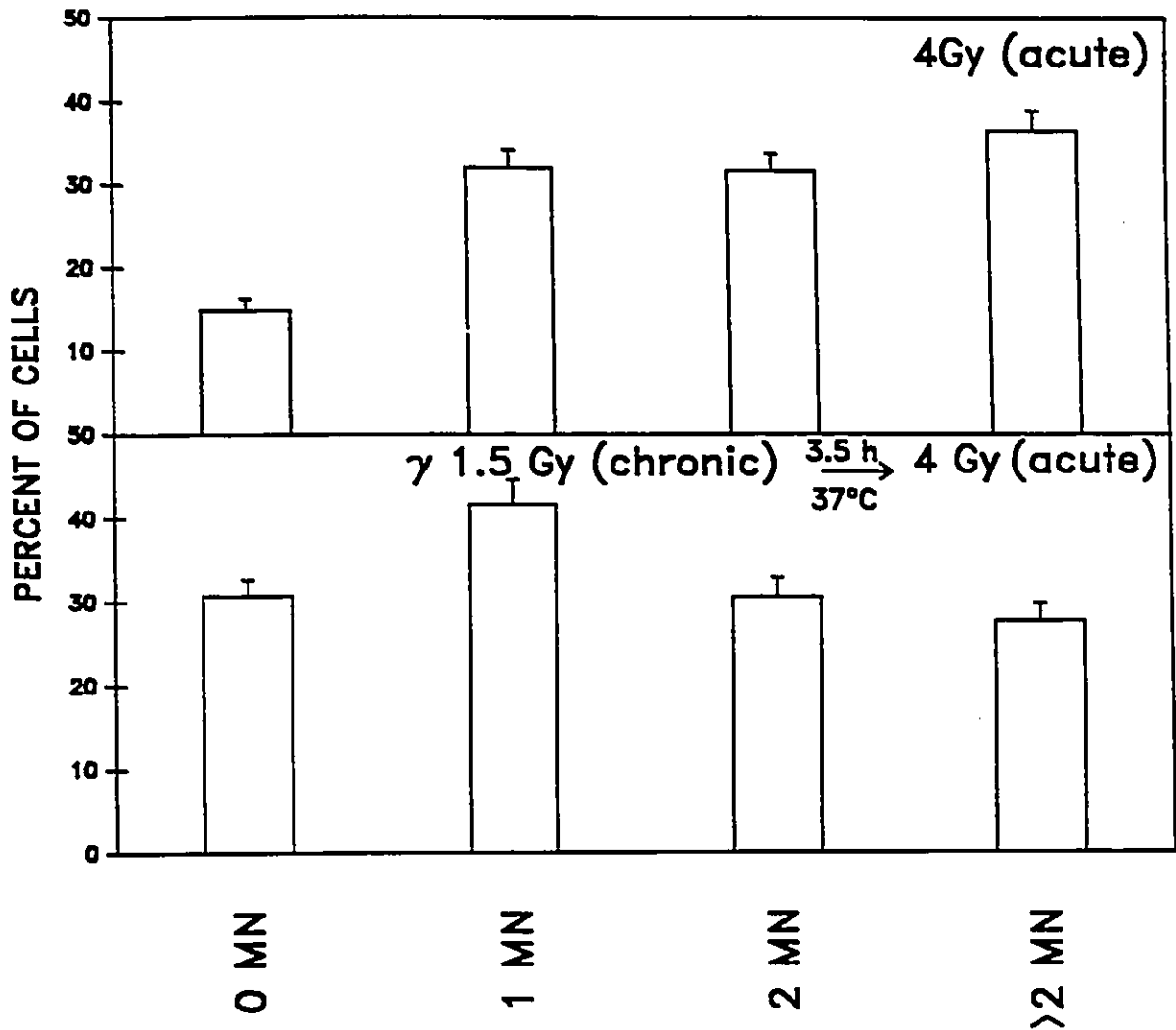


Figure 35. Shift in distribution of micronuclei in radiation-adapted C3H 10T1/2 cells. Plateau phase cells were exposed to an acute 4 Gy dose (2 Gy/min) and incubated at 37°C for 3.5 h, or to a chronic 0.65 Gy dose (0.0024 Gy/min) followed 3.5 h later by a 4 Gy acute dose and subsequent incubation at 37°C for a further 3.5 h.

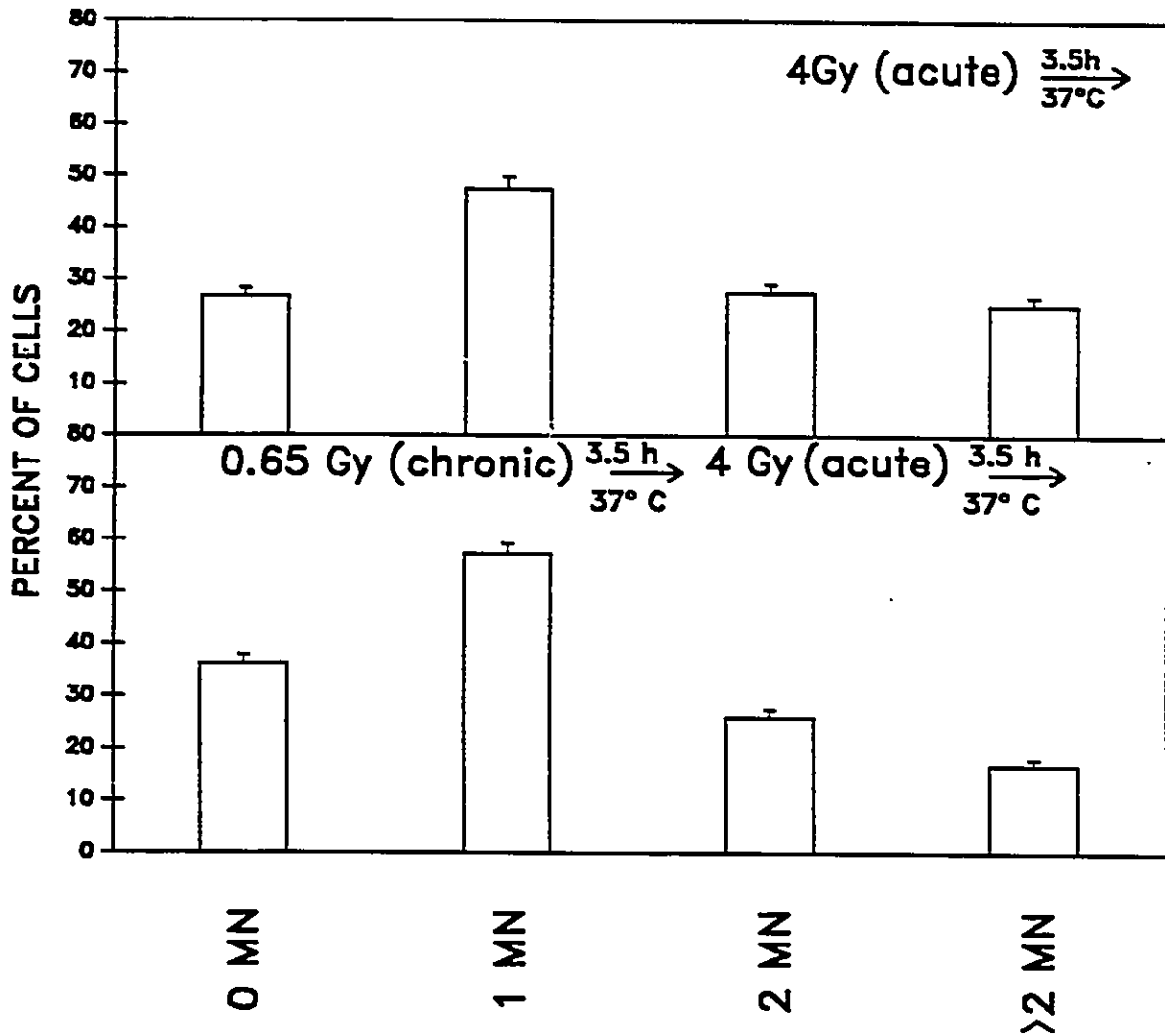
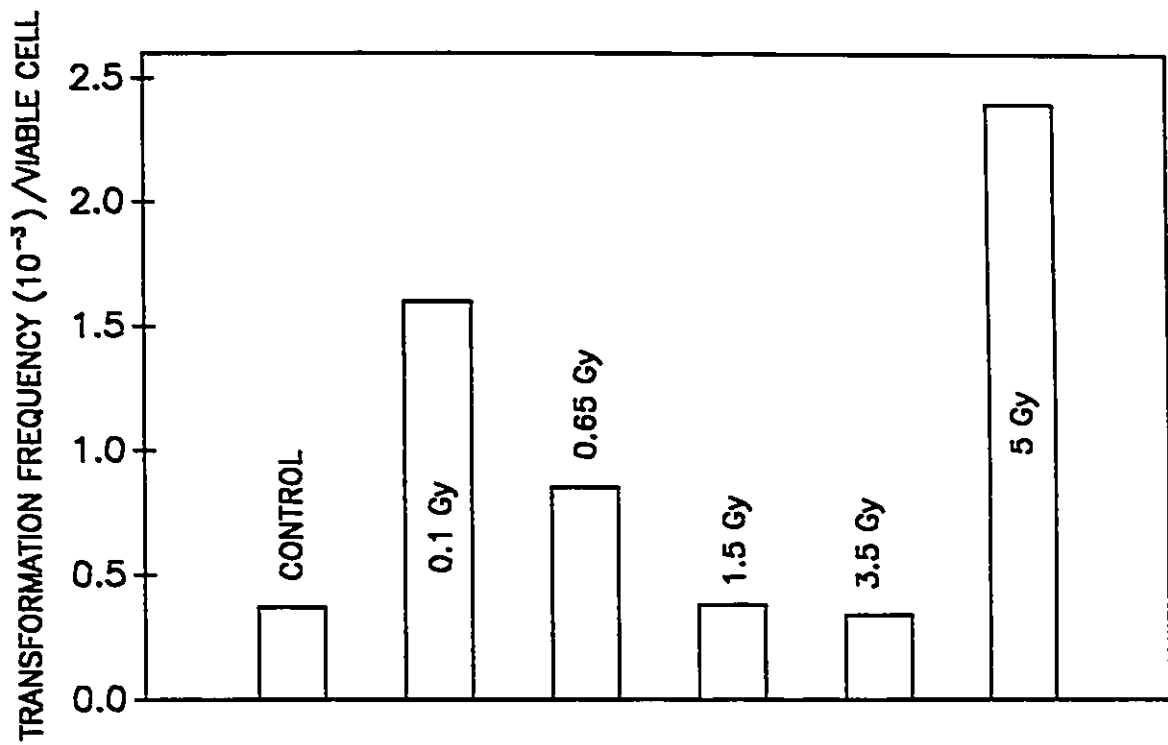


Figure 36. Transformation frequency per viable cell of plateau-phase C3H 10T1/2 cells exposed to γ -rays at low dose-rate (0.0024 Gy/min).



DISCUSSION

The data described in this chapter indicate that, in plateau-phase C3H 10T½ cells, a pre-exposure to low-dose-rate ionizing radiation can induce resistance to micronucleus formation and neoplastic transformation resulting from a subsequent acute dose of radiation. These data demonstrate that an AR to IR exists in these cells (Azzam et al. 1994a). The absence of an observable AR for cellular survival suggests that either cellular survival changes are too small to be detected reliably in this cell line, or that, as discussed previously, clonogenic survival is dependent on mechanisms different from or in addition to those leading to the AR observed for reduced neoplastic transformation and micronucleus formation. Other studies have addressed the question of whether one or more mechanisms lead to expression of the AR by measuring multiple biological end-points (Sanderson and Morley 1986, Rigaud et al. 1993, Shadley 1994). As reviewed, the results in general tend to support the presence of multiple mechanisms.

Similarly to our studies with AG1522 cells, the reduction in micronucleus formation and the shift in distribution of micronuclei in the case of pre-exposed C3H 10T½ cells, are consistent with the proposal that mechanisms preventing the formation and/or leading to repair of DNA dsb are enhanced as a result of pre-exposure to low-dose-rate-irradiation. Our data

indicate that the reduction in micronucleus frequency correlates with a reduction in the neoplastic transformation frequency. Several studies have demonstrated that radio-adapted cells are less susceptible to the mutagenic effects of IR (reviewed in Wolff 1992b, Shadley 1994). Moreover, Rigaud and Moustacchi (1994) have shown that pre-treatment with a low conditioning dose reduces the proportion of rearranged mutants due to a challenge dose. These studies along with the cell transformation results reported here suggest that an error-free process of damage repair may be enhanced by pre-exposure to an adapting dose of radiation, delivered in these studies at low-dose-rate. Experiments demonstrating that the adapting dose of radiation enhances the structural integrity of a gene(s) coding for a signal directly involved in the process leading to neoplastic transformation would confirm that the induced process(es) is error-free. A report by Coleman et al. (1994) on altered expression of the p53 tumor suppressor gene in morphologically transformed C3H 10T½ cells appeared in the course of writing this manuscript. Their study suggested that alteration of the expression of the p53 tumour suppressor gene may represent an early and necessary step in the morphologic transformation of 10T½ cells. Investigation of the effect of an adapting dose of radiation on such expression and on the mutation spectrum would be of interest.

The transformation frequency per viable cell due to a 4 Gy

dose was reduced by about 2-fold when the cells were pre-exposed to 0.1, 0.65 or 1.5 Gy adapting exposure. These data indicate that in the range of 0.1 to 1.5 Gy of low-dose-rate irradiation, the same protective effect against cellular transformation by a 4 Gy acute dose was induced. This result suggests that a maximum induction of the AR against cellular transformation occurs at these doses of radiation, and that adaptive responses less than maximum could occur at doses less than 0.1 Gy delivered at low-dose-rate (0.0024 Gy/min).

The data presented here confirm previous results (Raaphorst and Azzam 1986) showing that maintenance of cells in a quiescent state after a challenge dose, results in a decreased number of transformed cells. These data suggest the existence of repair processes analogous to the repair of sublethal or potentially lethal damage. Cellular transformation is likely a result of DNA damage and the repair of such damage, within a few hours as shown here, prevents the development of transformed foci. Exposure to an adapting dose of radiation, prior to the challenge dose, enhanced the rate of repair of such transforming damage.

The time between DNA damage and the next mitosis appears to be critical for expression of the transforming lesion. Proliferation after exposure can result in a higher number of transformed cells (Kennedy and Little 1984). This may be due to

the shorter time available for repair before the lesion is fixed during mitosis. As shown in table 9, the percentage of cells reaching the binucleate state was reduced when the cells were held in plateau phase following the challenge exposure. Even further reduction was observed when the cells were pre-exposed to a low-dose-rate adapting exposure prior to the challenge dose only, or the challenge dose followed by holding of the cells at 37°C for 3.5 h. These results suggest a greater division delay at G₁, S or G₂ phase of the cell cycle as a result of the adapting exposure. A molecular basis for cell cycle delays at these stages of the cell cycle following exposure to ionizing radiation has recently been described (Maity et al. 1994).

This ability to assay transformation frequencies of the order of 10⁻⁴ which can be produced or modulated by radiation doses of 0.1 Gy or less, provides a potentially useful in vitro model of the carcinogenic process with a sensitivity level adequate to contribute greatly to the problems of establishing human radiation safety standards (Hall and Miller 1981, Sinclair 1987). Epidemiological studies were not able to contribute in a definitive manner to the estimate of the risk of low doses of radiation (reviewed in Tubiana et al. 1990). The application of transformation assays provided few useful data for establishing human radiation safety standards, and unfortunately has also revealed paradoxical responses which have been difficult to explain

or to reproduce from laboratory to laboratory (reviewed in Calkins et al. 1991). While dose fractionation has been reported to result in a significant reduction in the neoplastic transformation frequency per viable cell (Hill et al. 1984), other reports indicated that incubation time between the fractionated X-ray doses led to increased neoplastic transformation (Miller et al. 1979). Clear differences exist between the various experimental protocols which traditionally used asynchronous cell populations and high dose-rate irradiations. Considering the conflicting results, two repair processes may possibly be involved in the repair of potentially neoplastic damage: one error-free and one error-prone. Our experimental treatments which were done on density-inhibited cells and where the adapting dose was delivered at low dose-rate generated data which suggest that, under these conditions, the adapting dose may enhance error-free repair. Molecular data are emerging whereby the expression of various genes is regulated following exposure to the same dose of radiation delivered at high or low dose-rate (de Toledo unpublished results). Different rates of energy deposition during an adapting pre-exposure may modulate differently the expression of genes involved in the response to oxidative stress. Alternate signal transduction pathways resulting in altered regulation of progression in the cell cycle, changes in chromatin conformation, antioxidation, induction or enhancement of already existing repair processes or apoptosis can modulate differently the various steps involved in carcinogenesis. The

result of such induced processes may be an enhanced or reduced transformation frequency due to a challenge dose of radiation. If an enhanced error-free repair were to be generally true in human cells, the results reported here would have important implications for the estimation of cancer risk resulting from multiple exposures to ionizing radiations. Caution has to be exercised in extrapolating neoplastic transformation data from rodent cells to humans, as studies indicate that the *in vitro* transformation process of rodent and human cells is different (reviewed in Kuroki and Huh, 1993). While rodent cells are easily transformed, *in vitro*, into malignant cells by exposure to carcinogens or transfection with oncogenes, this is a rare event with human cells where immortalization is a prerequisite for malignant cell transformation. The embryonic origin of the C3H 10T½ cells is of particular importance as the fate of cells derived from whole embryos is governed by specific cellular interactions which are highly regulated (reviewed in Alberts et al., 1989, chapter 16). The effects of radiation on these cells may be considerably different from the effects on differentiated cells.

In chapter 1, AG1522 cellular survival data showed an enhanced radiosensitivity at very low doses of radiation, followed by reduced rates of killing at higher doses. Similarly, the data in figure 36 show a maximal incremental rate of transformation at low doses of radiation delivered at low dose-rates. Within the

range of 0.1 Gy and 3.5 Gy the frequency of transformation was reduced as the dose was increased. These results could also be interpreted in terms of induction of an adaptive response following a dose of radiation of 0.1 Gy or less, at which dose enough damage is present to signal the induction of a radio-protective mechanism. The data indicate that with increasing doses at low dose-rate, the residual amount of expressed transforming damage per unit time is lower. The greater the dose, the more time is available for a radio-induced process to be expressed. The induced process is possibly able to protect the cells against subsequent events and to repair the damage caused by the previous events. The effect of such an induced radioprotective process was maximal following a dose of about 3.5 Gy delivered over 24 hours. The transformation frequency per viable cell following this latter dose was similar to background. At greater doses, the induced mechanism may have been saturated, and a net resulting transforming damage was expressed. These results are consistent with the report of Calkins et al. (1991) where the transformation frequency in C3H 10T½ cells was observed to plateau or diminish abruptly at relatively low dose levels and then increase with increasing doses, but at a reduced incremental rate. Calkins et al. (1991) had postulated that this discontinuity in the dose-response relationship corresponds to the induction of a repair system not functional below an induction threshold dose.

The process of oncogenic transformation is a multistep process where the initiating and promoting events are not well understood (reviewed in Tubiana et al. 1990, Cohen and Ellwein 1991). The process likely involves the activation of an oncogene(s), suppression of an anti-oncogene(s), or abnormal expression of growth factors/growth factor receptors. Such a process may occur by a mutational step, and irradiation at low dose-rate seems to protect against such events in the C3H 10T½ cell line maintained according to the culture conditions described previously. Further research using the transformation assay described here makes possible the study of the effects of radiation on initiation and promotion. *In vivo* animal studies by Mitchel and Trivedi (1992), and, Mitchel and McCann (1994) have shown that fractionated β irradiation can act not only as a tumor promotor of initiated cells but also is capable of acting as a tumor promotor when delivered prior to the initiating step. Clearly, much research is still needed before such studies can have a concrete impact on the field of human radioprotection. However, our results clearly suggest that the deleterious effects of consecutive doses of radiation are not always additive.

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APPENDIX 1THE USE OF SEMI-QUANTITATIVE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION TO STUDY GENE EXPRESSION IN NORMAL HUMAN SKIN FIBROBLASTS FOLLOWING LOW DOSE RATE IRRADIATION

In the course of this thesis, the existence of an adaptive response to ionizing radiation as revealed by different biological end-points was reviewed. Also, evidence that pre-exposure of normal human skin fibroblasts (AG1522) to a low-dose-rate irradiation renders these cells more radioresistant to the effects of a challenge exposure of high dose rate X-irradiation was presented (Azzam et al. 1992, 1994). Repair of DNA damage, cell cycle regulation and antioxidation have been postulated to influence the cellular response to ionizing radiation injury. However, the molecular mechanisms responsible for these effects, except antioxidation, are unknown (Datta 1992). For this study, we decided to monitor changes in the levels of messenger RNAs of genes involved in these processes following exposure to an adapting dose of radiation. It was hoped that modulation of the expression of these genes as a result of the adapting exposure may help to understand the mechanisms underlying the AR to IR. Oligonucleotides for genes possibly involved in the above processes and in the general response to radiation were synthesized and used to determine their respective transcript levels by reverse transcription - polymerase chain reaction (RT-PCR) analysis. We report here the effect of ionizing radiation on the expression of specific genes in AG1522 cells exposed to a 3.6 Gy adapting dose (0.0024 Gy/min).

The combined use of reverse transcription followed by polymerase chain reaction (RT-PCR) to detect and measure RNA levels has been extensively described under different names (reviewed in Larrick 1992). The exponential nature of the PCR amplification step confers a very high sensitivity to this method compared to traditional RNA analysis. The procedure requires only a few hours to be performed and less manipulation of the samples, as total RNA can be used. Protocols using internal standards have been developed in order to obtain quantitative measurements. The use of known amounts of an internal standard in competition with the target sequences makes it possible to determine the absolute amount of the target cDNA (Becker-André and Hahlbrock 1989, Gilliland et al. 1990, Siebert and Larrick 1992). This is feasible only when few genes are being analyzed, as the construction of specific standards for each gene is labour intensive. However, mRNA levels can be semi-quantified without the use of internal standards if the PCR product is measured in the exponential phase of the reaction (Murphy et al. 1990).

Recent studies of environmental stresses that produce physical and chemical damage to cells suggest that one common feature of the stress response is, partly, the induction or activation of proteins that protect the cell against specific external insults. Examples include the induction of heat shock proteins, which confer resistance to killing by heat, and induction by cadmium of proteins that reduce metal toxicity (reviewed in

Weichselbaum et al. 1991, Fornace 1992). However, little is known about induction by ionizing radiation of proteins that may protect against cell killing or mutagenesis. A number of possible regulatory mechanisms by which mammalian cells may control gene expression after ionizing radiation have been suggested (Sahijdak et al. 1994). They include binding of regulatory proteins to gene promoters or RNA polymerase, post-transcriptional modification, alterations in rRNA processing and post-translational modification. In recent years, specific genes have been shown to be induced or repressed by specific agents including various stresses (Fornace 1992). Initially, the immediate responses of cells were studied following supralethal doses of ionizing radiation (e.g. Sherman et al. 1990). While this project was in progress, the early transcriptional responses of cells following exposure to low doses of radiation were reported (Woloschak et al. 1990). In this study, the mRNA levels of genes possibly involved in the response to ionizing radiation were measured for the first time following exposure to an adapting dose of radiation delivered at low dose-rate. The endpoints of cell survival and micronucleus formation following the challenge of these cells with an acute dose of X-rays were simultaneously monitored.

As numerous genes are potentially involved in the multiple transduction pathways postulated to be induced by IR, a rapid semi-quantitative method to analyze as many genes as possible from a limited quantity of cell sample might be a method of choice.

MATERIALS AND METHODS

Cell Culture and Irradiation

AG1522 normal human skin fibroblasts were cultured and exposed to low dose-rate irradiation as previously described in chapter 1. The experiments were done with cells in passage 10 or 11. For the low dose rate irradiation, the cells were exposed at 37°C to 3.6 Gy from a ^{60}Co gamma beam 150 irradiator (Atomic Energy of Canada Ltd.) delivered at a dose rate of 0.0024 Gy/min. Cell cycle analysis indicated that about 94% of the cells were in G_0/G_1 , and less than 1.5% in S phase.

RNA Extraction

The cellular monolayers were rinsed with phosphate-buffered saline and RNA was extracted by the guanidinium thiocyanate phenol-chloroform method (Chomczynski and Sacchi 1987). The precipitate was collected, washed with 70% ethanol, resuspended in 10mM Tris, 1mM Na_2EDTA and 0.3 M sodium acetate pH 4.5, re-extracted with phenol-chloroform, followed by chloroform, and then precipitated with ethanol. The resulting pellet was resuspended in 10mM Tris pH 7.4 and 5mM MgCl_2 , and treated with 5 U/ μL of Rnase-free Dnase I (GibcoBRL) at 37°C for 30 min. After phenol-chloroform and chloroform extractions, ethanol/sodium acetate precipitation and centrifugation, the resulting RNAs were resuspended in water and

their concentration adjusted to 2 $\mu\text{g}/\mu\text{L}$.

Northern Analysis

Equal amounts of total RNA were electrophoresed in 1.3% agarose, 2.2 M formaldehyde gels (Sambrook et al. 1989) and transferred to a nylon membrane (ZetaProbe-BioRad or Flash-Stratagene). After pre-hybridization for 2-4 h at 65°C in 5x SSPE (0.75 M NaCl, 50 mM NaH_2PO_4 , 5mM EDTA) pH 7.2, 7% sodium dodecyl sulfate and 100 $\mu\text{g}/\text{mL}$ of denatured salmon sperm DNA, the blots were hybridized for about 17 h in the same solution containing 1-2x10⁶ dpm/mL of a ³²P-labelled probe. The blots were washed at 65°C in 1x SSC (150mM NaCl, 15mM sodium citrate), 0.1% sodium dodecyl sulfate and autoradiography was carried at -70°C using XAR-5 Kodak films.

RT-PCR Analysis

Semi-quantitative RT-PCR determines the relative amounts of transcript levels in different samples. This analysis is carried out during the exponential phase of the reaction which is characterized by the doubling of the amount of PCR product in each amplification cycle.

Reverse Transcription (RT)

Total RNA (5 μg or less) was reverse transcribed in a 5 μL

reaction for 1 hr at 37°C using 1 ng/ μ L oligo(dT)₁₂₋₁₈ primer, 10 U/ μ L of M-MLV reverse transcriptase (GibcoBRL) in 50mM Tris pH 8.3, 75mM KCl, 3mM MgCl₂, 10mM DTT (dithiothreitol), 5 U/uL RNAGuard (Pharmacia) and 50 μ M each of four deoxyribonucleoside triphosphates. In each experiment, controls for contamination were included for each RNA sample (C_{RT}) and for the solutions (S_{RT}). The RNA controls omitted the M-MLV reverse transcriptase and the solution control omitted the RNA. The resulting reaction was diluted to 25 ng/ μ L of RNA. In the case of RT reactions for the dilution curve (see next section), the reaction was diluted into 3 solutions of 50, 25 and 12.5 ng/ μ L. The amount of template for the PCR reaction is indicated as an amount of starting RNA, since the amount of cDNA formed in each experiment was not determined. In order to verify the efficiency of each RT reaction, the mRNA levels for the β -ACTIN gene were determined by RT-PCR, since we have determined by Northern analysis that β -ACTIN mRNA levels did not vary after the irradiation treatments.

Polymerase Chain Reaction (PCR)

Usually, cDNA template synthesized from 100 ng of RNA was amplified in a 25 μ L reaction mixture containing 10 pmol each of the 5' and 3' primers (Table 1) in 20mM Tris pH 8.3, 50mM KCl, the optimal MgCl₂ concentration (Table 1), 50 μ M each of four deoxynucleoside triphosphates, 100 μ g/mL gelatin and 1 U of Taq DNA polymerase (Amplitaq Perkin-Elmer Cetus). The product was labelled

by adding 1-5 μM of biotinylated-14-dATP. The reaction mix, without Taq DNA polymerase, was overlaid with light mineral oil (Sigma), heated at 95°C for 5 min and held at 80°C. Taq DNA polymerase was then added to the reaction. A Programmable Thermal Cycler (MJ Research) was used to carry amplifications in sequential cycles of 95°C for 60s, 55°C for 60s and 72°C for 60s. The required number of cycles varied for each gene (Table 1), and was established based on obtaining maximum amount of the PCR product while still in the exponential phase of the PCR. For each gene analyzed, a serial dilution curve was produced simultaneously, in order to monitor the exponential formation of the product. This dilution curve was generated from the RT reaction from RNA of control cells. In each PCR analysis, duplicates were run for experimental RT reactions (E_{RT}) and for the serial dilution curve. Single PCR reactions were run for the control RT (C_{RT}).

Electrophoresis, Blotting and Densitometry

A 4 μL sample of the PCR reaction was electrophoresed on a 1.5% agarose gel containing 0.2 $\mu\text{g/mL}$ of ethidium bromide, and then transferred to a membrane (Flash-Stratagene) by Southern blotting (Sambrook et al. 1989). After U.V. crosslinking, the membrane was submitted to chemiluminescent detection (Flash-Stratagene with Kodak XAR-5 films). The autoradiograms were analyzed by scanning densitometry using a StrataScan 7000 scanning system (Stratagene).

RESULTS

Levels of Gene Transcripts After Low Dose Rate γ -irradiation

Panel A in figure 1-1 shows the effect of 3.6 Gy of ^{60}Co γ -rays delivered at 0.0024 Gy/min on the transcript levels of some of the genes tested and panel B shows the corresponding serial dilution analysis. No changes were observed in the transcript levels for the genes: β -ACTIN, β -MICROGLOBULIN, SOD-1, SOD-2, CATALASE, GLUTATHIONE PEROXIDASE, C-JUN, JUNB, JUND, XRCC1, URACYL DNA GLYCOSYLASE, β -DNA POLYMERASE, TOPOISOMERASES I and IIB and CYCLINS C, D1, D2, D3 and E. However, increased levels for the GADD45 transcript and decreased transcript levels for the genes: TOPOISOMERASE II α , FACC-1 and CYCLINS A and B were observed.

Semi-quantification Analysis of Transcript Levels

A more detailed quantification of the transcripts that showed different expression levels and of the control gene β -ACTIN is shown in Figure 1-2. Serial dilutions of RT reactions corresponding to 6.25, 12.5, 25 and 50 ng of RNA from control and ^{60}Co -irradiated cells were analyzed by RT-PCR. Densitometric analysis of the autoradiograms showed that the levels for: CYCLIN A decreased 4 fold, CYCLIN B decreased 2 fold, TOPOISOMERASE II α decreased 4 fold, FACC-1 decreased 3 fold and GADD45 increased 2 fold. The results for β -ACTIN and GADD45 transcripts were also confirmed by Northern analysis (results not shown).

DISCUSSION

We observed that the treatment of normal human fibroblasts with 3.6 Gy of ^{60}Co - γ rays, delivered at low dose rate, affects the transcript levels of some genes. An increase (2 fold) was observed for *GADD45* and decreased levels were observed for the genes *CYCLIN A* (4 fold), *CYCLIN B* (2 fold), *TOPOISOMERASE II α* (4 fold), and *FACC-1* (3 fold). The modulation in the transcript levels of these genes by low dose rate irradiation has not been previously reported.

The cyclins are proteins whose levels vary throughout the cell cycle. They are responsible for conferring activity upon their associated partners, protein kinases (cdks), at the appropriate time during the cell cycle. Cyclin A plays a role in the execution of S phase as well as during the G_2 -M phase transition (Pagano et al. 1992). Cyclin B is involved in the control of the G_2 -M transition (reviewed in Xiong and Beach 1991). In mammalian cells three different classes of cyclins (C, D and E) are expressed in the G_1 phase (reviewed in Sherr 1993). The role of cyclin C is not clear yet but cyclin D1 and cyclin E seem to be involved in the G_1 to S phase transition (Resnitzky et al. 1994). Ionizing radiation treatments lead to cell cycle delays in G_1 and G_2 phases, and decreased levels of cyclin A protein and cyclin B protein and mRNA have been observed in G_1 and in G_2 arrested cells respectively

(Dulic et al. 1994, Mushel et al. 1991).

The decreased levels for the CYCLIN A and CYCLIN B transcripts observed in plateau phase AG1522 cells after irradiation may indicate that even in quiescent cells, ionizing radiation is able to induce a process whereby further down regulation of these cyclin transcript levels is achieved, probably leading to or as a consequence of repositioning of the cells at a different stage in G_0 - G_1 . This repositioning in G_0 - G_1 did not affect the regulation of the transcript levels of the G_1 cyclins (C, D1, D2, D3 and E). Also, the decreased levels of these transcripts may result from an enormous reduction in the levels of these messages only in the few cells in S or G_2 -M phases, resulting in an overall reduction of only 2-4 fold in the whole cell population analyzed. Only *in situ* experiments can differentiate between these two possibilities.

DNA Topoisomerases are a unique class of enzymes regulating DNA topology and therefore have been postulated to participate in many cellular processes including DNA repair (Stevnsner and Bohr, 1993). Type I topoisomerase makes transient single-strand breaks in DNA, its activity is stable during the cell cycle (Heck et al. 1988) and it is a cofactor for RNA polymerase II (Kretzschmar et al. 1993). Type II topoisomerases make transient DNA double-strand breaks and two distinct proteins (Drake et al. 1989) coded for by

two different genes (Tan et al. 1992) exist in mammalian cells. *Topoisomerase II α* (p170), coded for by the gene in chromosome 17q21-22, predominates in proliferating cells. Its protein levels vary during the different phases of the cell cycle, being maximum in G₂-M and undetectable in G₀ (Woessner et al. 1991). The synthesis of this protein is suppressed in HeLa cells blocked in G₂ phase following irradiation (Goswami et al. 1992) and the radioprotector WR-1065 inhibits its activity (Grdina et al. 1994). *Topoisomerase II β* (p180), coded for by the gene in chromosome 3 is the predominant form in quiescent cells and the protein levels do not vary during the cell cycle (Woessner et al. 1991).

The observed decreased levels of the cell cycle regulated *TOPOISOMERASE II α* transcript, may also be a consequence of the effect of radiation in repositioning the cells in the G₀-G₁ phase as the *TOPOISOMERASE II β* transcript, which is not cell cycle regulated was not affected.

Increased levels of *GADD45* (Growth Arrest and DNA Damage) mRNA, after ionizing radiation treatments (Papathanasiou et al. 1991), occur via a p53-dependent DNA damage response pathway (Kastan et al. 1992, Zhan et al. 1993). In this pathway, the increase in the p53 protein levels is associated with the occurrence of DNA strand breaks (Nelson and Kastan 1994).

The tumour suppressor p53 protein is a sequence-specific DNA binding protein (Kern et al. 1991) that can inhibit TATA-mediated transcription (Mack et al. 1993) and also the transcription of *CYCLIN A* (Yamamoto et al. 1994) *in vitro*. It can also activate transcription as in the case of *GADD45* (Kastan et al. 1992, Zhan et al. 1993), *MDM2* (Barak et al. 1993, Price and Park 1994), and *WAF1/CIP1* (El-Deiry et al. 1993, Harper et al. 1993).

In mammalian cells exposed to DNA-damaging agents, such as ionizing radiation, the induction of the wild type p53 protein is critical for the regulation of the G₁ cell cycle checkpoint and apoptosis. The occurrence of one or another will depend on the type and physiological state of the cell (Slichenmeyer et al. 1993). Fibroblasts will undergo an induced G₁ arrest (Kastan et al. 1992) while hematopoietic cells will undergo apoptosis (Clarke et al. 1993, Lowe et al. 1993, Lee and Bernstein 1993). The loss of wild type p53 activity will confer radioresistance to hematopoietic cells, as they will not undergo apoptosis, but in fibroblasts and other cell types, the loss of the G₁ checkpoint is not sufficient to alter their radiosensitivity (Slichenmeyer et al. 1993).

The p53-dependent G₁ arrest caused by ionizing radiation, has been correlated with a lack of activation of cyclin E-Cdk2 and cyclin A-cdk2 kinases (Dulic et al. 1994). The inactivation of the cyclin E-Cdk2 kinase is due to the binding of the p53 inducible

inhibitor WAF1/CIP1 to the complex. In the case of cyclin A-Cdk2 kinase, the inactivation occurs due to a decrease in the levels of cyclin A protein.

The increased levels in *GADD45* transcripts in normal human fibroblasts after low dose irradiation should be the consequence of a cell growth arrest and/or DNA damage via the p53 pathway which possibly is down regulating the *CYCLIN A* transcript also.

FACC-1 is a defective gene in Fanconi's anemia complementation group C (Strathdee et al. 1992). These cells are hypersensitive to DNA damage and it was postulated that *FACC-1* has a role in the protection against, or repair of, DNA damage (Strathdee and Buchwald 1992, Gordonsmith and Rutherford 1991).

The observed transcript level changes occurred after a low dose rate irradiation of plateau phase AG1522 which rendered these cells more radioresistant (adaptive response) to a second challenging dose (Azzam et al. 1992, 1994). Whether these changes are just a consequence of the radiation damage or they effectively participate in the adaptive response remains to be established.

Table 1-1 Primer Sequences, optimal Mg²⁺ concentration, product size and number of PCR cycles for the different genes

Gene	Primers	Mg ²⁺ mM	Size bp	Cycles
<i>β</i> -ACTIN	(5') ATTGCCGACAGGATG (3') CTTGCTGATCCACATCT	2.0	152	19
<i>β</i> -MICROGLOBULIN	(5') ACCCCCCACTGAAAAAGATGA (3') ATCTTCAAACCTCCATGATG	1.5	120	19
SOD-1	(5') GACTTGGGCAATGTGA (3') AATTACACCACAAGCCAA	1.5	200	21
SOD-2	(5') TCTTCAGCCTGCACTGAA (2') CCCAGTTGATTACATTCC	2.5	382	24
CATALASE	(5') TACTACCCCAACAGCTT (3') GCATTCTTAGGCTTCT	1.5	313	25
GLUTATHIONE PEROXIDASE	(5') GAAGAGATTCTGAATTC (3') CACCAGGAACCTTCTCAA	2.5	240	25
C-JUN	(5') AAGCGCATGAGGAACCG (3') TTTGCAACTGCTGCGTTA	2.5	204	25
JUNB	(5') AACATGGAAGACCAGGAG (3') GAAGGCGTCTCCCTTGAC	1.5	258	26
JUND	(5') AAATGCCGCAAGCGCAA (3') TGGCTGAGGACTTTCTG	2.0	142	28
GADD45	(5') GAAGACCGAAAGGATGG (3') GGGAGATTAATCACTGG	1.5	455	28
FACC-1	(5') AGAGAGCACAGACTATG (3') GCCAAGACGATTCCA	1.5	302	32
XRCC-1	(5') ACCATCTCTGTGGTCCTACA (3') TTCACACGGAACCTGGCCAAG	1.0	400	28
URACYL DNA GLYCOSYLASE	(5') CAGAACTCGAATGGCCTTG (3') TTAACAGCAGCTTCTCA	2.0	251	29
<i>β</i> -DNA POLYMERASE	(5') GAGACAAAGTTCATGGGT (3') GTGTACTCATTGATTGT	2.0	196	28
TOPOISOMERASE I	(5') GAACAGCTAGCAGATGC (3') CTCATAGTCTTCATCAG	1.5	330	27
TOPOISOMERASE II α	(5') AAGATGATCACACGTTG (3') TGTAGAATTAAGAATAGC	2.5	300	27
TOPOISOMERASE II β	(5') GTAACAACATTAACTTGC (3') GTTCTGTCCACTACAAA	1.5	423	25

<i>CYCLIN A</i>	(5') CAGACATCACTAACAGTA (3') AGGCTGCTGATGCAGAA	2.0	361	28
<i>CYCLIN B</i>	(5') GAAGTCACTGGAAACATG (3') AGTATGTTGCTCGACATC	2.0	478	25
<i>CYCLIN C</i>	(5') TCCCCACTATTTGCAATGG (3') TGCAAGGGGAAGCAACAT	2.0	493	26
<i>CYCLIN D1</i>	(5") AGGAGAACAAACAGATCA (3') TAGGACAGGAAGTTGTTG	2.0	162	25
<i>CYCLIN D2</i>	(5') TCATGACTTCATTGAGCA (3') CACTTCCTCATCCTGCTG	2.0	195	30
<i>CYCLIN D3</i>	(5') ACATGATTCCTGGCCTT (3') TGAGCTCATCCCCGACA	2.0	220	28
<i>CYCLIN E</i>	(5') AATAGAGAGGAAGTCTGG (3') AGATATGCAACCTGCATG	2.0	440	30

FIGURE 1-1 Messenger RNA levels of different genes in AG1522 cells exposed to 3.6 Gy ^{60}Co γ -rays. Human fibroblast cells in plateau phase were exposed to 0 or 3.6 Gy of γ -rays at 0.0024 Gy/min. RNA was extracted and analyzed by RT-PCR as described in Material and Methods. (A) Cells exposed to 0 or 3.6 Gy of γ -rays. (B) Serial dilution curve from 200, 100 and 50 ng of RNA from control cells. E: experimental RT reaction; C: control RT reaction.

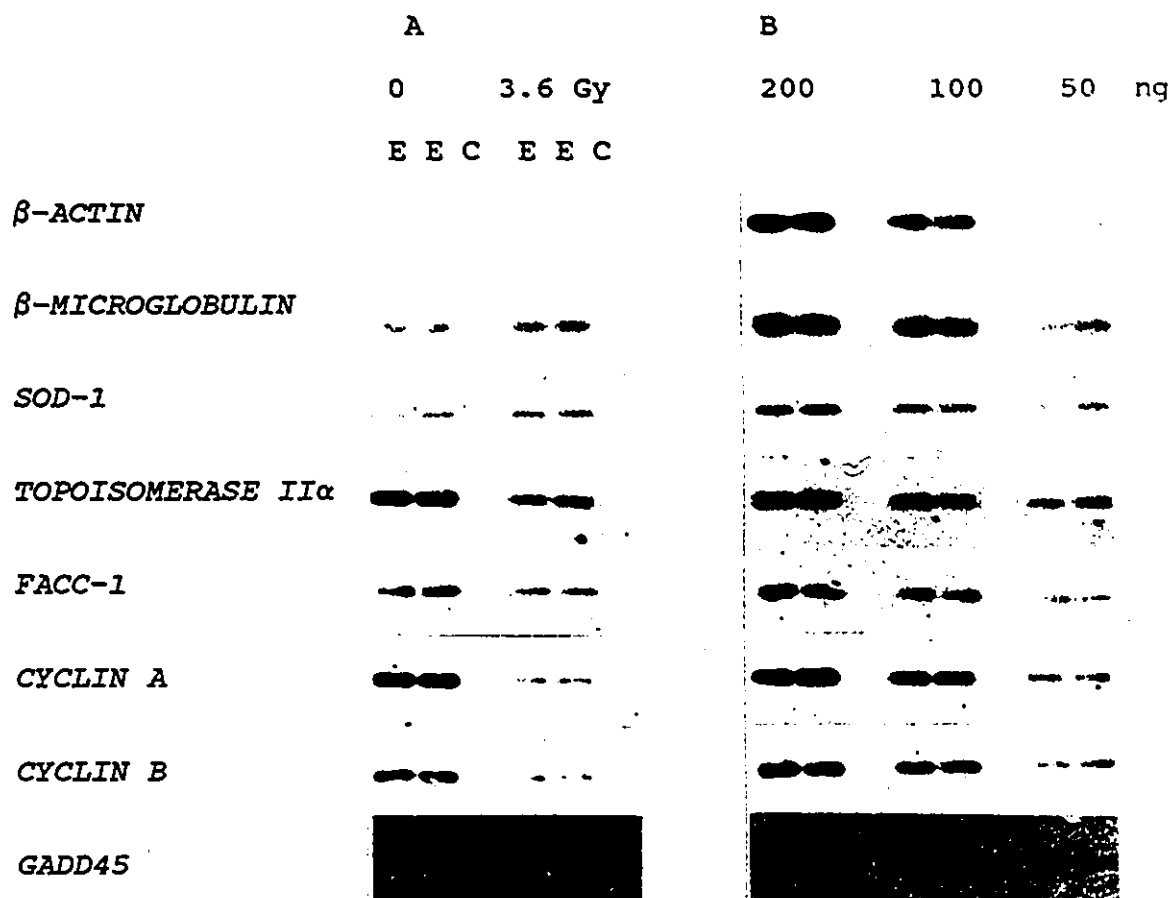
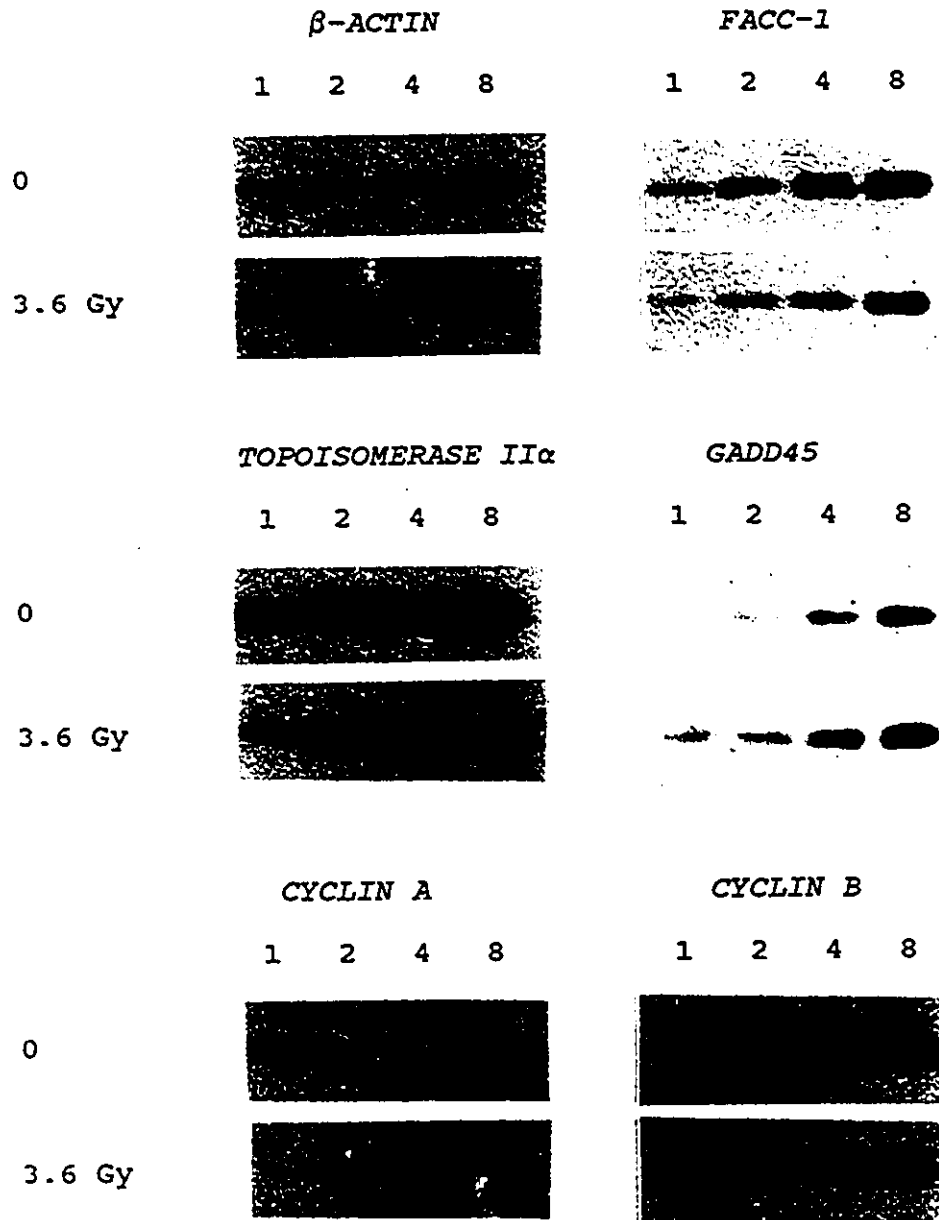


FIGURE 1-2 Analysis of expression levels of radiation modulated genes in AG1522 cells. Serial dilutions of the RT reactions corresponding to 25, 50, 100 and 200 ng (1,2,4 and 8x respectively) of total RNA from 0 and 3.6 Gy γ -irradiated cells were analyzed by RT-PCR.



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APPENDIX 2List of AbbreviationsUnits of measure

A	-	angstrom
°C	-	degree centigrade
cGy	-	centigray
dl	-	deciliter
Gy	-	Gray (1 Gy = 1 J/kg = 100 rad)
h	-	hour
kDa	-	kilodalton
keV	-	kiloelectron volt
kV _p	-	kilovolt peak
LD ₅₀	-	Dose to produce lethality in 50% of the subjects
LET	-	linear energy transfer
mA	-	milliampere
mGy	-	milligray
min	-	minute
ml	-	milliliter
mm	-	millimeter
mM	-	millimolar
MΩcm	-	mega-ohm-centimeter
N	-	normal (1 equivalent/liter)
nm	-	nanometer
ppm	-	part(s) per million
μCi	-	microcurie
μg	-	microgram
μGy	-	microgray
μm	-	micrometer

Miscellaneous

ADN	-	acide désoxyribonucléique
AR	-	adaptive response
ARN	-	acide ribonucléique
ADP	-	adenosine diphosphate
AT	-	ataxia telangiectasia
BCNU	-	1,3-bis (2-chloroethyl)-1-nitrosourea
BrdU	-	bromo-deoxy-uridine
cisplatin	-	cis-platinum (II) diamine dichloride
CO ₂	-	carbon dioxide
DF	-	1:1 mixture of Dulbecco's Modified Eagle Medium and Nutrient Mixture F12 (D-MEM/F12)
DNA	-	deoxyribonucleic acid
dsb	-	double-strand break
ERCC	-	excision-repair cross-complementing rodent repair deficiency
e ⁻ aq	-	aqueous electron

et al.-	et alii
<i>E. coli</i>	<i>Escherichia coli</i>
e.g. -	exempli gratia
EMS -	ethyl methanesulfonate
EPA -	Environmental Protection Agency
etc. -	et cetera (and so on)
FCS -	fetal calf serum
G ₀ -	cells that show no evidence of progressing through division for very long periods
G ₁ -	gap 1
G ₂ -	gap 2
H -	hydrogen radical
H ₂ -	molecular hydrogen
H ₂ O -	water
H ₂ O ₂ -	hydrogen peroxide
[³ H] -	tritium
[³ HdThd -	tritiated deoxythymidine
HPRT -	hypoxanthine phosphoribosyl transferase
IARC -	International Agency for Research on Cancer
IR -	ionizing radiation
M -	mitosis
MMC -	mitomycin C
MMS -	methyl methanesulfonate
MN -	micronucleus
mRNA -	messenger RNA
NCI -	National Cancer Institute
NaHCO ₃ -	sodium bicarbonate
No. -	number
NS -	non significant
·OH -	hydroxyl radical
O ₂ -	molecular oxygen
PBS -	phosphate buffered saline
PLD -	potentially lethal damage
PLDR -	potentially lethal damage repair
RH -	organic molecule
·RH -	organic free radical
RNA -	ribonucleic acid
RNase -	ribonuclease
S -	phase of cell after G ₁ period and prior to G ₂ period when DNA is duplicated
Sc -	<i>Saccharomyces cerevisiae</i>
SLD -	sublethal damage
ssb -	single-strand break
TdR -	thymidine deoxyriboside
UNSCEAR	United Nations Scientific Committee on the Effects of Atomic Radiation
UV -	ultra-violet
v/v -	volume to volume
w/v -	weight to volume
XPAC -	Xeroderma pigmentosum complementation group A