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The Adaptive Response: I. Assessing the Relative Biological Effectiveness of Low Doses of Tritium II. Determining its Effect on DNA Repair at a Chromosomal Level

Thesis submitted to the
School of Graduate Studies and Research
University of Ottawa
in partial fulfillment of the requirements for the
M.Sc. degree in the

Ottawa-Carleton Institute of Biology

Thèse soumise à
L'École des études supérieures et de la recherche
Université d'Ottawa
en vue de l'obtention de la maîtrise ès sciences

L'Institut de biologie d'Ottawa-Carleton

To my love, Terrie, without whose support this endeavour that sits before you would never have been.

Acknowledgements

I would first like to thank my supervisors, Dr. Ron Mitchel and Dr. David Brown, who believed in my abilities and gave me opportunity to pursue my goals and the guidance to achieve them-for that I am forever grateful.

To Shelley Maves whose humour, kindness and willingness to help knows no limits, I say go forth and play with the big boys, but always remember us...the little people. I would also like to express my gratitude to Kathy Gale whose expertise and friendship will not be forgotten. To Blake Smith, whose smile and wit few can match, I say thank you for all you have done. To Sean Cregan, Sarah Brewer, Allison Hunter and Miguel Providenti, my friends and fellow graduate students, thanks for keeping me in laughter and smiles. I would also like to acknowledge the CANDU Owners Group whose financial support via their COG Scholarship made it possible for me to attend the University of Ottawa's Graduate School and work at Atomic Energy of Canada Limited. Additional thanks goes out to Irene Langlois at Health Canada, Ottawa, for generating the flow cytometry data.

Finally to Theresa Lynn Faulkner, my friend and my love, thank you for your support and guidance over the last few years I know that were it not, this would not have been.

Abstract

The adaptive response, a phenomenon sensitive to low doses of ionizing radiation, was used to assess the relative biological effectiveness (RBE) of low doses of tritium. Normal human fibroblasts (AG1522) were grown in culture to confluence and exposed to tritium β-particles or ^{60}Co γ-ray adapting doses ranging from 0.10 cGy to 50 cGy. These cells, and other unexposed cells, were allowed to adapt during a 3 h, 37 °C incubation prior to a challenge dose of 4 Gy of ^{60}Co γ-rays. Cells were replated in the presence of cytochalasin B and adaption was assessed by measuring micronucleus frequency in binucleated cells. Exposure to any of the low doses from either radiation caused cells to adapt and reduced the micronucleus frequency that resulted from the subsequent 4 Gy exposure. The magnitude of the adaption that developed during the fixed 3 h incubation was the same regardless of the dose or the radiation type. These results demonstrate that the RBE for low doses of tritium is 1, using adaption as the endpoint. They also show that doses as low as (on average) one track per cell (0.10 cGy) initiate the same maximum rate of development of the adaptive response as do doses that produce many tracks per cell, and the two radiations are not different in this regard.

Fluorescence *in situ* hybridization using whole chromosome paints (WCP) was then employed to probe the chromosome content of radiation induced micronuclei in radiation-adapted and non-adapted fibroblasts. Cells grown to confluence were subjected a ^{60}Co γ-ray adapting dose of 10 cGy and allowed to adapt over the same 3 h period prior to a 4 Gy ^{60}Co γ-ray challenge dose. Cells were again incubated in the presence of cytochalasin B and, following fixation, a single WCP specific for either chromosome 2, 4, 7, 18 or 19, was hybridized and micronuclei were scored for chromosome specific content within both binucleate and mononucleate cell populations.

As similarly demonstrated in the RBE study, adaption to radiation resulted in a reduction in the frequency of micronuclei which appeared after the 4 Gy radiation exposure. Within the 4 Gy exposed, non-adapted cells, each chromosome tested was incorporated into micronuclei more frequently than would be expected based on its relative DNA content. However for chromosome 2 and 18 this bias of incorporation was decreased significantly in cells exposed to a 10 cGy adapting dose 3 h prior to the 4 Gy challenge dose. Conversely, in these radiation adapted cells, the appearance of micronuclei positive for chromosome 19 increased significantly while the frequency of micronuclei containing DNA originating from chromosomes 4 and 7 remained unchanged. These findings support the existing evidence for a naturally occurring chromosomal hierarchy for the repair of radiation damage and now further show that adaption to ionizing radiation can alter that hierarchy and additionally bias repair in some, but not all chromosomes.

Résumé

La réponse adaptive, un phénomène sensible aux doses basses, a été utilisée pour évaluer l'efficacité biologique relative (EBR) des doses basses de tritium. La culture de fibroblaste normal humain (AG1522) a été grandie à confluence et a été exposée aux doses adaptives des rayons-β du tritium ou des rayons-γ du ^{60}Co de 0,10 cGy à 50 cGy. Ces cellules, et autre cellules non-exposer, ont été permis de s'adapter pendant une period d'incubation de 3 h à 37°C antérieur à une dose défi de 4 Gy des rayons-γ du ^{60}Co. Les cellules ont été remis en culture dans la présence de cytochalasin B et l'adaptation a été évaluée par la fréquence des micronoyaux parmis les cellules avec deux noyaux (les bi-noyaux). L'exposition à toutes les doses basses des deux source de radiation a causé l'adaptation des cellules et a réduit la fréquence des micronoyaux résultant de la dose de 4 Gy subséquante. La magnitude de l'adaptation développée pendant le temps fixe d'incubation de 3h était le même peu soigneux de la dose ou le type de radiation. Ces résultats démontrent que l'EBR pour les doses basses de tritium est 1, utilisant l'adaptation comme le point final. Ils montrent aussi que les doses aussi basse (sur moyenne) qu'une trace par cellule (0.10 cGy) intente la même vitesse maximum du développement de la réponse adaptive que fait les doses qui produisent plusieurs traces par cellule, et les deux radiations ne sont pas différentes dans cette estime.

L'hybridation en situ fluorescent, utilisant les peints de chromosome entier (WCP), a été employé pour sonder la teneur en chromosome des micronoyaux provoqués par la radiation dans les fibroblastes adaptés et non-adaptés. Les cellules grandies à confluence ont été soumis à une dose adaptive de 10 cGy des rayons-γ du ^{60}Co et ont été permis de s'adapter pour la même période de 3h antérieurement à une dose défi de 4 Gy des rayons-γ du ^{60}Co. Les cellules ont été incubées de nouveau dans la présence de cytochalasin B et, suite à la fixation, un seul WCP spécifique pour un chromosome, soit le #2, 4, 7, 18 ou 19, a été hybridé et les

micronoyaux ont été énumérés, selon leurs contenu de chromosome spécifique, dans les deux populations de cellules, bi-noyaux et mono-noyaux.

De la même façon démontré dans l'étude d'EBR, l'adaptation à la radiation a résulté dans une réduction dans la fréquence de micronoyaux qui a paru après l'exposition à une dose de radiation de 4 Gy. Parmis les cellules non-adaptées, exposées à 4 Gy, chaque chromosome tester a été incorporé dans les micronoyaux plus fréquemment que serait attendu baser sur son contenu d'ADN relatif. Cependant pour les chromosomes 2 et 18 ce penchant d'incorporation a été diminué considérablement dans les cellules exposées à une dose adaptive de 10 cGy, 3 h antérieur à la dose défi de 4 Gy. Réciproquement, dans ces cellules adaptées à la radiation, l'apparence de micronoyaux positif pour le chromosome 19 a augmenté considérablement mais la fréquence de micronoyaux avec l'ADN provenant du chromosome 4 ou 7 est restée inchangée. Ces conclusions supportent les évidences existantes pour une hiérarchie chromatique naturelle pour la réparation de l'endommagement causer par la radiation et maintenant de plus démontrent que cette adaptation à la radiation ionisante peut changer cette hiérarchie et en outre peut influencer la réparation dans quelque, mais pas tous les chromosomes.

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Forward

The adaptive response is a phenomenon in which cells pre-exposed to a low dose of radiation exhibit resistance to a subsequent higher dose. Current research examining the phenomenon is proceeding under the hypothesis that the low adapting dose induces an increase in DNA repair capacity such that the cells are more resistant to a subsequent higher dose. The adaptive response to radiation demonstrates, therefore, that low doses induce a biological response and this response can be used to quantify the effects of low doses of radiation.

This concept formed the means to investigate the first question: **what is the relative biological effectiveness (RBE) of low doses of tritium beta radiation?** RBE is defined as the ratio of absorbed dose of a reference radiation, typically gamma rays or X-rays, to the absorbed dose of a test radiation, in this case the beta emission of tritium, required to produce the same biological effect. In terms of radiation protection, RBE studies form the basis for assigning radiation weighting factors (W_r) for the conversion of absorbed dose (Gray (Gy) J/Kg) to a biologically equivalent dose (Sievert (Sv)), as explained below.

Previous research has shown that identical doses of different types of radiation (i.e. alpha, beta, gamma, neutrons) cause different levels of biological effect. As the Gy is a physical unit describing the amount of energy absorbed per unit of material, it is not sufficient to predict the biological effects of such a dose. However, multiplying Gy by W_r produces a biologically equivalent dose (Sv):

Absorbed dose (Gy) x Weighting factor (W_r) = Equivalent dose (Sv)

Thus, when expressed as equivalent dose, different types of radiation produce the same biological effect.

For radiation protection, a W_r of one has been used for tritium beta radiation. However the RBE studies underlying this assignment have used high dose and dose rates. This is in contrast to occupational settings where atomic energy workers are exposed to tritiated water, a waste product of CANDU reactors, at low dose and dose rates. From this follows the question: what is the RBE of low doses of tritium? If found to be different than the assumed value of one, it would have important implications for radiation protection practices.

The question was addressed using the phenomenon of the adaptive response, a means to detect the biological effects of low doses. Specifically, the level of adaption induced by tritium beta radiation was compared to the level induced by ^{60}Co gamma radiation, measured by micronucleus frequency in binucleate fibroblast cells. This technique scores the frequency of micronuclei, which are small, spherical, DNA containing bodies found within the cytoplasm of interphase cells. Radiation induced micronuclei are primarily composed of acentric DNA fragments, the result of unrepaired DNA double strand breaks. Thus, micronuclei are simply one measure of unrepaired DNA damage. To increase sensitivity, the assay uses the fungal metabolite cytochalasin B which has the property of inhibiting cytokinesis while still allowing nuclear division. Cells, which have divided once, can therefore be identified by their binucleate appearance. Sensitivity is increased because a cell must divide at least once to express a micronucleus. By scoring only those cells that have divided (as indicated by their binucleate state) the assay becomes a sensitive tool for biological dosimetry.

Cells are said to have adapted if they show a reduction in micronucleus frequency relative to those cells that received only the high challenge dose. Using this assay, the level of adaption was assessed for equal doses, at approximately occupational dose levels, of the

reference radiation, gamma rays and the test radiation, tritium beta rays. Equal levels of adaption for equal doses of the two types of radiation would suggest that the RBE of low doses of tritium is one. Alternatively, unequal levels of adaption would indicate an RBE of greater than or less than one.

The second question investigated was: **does an adapting dose of radiation alter the risk of inclusion of a specific chromosomes in a micronucleus?** This examines radiation risk at a chromosomal level and asks whether a prior low dose alters that risk. It has been shown previously that the phenomenon of the adaptive response is the result of an upregulation in DNA repair capacity. This upregulation may be directed uniformly across the genome or it may be targeted to specific chromosomes. If repair is shown to be biased to specific chromosomes then this could have important implications with respect to radiation risk assessment and radiation carcinogenesis. For example, if there is bias in repair such that some chromosomes are preferentially repaired, then it follows that lesions in genes residing on those chromosomes would be preferentially repaired over lesions in genes residing on less favoured chromosomes. If those genes were, for example, tumour suppressor genes or proto-oncogenes, then a prior low dose could affect the risk of carcinogenesis.

To address this question, whole chromosome paints were employed to probe the chromosome specific content of micronuclei in adapted and non-adapted cells and the resultant frequencies were compared. A reduction in the frequency of inclusion of a chromosome in micronuclei from adapted cells versus micronuclei in non-adapted cells would suggest a greater frequency of repair of that chromosome induced by the adapting treatment. Conversely, an increase in micronucleus frequency would indicate that the increased DNA repair capacity was directed away from those chromosomes.

Literature Review

1. Linear No-Threshold Hypothesis

The effects of exposure to ionizing radiation can be grouped into two distinct categories termed, ***deterministic*** and ***stochastic***. Deterministic effects are somatic in nature and require large acute exposures (i.e. 100-600 cGy over a few hours) to cause significant incidence. They are the direct consequence of non-repairable cellular damage causing cell death at levels that inhibit tissue or organ function temporarily or permanently; examples include, nausea (100 cGy), vomiting (200 cGy), haemorrhage (600 cGy), and death (>600 cGy).

One important and defining characteristic of deterministic effects is that a practical threshold dose exists for each effect, such that below a given dose the effect will not occur. For example, doses of ionizing radiation to a certain level will not produce the deterministic effect, skin erythema. Beyond this threshold, however, the severity of the response in the individual is directly proportional to the dose received.

In contrast to the radiation type injury of deterministic effects, stochastic effects arise not as the result of non-repairable lethal DNA damage, but instead from the misrepair of DNA lesions with concomitant maintenance of cell viability. If the lesion detrimentally affects a critical target gene (e.g. tumour suppressor gene), following some latency period (months-years) in association with promoting factors, these cells may develop into cancerous tumours. In addition to this process of carcinogenesis, hereditary effects are also considered stochastic. Such effects occur when a mutation event or events affect germ-line tissue and therefore possible detrimental effects may be seen in the descendants of exposed individuals.

It is important to note that stochastic effects can arise not only from injury to multiple cells but also to a single cell. Thus, and in contrast to deterministic effects, stochastic effects are assumed to have no apparent dose threshold. Put another way, no matter how small the dose of ionizing radiation, it is assumed to carry with it a corresponding probability of affecting a

cell or cells and generating the effect. This points to a defining characteristic of stochastic effects; increasing dose does not increase the severity of the effect. For example, radiation induced myeloid leukemia may result from a single exposure of 0.10 cGy or 500 cGy, but the affected individual still has the condition; increasing the dose does not increase the conditions severity. However, increasing dose does increase the ***probability*** the exposed individual will contract the condition. Thus, for stochastic effects, it is the probability of the biological effect occurring, not the severity, that is proportional to the dose.

In terms of radiation protection, regulatory dose limits have been established to preclude deterministic effects while minimizing the probability of stochastic effects. For atomic radiation workers a maximum yearly permissible dose of 50 mSv (5000 mRem) (whole body, gonads and bone marrow) has been recommended by the International Commission on Radiological Protection (ICRP 1991), and generally adopted by national regulatory agencies. Such regulatory levels are based on the linear back extrapolation of data from epidemiological studies examining the incidence of cancer in a large population, with the most important being those who received a high dose of ionizing radiation at a high dose rate from the atomic bombing of Hiroshima and Nagasaki (UNSCEAR 1986; Pierce *et al.* 1996).

Inherent in this model of radiation risk assessment are the following fundamental assumptions: (1) The effects of sequential doses of radiation are additive; that is, there is always some DNA damage by ionizing radiation that is not repairable, (2) Any radiation dose, no matter how small, is potentially harmful (i.e., there is no threshold), (3) The probability of adverse biological effects is linearly proportional to dose (UNSCEAR 1988). Appropriately, this model, which forms the foundation for current radiation protection practices, is termed the ***linear no-threshold hypothesis***. While the linear no-threshold hypothesis is assumed to

provide an extremely conservative estimate of risk, its appropriateness, on a number of fronts, is currently being debated (reviewed by Mossman 1998; Nussbaum 1998; Sinclair 1998).

It has been accepted by most national regulatory agencies (ex. ICRP) that the stochastic risks predicted by the linear no-threshold model are accurate down to the 100 mSv level, with a two fold reduction in risk for doses received at low dose rate (HPS 1996, Sinclair 1998). However, at doses lower than 100 mSv, which fall into the range of occupational exposures, Mossman (1998) and others (ACRP 1996) have questioned the validity of the linear no-threshold model because of a lack of supporting data. They have highlighted three issues driving the debate: (1) Regulatory compliance costs too much in terms of benefits gained, (2) Acceptance of the linear no-threshold hypothesis has led to the widespread fear that no dose of radiation is safe, possibly affecting medical applications of radiation, (3) An increasing amount of data that suggest the linear no-threshold model oversimplifies the dose-response relationship in the low dose region where occupational and public exposures actually occur, and that it may in fact overestimate risk, (4) No evidence in the literature exists for risk associated with doses less than 50 cGy (reviewed by Mossman 1998).

In an attempt to address this apparent oversimplification at low doses, alternate models have been suggested to replace the linear no-threshold hypothesis. These include: (1) Low dose hormesis, where the calculated risk is less than zero (i.e. beneficial) up to a certain threshold dose, beyond which the dose-effect curve is linear, (2) The convex supra-linear model, in which the risk per unit dose is actually greater at lower doses than higher, (3) Zero-effect threshold, where to a certain threshold the risk remains zero, and beyond which the dose-effect curve is linear, (4) Models which incorporate a two fold or greater reduction for low dose effects (reviewed by Nussbaum 1998; Sinclair 1998).

Evidence within the literature can be found for each dose-effect curve listed above at doses greater than 50 cGy. Attempts have also been made to fit pre-existing epidemiological data to each model. However, because of factors such as: (1) Non-specificity of radiation carcinogenesis, (2) Long latency period for the onset of cancer, (3) High background rate of cancer induction due to factors other than ionizing radiation, such as spontaneous events and promotion by other human carcinogens (ex. cigarette smoking), epidemiological studies simply lack the statistical power necessary to demonstrate a radiogenic effect and thus differentiate between proposed models.

As a result of this and additional factors, many researchers have turned to animal and cellular systems in an attempt to gain a better understanding of the effects of low doses of ionizing radiation. One area that has received considerable interest over the past twenty years has been the assumption of the linear no-threshold hypothesis that radiation damage is cumulative and, as such, each sequential dose is not only deleterious but also additive. Implicit in this assumption is the notion that DNA repair is a constant in radiation carcinogenesis and not an important variable determinant of risk.

However one important line of evidence that suggests that DNA repair is indeed an important variable determinant of risk, thereby contradicting the linear no-threshold hypothesis, is the phenomenon of the adaptive response.

2. The Adaptive Response

A small amount of a given stress agent administered at the cellular, tissue or organism level will often confer a level of protection in that system to a subsequent higher challenging stress (reviewed by Lindahl *et al.* 1988; Luckey 1991). This phenomenon has been termed the ***adaptive response*** and been characterized for a number of different stresses such as heat, alkylating agents and radiomimetic drugs. However, in terms of identifying the phenomenon and characterizing the underlying molecular mechanisms, the agent that has arguably received the most attention has been ionizing radiation.

Initial insights and direction to future investigations came from experiments in which novel dose-survival curves for the protozoan *Tetrahymena pyriformis* showed an ***increase*** in cell survival with ***increasing dose*** over a specific range of low doses of X-rays (Calkins 1967). From this observation Calkins (1967) hypothesized that at, and above, a certain threshold dose, ionizing radiation induces processes that produce radioresistance in those cells.

With studies such as this and others forming a basis for investigation, in addition to the established background of adaptive responses for other stress agents such as alkylating compounds (reviewed by Lindahl *et al.* 1988) and heat (Mitchel and Morrison 1982, 1984a), researchers began to examine whether low doses of ionizing radiation could induce similar responses in eukaryotic systems. That is, could a low dose of ionizing radiation induce resistance against the effects of a subsequent larger dose of ionizing radiation? If found to be true such findings, researchers argued, would contradict long held assumptions implicit in the linear no-threshold hypothesis which is the basis for both radiation protection practices and cancer risk estimates. Specifically, that sequential doses of ionizing radiation are additive, always deleterious, and finally, that repair is not a determinant of risk.

Indeed, initial studies using the yeast *Saccharomyces cerevisiae* demonstrated just such a radiation induced adaptive response to a subsequent larger dose of ionizing radiation (reviewed by Boreham *et al.* 1991). With respect to mammalian cells, the first demonstration of a radiation induced adaptive response came when Olivieri *et al.* (1984) observed that human lymphocytes cultured with tritiated thymidine, which provided a chronic low adapting dose at a level that did itself not produce aberrations, had significantly fewer chromatid aberrations after a subsequent challenge dose of 150 cGy of X-rays than those that did not receive the adapting dose.

Subsequent to this first report of a radiation induced adaptive response in a mammalian cell line, many research groups have further investigated the phenomenon using other biological endpoints, cell types and whole organisms. For a listing of demonstrations of the adaptive response in a wide variety of cell lines, the reader is asked to refer to the *Introduction* section of *Chapter III*.

In terms of the scope of the review presented here, the remainder will emphasize findings that illuminate some aspect of the molecular mechanisms and cellular processes that underlie the adaptive response in mammalian cell lines and animal systems, which just recently have begun to be elucidated. It should be noted that the processes that produce the adaptive response to ionizing radiation in prokaryotes (reviewed by Walker 1984; 1985), lower eukaryotes such as yeast (reviewed by Boreham *et al.* 1991) and non-mammalian cell types (reviewed by Koval 1986) are reasonably well understood. If the reader wishes to educate himself or herself on these specific topics he or she is referred to these references and references contained within.

a. *Characteristics of the adaptive response*

The phenomenon of the adaptive response is characterized by several distinct observations. Firstly, manifestation requires that the adapting dose precede the challenge by a number of hours. For example, lymphocytes appear to require a minimum of 4 hours to develop full activity against the challenge exposure (Shadley *et al.* 1987). This lag in expression was interpreted as evidence of a *de novo* protein synthesis requirement in the development of the adaptive response. This hypothesis was later confirmed when Youngblom *et al.* (1989) demonstrated that the addition of the protein synthesis inhibitor cycloheximide during the adapting period abolished the adaptive response in human lymphocytes. In addition, low dose radiation has been shown to induce alterations in the protein profiles of 2D gels in the first few hours following exposure (Wolff *et al.* 1989). Interestingly, one of the induced proteins was shown to bind specifically to radiation-damaged DNA (Wolff 1992).

If *de novo* protein synthesis is necessary then transcription may also be a requirement. Indeed, Ikushima (1992) demonstrated that the *de novo* synthesis of transcripts is also necessary by showing that the addition of the transcription inhibitor actinomycin D during the period between the adapting and challenge doses abolished the adaptive response.

The adaptive response is thought, by many investigators, to involve principally the low dose induction of DNA repair mechanisms, so that damage resulting from a subsequent high dose of ionizing radiation is better repaired. This is supported, at least indirectly, by the findings presented above. However, additional lines of evidence also support this assertion. 3-Aminobenzamide, an inhibitor of poly(ADP-ribose) polymerase (PARP), was shown to prevent adaption when applied during a 2 hour period following the adapting dose (Wiencke *et al.* 1986; Shadley and Wolff 1987; reviewed by Kleczkowska and Althaus 1996). This suggested that PARP participates in the early steps of the adaptive response. Although the exact molecular

function of PARP has not been determined, it is known to be an abundant nuclear protein implicated in DNA repair by its early activation and DNA strand break binding activity following genotoxic insult by DNA damaging agents (Lindahl *et al.* 1995). Recently, Rudat *et al.* (1998) demonstrated that trans-dominant inhibition of PARP by the PARP DNA binding domain had significant radiosensitizing effects and suggested that PARP may be involved in the repair of DNA double strand breaks.

Other studies have examined the role of DNA repair in the adaptive response. Ikushima *et al.* (1996), using neutral single-cell gel electrophoresis, demonstrated that an observed adaptive response in Chinese hamster cells is due, a least in part, to an increased rate of DNA double strand break repair resulting in less residual damage, rather than from the induction of protective mechanisms that reduce initial DNA damage. Similar findings were also reported by Zhou *et al.* (1992). An increased rate of DNA double strand break repair in adapted fibroblasts was also noted by Azzam *et al.* (1994a), as measured by micronucleus frequency in normal human fibroblasts. However, others using similar assays and cell systems have failed to observe such an effect (Wojcik *et al.* 1996, Wojewodzka *et al.* 1994; 1996; 1997).

Studies examining repair fidelity in adapted cells have also lent support to the hypothesis that the induction of DNA repair mechanisms is involved in eliciting the adaptive response. This specific area of research into the adaptive response has been termed ***mutagenic adaption*** and has typically involved analysis of mutations at the hypoxanthine phosphoribosyl transferase (*HPRT*) locus. Briefly, the assay utilizes the fact that cells which have undergone a mutation event within the *HPRT* locus become resistant to the otherwise toxic guanine analog, 6-thioguanine (6-TG). As a result, $HPRT^-$ mutation frequency can be determined by simply dividing the number of mutant colonies growing on a selective medium containing 6-TG by the total number of cells plated and correcting for plating efficiency. Using this technique Sanderson and Morley (1986) were the first to demonstrate that human

lymphocytes incubated with tritiated thymidine for 6 hours became less susceptible to the induction of mutations at the *HRPT* locus when challenged with either 1.5 or 3 Gy of X-rays. Other researchers followed their example and, using gamma- and X-ray adapting doses, demonstrated similar responses in **human lymphocytes** (Kelsey *et al.* 1991), **human lymphoblastoid cells** (Rigaud *et al.* 1993), **human T-cell leukemia cells** (Zhou *et al.* 1994), **Chinese hamster ovary cells** (Laval 1988), **mouse mammary carcinoma SR-1 cells** (Zhou *et al.* 1993) and **mouse embryonic skin M5-S cells** (Sasaki 1995).

A number of studies have examined the molecular spectrum of the induced *HPRT* mutants. Rigaud *et al.* (1993), Rigaud and Moustacchi (1994) and Zhou *et al.* (1994) each demonstrated that the proportion of deletion mutations at the *HPRT* locus decreased when cells were pre-exposed to a low dose of ionizing radiation prior to a high challenge dose. As stated by Rigaud and Moustacchi (1994) this suggests that "...cellular mechanisms triggered by the low dose thus prevented preferentially a certain class of pre-mutagenic lesions, i.e., those leading to deletions...these lesions are likely to be the [result of DNA] double strand breaks. This implies that adapted cells can deal efficiently with this type of damage". That is, it appears that the reduction in *HPRT* mutation frequency is the result of an error-free DNA double strand break repair system that is induced by low doses.

Azzam *et al.* (1994b) have also observed an increased DNA double strand break repair capacity in mouse embryo C3H 10T½ cells adapted with low doses of γ-rays as measured by a reduction in micronucleus frequency. In addition, the adapted cells were protected against transformation to malignancy, suggesting that adaption enhances error-free repair. Repair of DNA double strand breaks in lower eukaryotic cells such as yeast is known to proceed via error

free homologous recombination and this system has been shown to be induced by exposure to ionizing radiation such that adaptive responses are observed (Mitchel and Morrison 1982; 1984a; 1984b; 1987).

While an increase in DNA repair capacity has been implicated in the adaptive response, researchers have examined whether other cellular processes could also be contributing to the overall effect. For example, cell cycle regulation has been postulated as having an influence on the adaptive response. It should be noted that it has been known for more than 30 years that exposure to ionizing radiation causes delays in cell cycle progression (Puck and Steffen 1963). However, the important question, as this relates to the adaptive response, is whether a low adapting dose is able to induce such delays or alter delays induced by the challenge dose. Mitchel (1995) observed that adapted fibroblasts took longer to reach a binucleate state than non-adapted and suggested that this delay in cell cycle progression may be a second adaption mechanism by allowing more time for DNA repair. Consistent with this hypothesis, mRNA analysis of the same adapted cells revealed decreased levels of cyclin A and B transcripts, encoded proteins known to be involved in regulation of the cell cycle (de Toledo *et al.* 1995). Additional studies have also confirmed that adapting doses are able to influence cell cycle progression (Meyers *et al.* 1995; Salone *et al.* 1996a; 1996b).

Recently the ability of low dose ionizing radiation to induce changes in chromatin conformation was observed using the technique of anomalous viscosity time dependence. Belyaev *et al.* (1996) and Belyaev and Harms-Ringdahl (1996) demonstrated that doses as low as 0.5-2 cGy were able to alter the viscosity of human fibroblasts cell lysates significantly, suggesting altered chromatin conformation. Using the same method Belyaev *et al.* (1993) showed a similar effect in human leukocytes. These results are also in agreement with Wojewodzka *et al.* (1994), who showed a similar response in human lymphocytes irradiated with 1 cGy.

Such alterations in chromatin conformation could have significant implications for the adaptive response. For example, it may be the reflection of an active process to increase the accessibility of DNA repair proteins to damaged sites, as hypothesized by Mitchel (1995). As well changes in chromatin conformation have been implicated in reducing DNA damage caused by the indirect effects of a challenge dose of ionizing radiation (Ljungman 1991).

An upregulation of antioxidation mechanisms has also been implicated in cellular responses to ionizing radiation and is suggested as having a role in the adaptive response by protecting cells from oxidative damage (de Toledo *et al.* 1995). *De novo* synthesis or post-translational activation of antioxidant enzymes (superoxide dismutase, catalase, and peroxidases) could minimize the deleterious effects of highly reactive free radicals produced by the radiolysis of water by ionizing radiation. This process is defined as ***indirect damaging effects of ionizing radiation***, see section 1.4.2. *Insights into the adaptive response*. Zhang *et al.* (1998) recently demonstrated such an induction in mouse testis; they observed that a dose of 5 cGy γ-rays not only protected the testis from a subsequent 2 Gy exposure, as measured by weight loss, sperm count and sperm abnormalities, but also significantly increased superoxide dismutase activity and decreased lipid peroxidation levels. Such induction of increases in antioxidant potential following a whole body dose of 25-50 cGy has also been shown in the cytosolic fractions of various other tissues of mice, such as the liver, pancreas and brain, but not the lung, heart, spleen or kidney (Kojima *et al.* 1997). Kojima *et al.* (1997) also noted that the phenomenon was observed soon after irradiation and persisted for approximately 24 hours, suggesting "an adaptive response to the active oxygen species generated by the radiation". Chinese hamster ovary cells pre-treated with xanthine-xanthine oxidase have also been shown to be less susceptible to the mutagenic and killing effects of a subsequent high challenge dose of γ-radiation. This adaptive response was also shown to be correlated with an increase in superoxide dismutase activity but unchanged catalase activity (Laval 1988).

Apoptosis, or programmed cell death, has also been considered as a possible adaptive mechanism for induction of the adaptive response (Mitchel *et al.* 1997). A 10 cGy dose of γ-radiation given to human lymphocytes 6 hours prior to a 3 Gy challenge exposure was found to increase significantly the frequency of apoptosis (Cregan *et al.* 1994). Mitchel *et al.* (1997) argue that this finding, although appearing to contradict previously well characterized cytogenetic adaptive responses in human lymphocytes that show protective effects (review by Shadley 1994) makes sense if the whole organism is considered. That is, if cells experience problems in DNA repair or if repair fidelity is somehow compromised the cell might initiate programmed cell death and remove itself for the benefit of the whole organism, rather than continue to survive with a possible tumour-initiating mutation.

Thus it appears that manifestation of the adaptive response is a highly active and integrated process involving not only a simple increase in protective proteins and DNA repair enzymes but also activation of various repair pathways, induction of antioxidant defense mechanisms, delays in cell cycle progression, initiation of apoptosis and alterations in chromatin conformation, all of which act in concert to increase the overall DNA repair capacity while maintaining repair fidelity.

b. *Signal transduction pathways involved in the adaptive response*

One hypothesis that has been put forward to explain the underlying molecular mechanisms of the adaptive response is the ***signalling loop model*** (Weichselbaum *et al.* 1991). It proposes that upon treatment with a low adapting dose of ionizing radiation an "alarm signal" is generated in the nucleus. This signal is then transferred out to the cytoplasm of the cell and, via further signal transduction pathways, transferred back to the nucleus. Upon returning to the nucleus a number of specific genes are activated with concomitant *de novo* protein synthesis. The ultimate consequence of the activation of these cellular processes is an

overall increase in the DNA repair capacity resulting in more efficient repair of DNA damage caused by a subsequent high dose of ionizing radiation.

In an attempt to examine the specifics of this model, a number of researchers initially investigated whether the hypothesized signalling pathways of the adaptive response were mediated by protein kinase C (PKC), a kinase known to be a key regulatory element in a variety of signalling pathways (Liu 1996). Ikushima (1992), Ibuki and Goto (1994) and Wojewodzka *et al.* (1995; 1997) each demonstrated that the development of the adaptive response to ionizing radiation was blocked by administration of a PKC inhibitor prior to and during the adapting dose. In addition, Woloschack *et al.* (1990), and Liu (1992), have also demonstrated that low doses of ionizing radiation lead to the activation of PKC immediately following the exposure. In a thorough examination of the role of PKC in the adaptive response, Sasaki (1995) reported that both the tumour promoter TPA and H7, known PKC activators at low concentrations, were able to mimic a low dose treatment and elicit the adaptive response in confluent mouse fibroblasts. Sasaki (1995) further showed that a high dose of TPA, which down-regulates PKC, was not only ineffective in inducing the adaptive response but in fact abolished the protective effects of a previous adapting dose.

Further studies have examined the role of second-messengers in signal transduction pathways with respect to the initiation and expression of the adaptive response. Wojewodzka *et al.* (1994) demonstrated that administration of EGTA, a calcium chelator, during the adapting dose, but not psi-tectorigenin, an inhibitor of phosphatidylinositol turnover, prevented the development of the adaptive response as measured by micronucleus frequency in binucleate human lymphocytes. For a description of micronucleus frequency within binucleate cells as an indicator of adaption, please refer to the *Introduction* section of *Chapter III*. Interestingly, while this implicates free calcium ions, but not inositol triphosphate, as having a role in adaptive response. Wojewodzka *et al.* (1994) also showed that the adapting dose of radiation does not

actually cause the release of calcium ions from intracellular stores. Hallahan *et al.* (1994) further demonstrated that low doses of ionizing radiation do not induce increases in calcium ion concentrations and further showed that the addition of the calcium chelator AM-BAPTA prevented the ionizing radiation induced activation of PKC.

Cyclic ADP-ribose is a third second-messenger whose role in adaptive response has been examined. It is both synthesized and hydrolyzed via the dual enzymatic action of the CD38 lymphocyte surface antigen, and upon production has the role of calcium mobilization. Inhibition of CD38 using an anti-CD38 antibody administered one hour prior to an adapting dose of 1 cGy (X-rays) inhibited the development of the adaptive response as measured by micronucleus frequency in binucleate human lymphocytes (Wojewodzka *et al.* 1996). This result not only directly implicates cyclic ADP-ribose in the adaptive response, but also indirectly supports the importance of calcium.

Both calcium ions and TPA have also been shown to affect intercellular communication via gap junctions (Loewenstein 1981; Oh *et al.* 1988). Recently, Ishii and Watanabe (1996) investigated whether expression of the adaptive response involves intercellular communication via gap junctions by examining confluent human embryonic fibroblasts (HEF) placed in either calcium free, or TPA containing medium at the time of low dose irradiation. When HEF cells were given a low dose of ionizing radiation in a calcium free medium an adaptive response was not observed. Similar results were shown for high concentrations of TPA as well; specifically 100 ng/mL, which corresponded to complete gap junction inhibition as indicated by Lucifer Yellow transfer.

Interestingly, Ishii and Watanabe (1996) failed to examine or note the possible involvement the down-regulation of PKC may have had in the abolishment of the adaptive response via exposure to high levels of TPA. Conversely, Sasaki (1995) who, as outlined above, examined the elimination of the adaptive response in confluent mouse fibroblasts via TPA induced down-regulation of PKC did not mention or investigate the possible involvement of inhibition of gap junctional communications in that abolishment.

Taken together, these findings suggest that: (1) Low dose ionizing radiation induces signal transduction pathways, (2) One signalling pathway involved in eliciting the adaptive response to ionizing radiation may be mediated by PKC, (3) While inositol triphosphate is not involved, calcium, possibly as a second messenger in the activation of PKC, and cyclic ADP-ribose are candidates as second-messengers in the adaptive response and (4) The adaptive response, at least in confluent fibroblasts, may involve intercellular communication via gap junctions (reviewed by Wojewodzka *et al.* 1997).

Activation of kinases, such as PKC, produces a cascade of phosphorylation events which eventually lead to the activation of immediate early response genes such as c-*fos*, c-*jun* and nuclear factor-κB (NF- κB). Many of the early response genes encode transcription factors whose expression has been suggested to have a role in regulating the adaptive response to ionizing radiation through induction and repression of further specific gene products necessary to elicit the response. Clearly then, one element necessary to understand the phenomenon of the adaptive response is the identification of those genes whose expression is altered following low dose exposure.

While the majority of studies have focused on changes in gene expression induced by relatively high doses of radiation (reviewed by Fornace 1992), over the last few years a number of researchers have begun to focus on lower doses in the adapting range. For example, it has

been shown that a localized (5 cGy) or whole body dose (12 cGy) of gamma-rays was able to activate the immediate early response gene c-*fos* in pig skin cells (Martin *et al.* 1995). Prasad *et al.* (1994) demonstrated activation of NF-κB over a range of 0.25-2 Gy with a maximal response at 50 cGy using a human lymphoblastoid B cell line.

In a recent paper, Robson *et al.* (1995), highlighted the importance of the repression of specific genes. The authors suggested that this process, in conjunction with induction, plays an equally important role in the signal transduction pathways generating the adaptive response. By screening a cDNA library the researchers identified a gene that was maximally repressed by 4 fold following exposure to 20 cGy. Further, repression was found to be confined to low doses (less than 1 Gy), with doses of 2 and 4 Gy failing to repress expression. In addition they observed other genes, shown previously to be induced by high doses, failing to be induced by low doses. From this the authors concluded that there may be two distinct mechanisms that respond to ionization radiation, one induced by low doses and the other by high, each utilizing specific genes.

It is important to note that exposure to low doses of ionization radiation induces the expression of additional types of proteins both directly and indirectly via previously mentioned transcription factors. This is not surprising considering the wide variety of cellular processes, from DNA repair and synthesis to apoptosis and cell cycle control, that are required to elicit the phenomenon of the adaptive response. Recently, Sadekova *et al.* (1997) demonstrated that 25 cGy induced the expression of peptide-binding protein 74 (PBP74) (a member of the heat shock 70 protein (hsp 70) family) in a human colon carcinoma cell line. The researchers reported a 15 min lag in expression following exposure which they interpreted to be the result of a *de novo* mRNA synthesis requirement. But perhaps most importantly they found that administration of an antisense oligonucleotide directed towards the initiation codon of PBP74

sensitized the cells to subsequent exposure of ionizing radiation. In addition Ibuki *et al.* (1998) have also shown induction of hsp 70 mRNA following exposure of a mouse myeloid leukemia cell line to 4 cGy.

c. *Low dose hypersensitivity and increased radioresistance*

Until recently, assays used to determine the biological effects resulting from exposure to ionizing radiation have been unable, with any degree of certainty, to identify radiogenic effects in the low dose range. This is because they lacked the necessary precision to identify induced events against the natural background of such events. However, the development of the Dynamic Microscope Image Processing Scanner (DMIPS) cell analyzer overcame this limitation (Palcic and Jaggi 1986; Spadinger and Palcic 1993). This fully automated device allows the specific identification of individual living cells and can revisit their location, thereby allowing their reproductive history to be followed. As a result extremely precise survival curves can be generated following low doses of ionizing radiation.

Using the DMIPS assay, Marples and Joiner (1993) found that single X-ray doses in the range of 1-20 cGy were ***more efficient*** per unit dose in killing Chinese hamster fibroblasts than doses greater than 1 Gy. Thus the cells exhibited a ***low dose hypersensitivity***. Interestingly, following this hypersensitivity the survival curve turned ***upwards***, continuing until about the 100 cGy level. That is, ***survival increased*** per unit dose; put another way single doses of X-rays, although increasing in magnitude, were less effective in killing cells per unit dose. The response to doses greater than 100 cGy was typical in nature with the curve showing a characteristic smooth downward–bend. These findings demonstrated that the low dose survival response to X-rays can not be accurately determined by the simple back extrapolation of survival data obtained following high dose exposure, as would be predicted by the linear no-threshold hypothesis. As well, Marples and Joiner (1993) hypothesized that the multiphasic

nature of the survival curve, specifically the hyper-radiosensitivity (**HRS**, less than 20 cGy), increased radioresistance (**IRR**, 20 cGy to 100 cGy) and typical curvilinear response (greater than 100 cGy), reflects the induction of radioprotective mechanisms triggered by increased levels of damage. Further work demonstrated the HRS/IRR phenomenon in a variety of cell types including a **human colorectal carcinoma cell line** (Lambin *et al.* 1993; 1994a), **normal human lung epithelial cells** (Singh *et al.* 1994), a **human bladder carcimoma cell line** (Lambin *et al.* 1994b) and a **melanoma cell line** (Lambin *et al.* 1994b). In addition, the existence of the HRS/IRR phenomenon has been confirmed using an alternate assay with similar levels of sensitivity, specifically the flow cytometry survival assay (Wouters and Skarsgard 1994; Wouters *et al.* 1996).

To test the hypothesis that the observed IRR was the result of inducible DNA repair processes, Skov *et al.* (1994a) examined low dose survival responses using the DMIPS assay in three hamster cell lines known to be deficient in some form of DNA repair. The following cell lines were used: (1) **UV20**, defective in base repair and extremely sensitive to crosslinking agents such as UV radiation. UV20 is slightly more radiosensitive than wildtype, (2) **EM9**, a radiosensitive cell line found to have a slower rate of single strand break repair as compared to wild type and (3) **XR-V15B**, a radiosensitive cell line found to have a slower rate of double strand break repair as compared to wild type.

The double strand break repair deficient cell line XR-V15B failed to exhibit IRR as compared with the parental wild type line. As well XR-V15B had a more pronounced HRS with the survival curve continuing on with the same slope as the hypersensitive region. In contrast the cell line deficient in single strand repair, EM9, eventually exhibited IRR following a somewhat more pronounced and extended HRS as compared to wild type. Finally, IRR was observed in the base repair defective cell line, UV20. Skov *et al.* (1994a) point out that these

findings are open to a number of interpretations. For example, simply failing to observe IRR in a cell line deficient in double strand break repair does not mean that IRR is not activated. In fact IRR may be triggered in the XR-V15B cell line but, because of a failure in double strand break repair, HRS is extended preventing the manifestation of IRR. With this and similar considerations in mind, they state that their findings simply implicate DNA repair in the HRS/IRR phenomenon.

A further approach used to provide evidence implicating the induction of DNA repair processes in IRR has been the application of inhibitors that block specific components of DNA repair processes. For example, 3-aminobenzamide (3-AB), an inhibitor of PARP, blocks the development of IRR in Chinese hamster cells so that the HSR observed in the low dose range extends out to high doses (Marples *et al.* 1997). Although the exact function of PARP has not yet been determined, it is known to represent an immediate-early response to DNA damage and has been shown to bind to DNA strand breaks and catalyze the production of ADP-ribose polymers covalently linked to proteins (Lindahl *et al.* 1995). As well, the topoisomerase I inhibitor, camptothecin, has been shown to produce a similar inhibition of IRR, also in Chinese hamster cells (Skov *et al.* 1994b). Topoisomerases have been implicated in cellular processes such as DNA repair, as well as in DNA replication and transcription, by regulating DNA helical conformation. Application of the protein synthesis inhibitor, cycloheximide, has also been investigated. As observed with 3-AB and camptothecin, the IRR response was abolished, suggesting a role for *de novo* protein synthesis in the development of IRR.

Clearly, the HRS/IRR phenomenon and the adaptive response share a number of characteristics, and may in fact result from the induction of the same underlying molecular mechanisms. This has been investigated by Lambin *et al.* (1994a) who suggested "If induced radioresistance is the explanation for the low dose substructure in the response of...cells to single doses, then it should be possible, in split-dose experiments, for the first dose to initiate

an adaptive response that provides protection against a subsequent exposure...". Indeed, they demonstrated that human colorectal carcinoma cells irradiated twice with 25 cGy had a significantly greater surviving fraction than those irradiated once with 50 cGy. In addition it was noted that the responses to the split-doses were unequal. That is the second 25 cGy dose had significantly less impact than the first, consistent with an induced adaptive response.

If both the adaptive response and HRS/IRR share underlying mechanisms, then it follows that the induction of radioresistant mechanisms that elicit the adaptive response should abolish hyper-radiosensitivity, via a "pre-emptive" up-regulation of DNA repair capacity, resulting in an "early onset" of increased radioresistance. This hypothesis was investigated by pre-exposing Chinese hamster cells to a single priming dose of X-rays (5 cGy, 20 cGy or 100 cGy) or a non-cytotoxic dose of hydrogen peroxide [10^{-4}], both known inducers of the adaptive response in this and other cell systems, six hours before a "challenge dose"; in this case, a single low dose of X-rays in the hyper-radiosensitive range. Cell survival was then assessed using the DMIPS assay and the resultant curves showed that the priming treatment eliminated HRS (Marples and Joiner 1995; Marples and Skov 1996). This strongly suggests that HRS/IRR and the adaptive response are both manifestations of the same, although as of yet still undetermined, underlying molecular mechanisms.

d. *In vivo demonstrations of the adaptive response*

The adaptive response is not solely an *in vitro* phenomenon, but has also been demonstrated *in vivo*. Cai and Liu (1990) observed that bone marrow cells of Kunming male mice exposed to 10 cGy *in vivo* and challenged 3 hours later with 75 cGy of X-rays exhibited reduced levels of chromatid aberrations as compared to cells taken from mice that had only received the challenge dose. Farooqi and Kesevan (1993) confirmed this observation in mouse bone marrow cells taken from Swiss albino male mice that were pre-exposed to low priming

doses of γ-rays (2.5 and 5 cGy) and challenged with a subsequent dose of 1 Gy γ-rays. When the adapting period was 2 hours both adapting doses were found to be effective at reducing micronuclei and chromosomal aberrations induced by the challenge exposure.

Research has also focused on the effects of low adapting doses on germ line tissue. Cai *et al.* (1993) confirmed and extended an earlier report demonstrating an adaptive response to low dose ionizing radiation in mouse germ cells (Cai and Liu 1990). They showed that whole-body exposures ranging from 1-20 cGy induced a significant reduction in chromatid and isochromatid breaks found in spermatocytes 4 hours after a 1.5 Gy challenge dose; and for reciprocal translocations, 60 days after the challenge dose. In addition, they demonstrated that a whole-body dose of 5 cGy induced an adaptive response to radiation induced dominant lethality in pre-meiotic cells (stem spermatogonia, differentiated spermatogonia, and spermatocytes) but not post-meiotic cells (spermatid and spematozoa). Cai and Wang (1995) subsequently demonstrated that low dose γ-rays, shown to be effective at inducing the adaptive response in mouse germ cells, do not change the sensitivity of the irradiated offspring bone marrow or germ cells to radiation induced cell killing, nor did it alter offspring splenocyte capacity for constitutive and post-radiation DNA repair.

Following their study demonstrating the induction of the adaptive response in rabbit lymphocytes irradiated *in vitro* (Cai and Liu 1990), Liu *et al.* (1992) demonstrated the induction of the same cytogenetic adaptive response by exposing rabbits to whole-body low-dose rate γ-radiation. In a similar study Wojcik and Tuschl (1990) reported an adaptive response induced in mouse lymphocytes irradiated *in vivo* by 5 cGy of γ-rays per day for 4 consecutive days. Lymphocytes were then isolated from the spleen up to 26 days following the initial exposure and challenged *in vitro* with UV light or mitomycin C (MMC). Using the endpoints of UV-light-induced unscheduled DNA synthesis and MMC-induced sister-chromatid exchanges, a

protective effect was observed in the pre-exposed mice for approximately 12 days post-exposure. A related study reported that T-lymphocytes isolated from the spleen of mice that had received a dose of 5-10 cGy of X-rays 7 hours prior to a challenge dose of 3 Gy of X-rays exhibited greater survival than those mice only receiving the high challenge dose (Yoshida *et al.* 1993). Survival was also shown to be enhanced in splenocytes and blood forming stem cells isolated from mice irradiated with low doses (5-10 cGy) of X-rays 2 months prior to a 7 Gy challenge dose (Yonezawa *et al.* 1990).

Recently Wang *et al.* (1998) demonstrated the existence of radioadaption during mouse embryogenesis. They showed that whole body irradiation of pregnant mice with 30 cGy (X-rays) on day 11 of gestation significantly reduced fetus malformations and mortality induced by a challenge dose of 5 Gy of X-rays on day 12. Tempel and Schleifer (1995), also working with embryos, demonstrated that brain and liver cells isolated from chicken embryos X-irradiated *in ovo* (5-30 cGy) showed resistance to a subsequent *in vitro* dose of X-rays (4-32 Gy). Bhattacharjee (1996) examined the role of the adaptive response on radiation induced thymic lymphoma (LT) in mice. Mice irradiated with 1 cGy of γ-rays 24 hours prior to a challenge dose of 2 Gy showed similar levels of LT to those just irradiated with 2 Gy. However, when multiple adapting doses, administered for 5 or 10 consecutive days, preceded the 2 Gy challenge dose, the yield of LT was significantly lower.

Other studies have used occupational and accidental exposures as a means to examine *in vivo* adaptive responses in human cell. Barquinero *et al.* (1995; 1996) showed that human lymphocytes isolated from individuals occupationally exposed to ionizing radiation exhibited fewer dicentrics than lymphocytes taken from non-occupationally exposed individuals following a challenge dose of 2 Gy γ-rays or exposure to the radiomimetic agent bleomycin. From this the authors suggest that "an adaptive response can...be induced in lymphocytes *in vivo* by very

low occupational doses of radiation". In contrast studies of lymphocytes isolated from children living in regions contaminated by fallout from the Chernobyl accident failed to demonstrate an adaptive response to a subsequent high challenge dose of ionizing radiation (Padovani *et al.* 1995) but did show a reduction in bleomycin sensitivity as compared with lymphocytes from control children (Tedeschi *et al.* 1995). In a follow up study the same group demonstrated that this adaptive response to bleomycin was related to continuous internal contamination rather than past acute exposure (Tedeshi *et al.* 1996).

Chapter I

Determining the relative biological effectiveness of low doses of tritium using the adaptive response in normal human fibroblasts

1.1. Introduction

In 1977 researchers Leona Samson and John Cairns observed, unexpectedly, that the mutation frequency of *E. coli* growing in the continual presence of low concentrations of the known mutagen, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) increased only in the first hour of treatment. For the remaining seven days of the experiment no further increase in the frequency of mutation was detected. Samson and Cairns next treated the cells with a low dose of MNNG and then challenged them with a subsequent dose which was 100 times greater. They found that the pre-adapted cells exhibited a significantly greater level of survival and a much lower mutation rate as compared to the non-adapted cells. Considering further findings that *de novo* protein synthesis was required to elicit the response, and a reduction in the rate of mutation rate was observed, the authors concluded that the adapting dose induced a previously uncharacterized error-free DNA repair pathway (Samson and Cairns 1977). Subsequent research revealed this to be an unusual mode of DNA repair which involved a number of specific alkyltransferases (reviewed by Frosina and Abbondandolo 1985). This initial discovery stimulated further study into this phenomenon, termed the adaptive response to alkylating agents, in mammalian (Frosina and Abbondandolo 1985; Lindahl *et al.* 1988) and plant cells (Heindorff *et al.* 1987a; Heindorff *et al.* 1987b; Rieger *et al.* 1990).

These preliminary findings prompted researchers to examine whether this phenomenon of the adaptive response could be induced by other DNA-damaging agents such as ionizing radiation. Initial studies carried out by Mitchel and Boreham, using yeast as a model system, were able to demonstrate similar adaptive responses to ionizing radiation and also showed that ionizing radiation resistance could be induced by prior exposure to heat or to UV radiation (Mitchel and Morrison 1982, 1984a, 1984b; Boreham *et al.* 1990; Boreham and Mitchel 1991, 1994; reviewed by Boreham *et al.* 1991). In 1984 Olivieri *et al.* were the first to demonstrate

reproducibly that low doses of ionizing radiation could induce apparent DNA repair mechanisms which rendered human cells, specifically lymphocytes, better able to cope with subsequent higher challenge doses. Olivieri's hypothesis was strengthened by the discovery that the adaptive response to ionizing radiation was inhibited by both cyclohexamide (Youngblom *et al.* 1989), which suggested the involvement of *de novo* protein synthesis and 3-aminobenzamide (Wiencke *et al.* 1986), an inhibitor of poly (ADP-ribose) polymerase, a protein known to be induced during the repair of DNA strand breaks (Benjamin and Gill 1980; Durkacz *et al.* 1980).

Although the majority of adaptive response experiments in human cells have used **lymphocytes** as their model system (Sanderson and Morley 1986; Moquet *et al.* 1987; Shadley *et al.* 1987; Wolff *et al.* 1988; Bosi and Olivieri 1989; Sankaranarayanan *et al.* 1989; Shadley and Wiencke 1989; Cortés *et al.* 1990; Fan *et al.* 1990; Khandogina *et al.* 1991; Wang *et al.* 1991; Wolff *et al.* 1991; Shadley and Dai 1992; Bai and Chen 1993; Domínguez *et al.* 1993; Shadley and Dai 1993; Kalina *et al.* 1994; Némethová *et al.* 1995; Vijayalaxmi *et al.* 1995; Wojcik and Streffer 1995; Wojcik *et al.* 1996; Wojewodzka *et al.* 1997; Gadhia 1998), the phenomenon has also been demonstrated in **Chinese hamster V79 cells** (Ikushima 1987; Osmak 1988; Sengupta and Bhattacharjee 1988; Sengupta and Bhattacharjee 1990; Ikushima 1989; Marples *et al.* 1994; Marples and Skov 1996), **rabbit lymphocytes** (Cai and Cherian 1996; Flores *et al.* 1996), **mice and mouse cell lines** (Fomenko *et al.* 1991; Farooqi and Kesavan 1993; Wang *et al.* 1998), **fish cells** (Kurihara *et al.* 1992), **Chinese hamster ovary cells** (Samson and Schwartz 1980; Laval 1988), ***Drosophila melanogaster* oocytes** (Fritz-Niggli and Schaeppi-Buechi 1991), **lepidopteran cells** (Koval 1984, 1986, 1988), **cervical carcinoma cells** (Kim *et al.* 1995), **C3H 10T1/2 mouse embryo cells** (Azzam *et al.* 1994b), a **human hybrid cell line** (Redpath and Antoniono 1998) and **human fibroblasts** (Azzam *et al.* 1992, 1994a, 1994b; Belyaev *et al.* 1996; Ishii and Misonoh 1996). To quantify adaption researchers have used several biological end points including: micronucleus formation, survival,

chromosome and chromatid aberrations, mutations and sister chromatid exchanges and neoplastic transformation.

Although the precise underlying mechanisms of the adaptive response remain elusive, research is proceeding under the hypothesis that low priming doses induce a number of cellular processes which have the cumulative effect of making cells refractory to subsequent high doses of radiation. These include cell cycle delays (Mitchel 1995), chromatin reorganization (Belyaev *et al.* 1996), and the induction of DNA repair, radical detoxification and various signal transduction pathways (reviewed by Stecca and Gerber 1998).

In terms of low level occupational exposure to radiation, one important source for Canadian atomic radiation workers to consider is tritium, a radioactive isotope of hydrogen that decays by β-emission and is a principal waste product generated by the fission process in CANDU Nuclear Reactors (NCRP 1979). Tritium is found in the form of tritium oxide (HTO) and is of particular importance as its biochemical nature is virtually indistinguishable from water. As a result it is readily absorbed through the lungs or skin with subsequent metabolization and distribution throughout the body. Consequently, gaining an understanding of the biological consequences of doses at levels experienced by atomic radiation workers is of clear importance. To that end numerous research groups have attempted to determine the relative biological effectiveness (RBE) of tritium. The results of these studies have been reviewed by Straume and Carsten (1993) and they show a wide discrepancy in values (1 - 5.9).

The purpose of the study presented here is to determine the RBE of occupationally relevant doses of tritium, specifically, low levels administered chronically. The endpoint measured was the level of adaption, as indicated by the radiation induced micronucleus frequency within radiation adapted cells. The adaptive response is a particularly suitable endpoint to follow because low doses can be seen to have significant biological effect.

Specifically, the level of adaption induced by tritium β-particles was compared to the level induced by the reference radiation, ^{60}Co γ-rays, in normal human fibroblasts at confluence.

1.2. Materials and methods

1.2.1. *Cell culture, irradiation and fixation*

The normal human male cell strain AG1522 (N.I.A. Aging Cell Culture Repository, Coriell Institute for Medical Research, Camden, NJ, USA) was maintained at 37 °C in D-MEM:F-12 culture medium (Gibco, Grand Island, NY, USA) supplemented with 15% fetal calf serum (Gibco), 2 mM glutamine and 25 μg/mL gentamicin sulfate (Gibco) and incubated in a humidified 2% CO_2/98% air atmosphere.

Initially cells, in passage 9, were seeded at $2x10^5$ cells/25 cm^2 flask (Nunc, Naperville, IL, USA). Seven days after the initial seeding, with one refeeding on day 4, the now confluent cultures (91% G_0/G_1, 7% G_2/M and 2% S, as determined by flow cytometry) were exposed to a single adapting dose (0.10, 0.50, 2.5, 10, or 50 cGy) of ^{60}Co γ-irradiation (GammaCell 200, Atomic Energy of Canada Ltd., Chalk River, ON, Canada) or tritium β-irradiation (see section 1.2.2.). Control samples were held in complete culture medium for an equivalent period of time. Adapting irradiations were performed in complete culture medium at 0 °C or 37 °C with a dose rate of 0.001 Gy/min or 0.003 Gy/min for the 0.10 cGy dose or remaining adapting doses, respectively. The cells were then washed six times in phosphate buffered saline (PBS) at 37 ° C, the medium replaced, and the cells allowed to adapt during a 3 h, 37 °C incubation prior to a challenge dose of 4 Gy of ^{60}Co γ-rays at 0 °C or 37 °C (dose rate: 1.29-1.07 cGy/sec depending on the activity of the ^{60}Co source). In some experiments, additional adapting periods of 6, 12 and 24 h were examined. It is important to note that doses administered at 0 °C, although given at the same dose rate, are considered acute. This is because damage accumulates throughout the exposure; after which the cells are released from the 0 °C block and return to normal cellular function.

Immediately following the 4 Gy challenge dose, the cells were detached using 1x trypsin-EDTA solution (Sigma, St. Louis, MO, USA) and $3x10^4$ cells were seeded into chambered slides (Nunc) containing 5 μg/mL cytochalasin B (Sigma), a fungal metabolite which inhibits cytokinesis while allowing nuclear division. As a result, those cells which have divided once are easily identified by their binucleate state. It is within there cells that micronuclei were enumerated for the purpose of establishing the level of adaption. After 72 h incubation at 37 °C the medium was removed and the cells fixed. First, a hypotonic treatment of 1% sodium citrate (w/v) was added for 5 min to swell the cells. An equal volume of 3:1 methanol:glacial acetic acid (v/v) was then added and the cells were incubated for an additional 5 min. The mixture was removed and replaced by 3:1 methanol:glacial acetic acid (v/v) for 10 min. All solutions and incubations were at room temperature. The chambers were removed and the slides air dried. Slides were stored at -20 °C and cells were stained with 1:1 4',6-diamidino-2-phenylindole (DAPI, 0.1 μg/mL):propidium iodide (2.5 μg/mL) (both Oncor, Gaithersburg, MD, USA). Micronuclei in 500 binucleate cells per treatment were counted, using an epifluorescence microscope equipped with the appropriate single-bandpass filters, according to criteria previously described (Fenech 1993). In the experiment to determine the kinetics of the response, 1000 binucleate cells per treatment were scored.

1.2.2. *Tritium dosimetry*

Activity in the medium used for tritium β-irradiation was assessed using a liquid scintillation counter. Dose rate, in cGy/min, was then determined by multiplying the resultant mCi/mL output by a tritium energy conversion constant (0.2027) determined by the following:

(1Ci/1000mCi) ($37x10^9$Bq/1000mCi) (1decay/sec/Bq) ($5.7x10^{-3}$MeV/1decay)

($1.602x10^{-6}$erg/1MeV) (1rad/100erg/g) (60sec/1min) (1cGy/1rad) (1mL/1g)

and a correction factor for the water content of a cell (**0.838**, Liber *et al.* 1985). Note that the above calculation assumes the average energy of a tritium decay to be 5.7×10^{-3}MeV.

1.2.3. *Cell cycle analysis*

The protocol used was a modification of Vindelov's *pH 10 procedure* (Vindelov 1977) and is described by Szekely *et al.* (1989). Briefly, 10^6 cells, detached by trypsinization and washed by centrifugation in phosphate buffered saline (PBS), were resuspended in 200 µL pH 7.6 TRIS buffer at 0 °C. The entire volume was then added to 4 mL *Solution A* (0.075% glycine (w/v), 1.753% NaCl (w/v), 0.10% Triton-X-100 (v/v), 0.05% RNase A (w/v) and 5 µg/mL ethidium bromide, pH 10) at 0 °C. The solution was mixed by inversion, incubated for 10 min at 0 °C and stored at 4 °C until required for flow cytometry analysis.

All measurements were performed on a Coulter EPICS 752 flow cytometer/sorter in the laboratory of Dr. B. Boyes of the Toxicology Department at Health Canada in Ottawa. The flow cytometer was fitted with a 5 W water-cooled argon laser set at 488 nm wavelength. The samples were run at 150 mW laser output. Red fluorescence (the ethidium bromide signal) was distinguished by a 635 nm bandpass filter. All signals collected were manipulated through the MDADS (Multiparameter Data Acquisition and Display System) installed in the flow cytometer. The average flow rate was maintained between 100 and 300 events/second.

1.2.4. *Statistical analysis*

The micronucleus frequency was calculated as:

$$\mathbf{F_m = (\Sigma MN/\Sigma BNC)(100)}$$

where F_m is the frequency of micronuclei as a percentage; ΣMN is the total number of micronuclei scored within the binucleate cell population; ΣBNC is the total number of binucleate cells scored in a given treatment.

To determine whether the micronucleus frequency within adapted cells was significantly different from the frequency within non-adapted cells, a one tailed single factor analysis of variance (ANOVA) was used. This assessed whether all means were equal (the null hypothesis) or, alternatively, not equal (the alternate hypothesis). Upon rejection of the null hypothesis and acceptance of the alternate, the Tukey test, a multiple pairwise comparison procedure, was employed to determine those means significantly different from each other.

To assess whether the level of adaption was equal for tritium β-irradiation and ^{60}Co γ-irradiation (in those samples shown to have a statistically significant adaption) a ***two*** factor (***radiation type***: β and γ; ***dose***: 0.10, 0.50, 2.5, 10, and 50 cGy) ANOVA with unequal but proportional replication was employed. For this analysis only the micronucleus frequencies within the adapted treatments for each radiation type and dose at *either* 0 °C or 37 °C were used. That is non-adapted cells exposed to the 4Gy challenge dose were excluded from analysis.

It should be noted that according to statistical theory, percentage data, such as is presented here, form a binomial rather than a normal distribution. Consequently, since the tests employed have an assumption of normality, all data were transformed to their arcsine prior to analysis. The resultant data form a distribution that is nearly normal (Zar 1984). In all cases significance was assessed at the 5% level (i.e. α=0.05).

1.3. Results

1.3.1. *Growth characteristics of AG1522 human fibroblasts*

The AG1522 fibroblast culture was initiated in 1976 from explants of minced foreskin obtained from a clinically normal 3 day old male. Cell morphology is fibroblast-like with exponentially growing cells assuming a well spread out multipolar shape. Proliferation of AG1522 proceeds in a density limited anchorage-dependent manner that generates, at confluence, a monolayer of bipolar or spindle shaped cells attached to the growth surface. This confluent state is seen in the plateau phase of the growth curve illustrated in Figure 1.1 which depicts cell number as a function of time after seeding.

The cell cycle distribution at various times after seeding is given in Table 1.1. Seven days post-seeding was chosen as the treatment day for studies presented here and in the subsequent chapters, as the cells, upon visual inspection, appeared highly confluent and contained the maximal percentage of G_0/G_1 (Table 1.1). An example of a Day 7 cell cycle distribution is illustrated in Figure 1.2. In this example the G_0/G_1 subpopulation comprised 90.54% of the total population.

Once the growth regime had been established, the required concentration of cytochalasin B was determined. This was significant for two reasons. Firstly, it was important to ensure that cells were not escaping the action of the cytochalasin B and consequently being lost to analysis. As well, maximizing the percentage of binucleate cells enhances scoring efficiency. Figure 1.3 shows that the cytochalasin B dose response curve initially plateaued at 4-5 μg/mL. As a result 5 μg/mL was the concentration of cytochalasin B chosen for this and future studies. Higher concentrations were excluded as the frequency of nuclear extrusion, a phenomenon previously reported to be induced by cytochalasins (Carter 1972), was significantly enhanced and as a result greatly impeded scoring.

To determine a sufficient period of incubation in cytochalasin B, a time course was set up using the pre-established 5 μg/mL concentration. Figure 1.4 shows that incubation for 72 h yielded the greatest percentage of binucleate cells (35%) of the time points examined and therefore was chosen as the length of cytochalasin B incubation for this and subsequent studies (unless otherwise noted). It should be noted that up to and including 72 h, tri- and tetranucleate cell levels were negligible and therefore are not shown.

1.3.2. *Determination of the RBE of low doses of tritium using the adaptive response*

The adaptive response is a phenomenon which has been shown to be sensitive to low doses of ionizing radiation. It is that sensitivity which was used here as a means of assessing the RBE of low doses of tritium β-irradiation as compared to a ^{60}Co γ-ray reference radiation. The level of adaption induced by the low adapting dose of ionizing radiation was measured by the micronucleus frequency within binucleate cells. An example of binucleate cells with and without an associated micronucleus, as well as a mononucleate cell, are illustrated in Figure 1.5.

The level of adaption induced by ^{60}Co γ-rays and by tritium β-particles at 37 °C is illustrated in Figures 1.6 and 1.7, respectively. As determined by the Tukey test, cells adapted by exposure to either radiation at any of the doses tested were significantly different from non-adapted cells, as measured by their response to a subsequent dose of 4 Gy (P<0.05). Considering only the adapted cells, a two factorial ANOVA (Table 1.2) demonstrated that the level of adaption was the same regardless of radiation type (i.e. γ-rays or β-particles) or radiation dose (0.10, 0.50, 2.5, 10 and 50 cGy) (P>0.25). This same statistical analysis applied to the results from cells adapted to radiation by exposure at 0 °C produced an equivalent result (Figure 1.8, 1.9 and Table 1.3). Thus the level of adaption was the same, whether induced by the reference radiation γ-rays or the test β-particles. This demonstrates that, using adaption as the endpoint,

the RBE for low doses of tritium is 1. In these experiments adaption was allowed to develop during a fixed 3 h incubation. The results also show, therefore, that doses of γ- or β-irradiation as low as (on average) one track per cell (0.10 cGy) (Bond *et al.* 1988) initiated the same maximum rate of development of the adaptive response as did doses that produced many tracks per cell (Figure 1.6-1.9).

1.3.3. *Kinetics of the adaptive response*

The kinetics of the adaptive response were examined using 10 cGy ^{60}Co γ-irradiation as the adapting dose followed by incubation for 3, 6, 12 or 24 h prior to a 4 Gy test dose (Figure 1.10 A). The magnitude of adaption was found to peak at 3 h and was not observable after 6 h incubation. Figure 1.10 B illustrates the results of holding adapted and non-adapted cells in confluence for an additional 24 h after the 4 Gy challenge dose. Comparing the micronucleus frequency between the two treatments, there was no observed difference. However, it is interesting to note that the level of induced adaption (Figure 1.10 A, adapted treatments) does not reach or surpass the reduction observed after simply holding the cells in confluence for an additional 24 h (Figure 1.10 B, 4 Gy non-adapted treatment).

1.3.4. *Tritium dosimetry*

Figure 1.11 shows one example of the efficiency of the washing procedure for the removal of tritium from the cells. After each wash there was an approximate 70-75% reduction in activity such that after the 6 washes only background levels of activity were detectable within the cells (one trial, remainder of data not shown).

Figure 1.1 Growth curve of AG1522 normal human fibroblast cells. The increase in cell number is shown as a function of time after seeding. The curve represents one growth experiment.

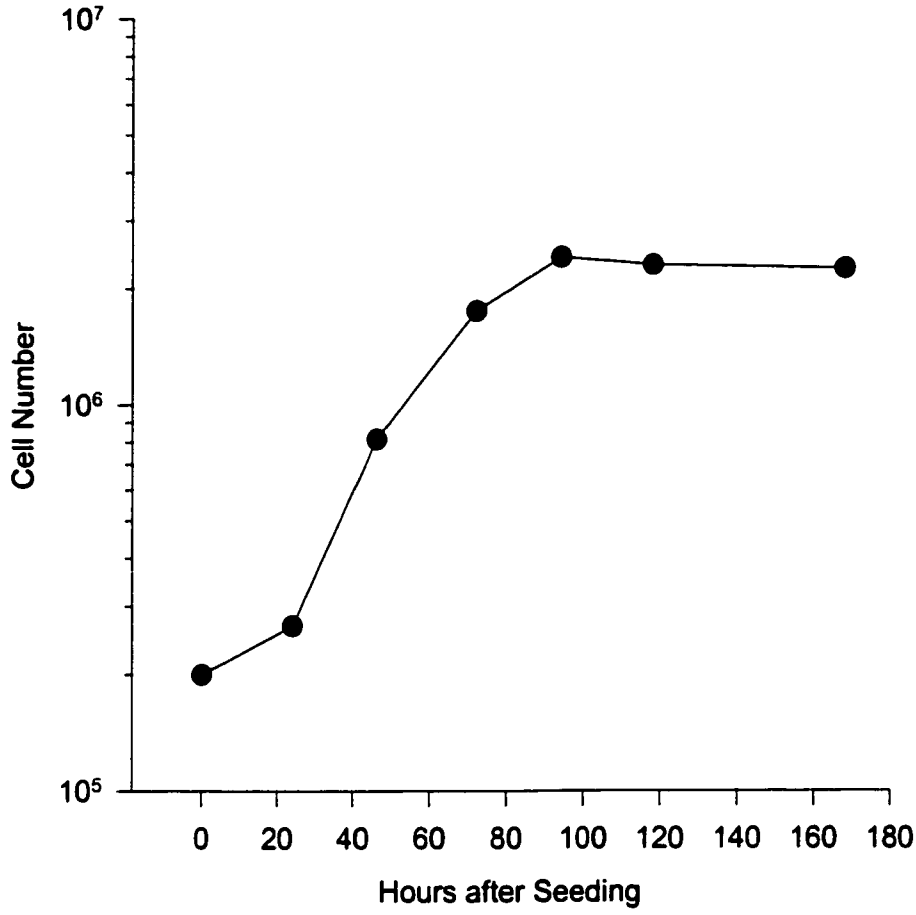

10^7
10^6
10^5
Cell Number
0
20
40
60
80
100
120
140
160
180
Hours after Seeding

Table 1.1 Cell cycle distribution for AG1522 fibroblasts after initial seeding. Mean results are reported ±standard deviation, n=2.

	Day 3	Day 5	Day 6	Day 7
% G1/G0	77.07±0.55	87.79±1.49	88.49±0.45	90.90±0.52
% S	11.46±1.05	0.92±1.30	2.32±1.66	1.80±0.60
% G2/M	11.46±1.60	11.32±2.79	9.20±2.11	7.29±0.08
Time (h) of refeeding prior to cell cycle analysis		48	72	91 25.5

Figure 1.2 Flow cytometric analysis of confluent AG1522 fibroblasts 7 days after seeding, with one refeeding on day 4.

G_0/G_1:	90.54%	**X axis**:	DNA content
G_2/M:	7.23%	**Y axis**:	Granularity
S:	2.23%	**Z axis**:	Number of counts

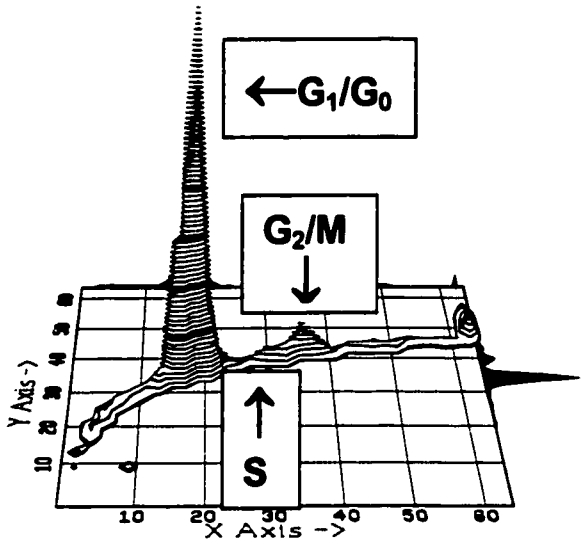


Figure 1.3 Cytochalasin B dose response curve for AG1522 fibroblast cells. Binucleate cell frequency was assessed 48 h following seeding. Mean results are reported ±standard deviation, n=2.

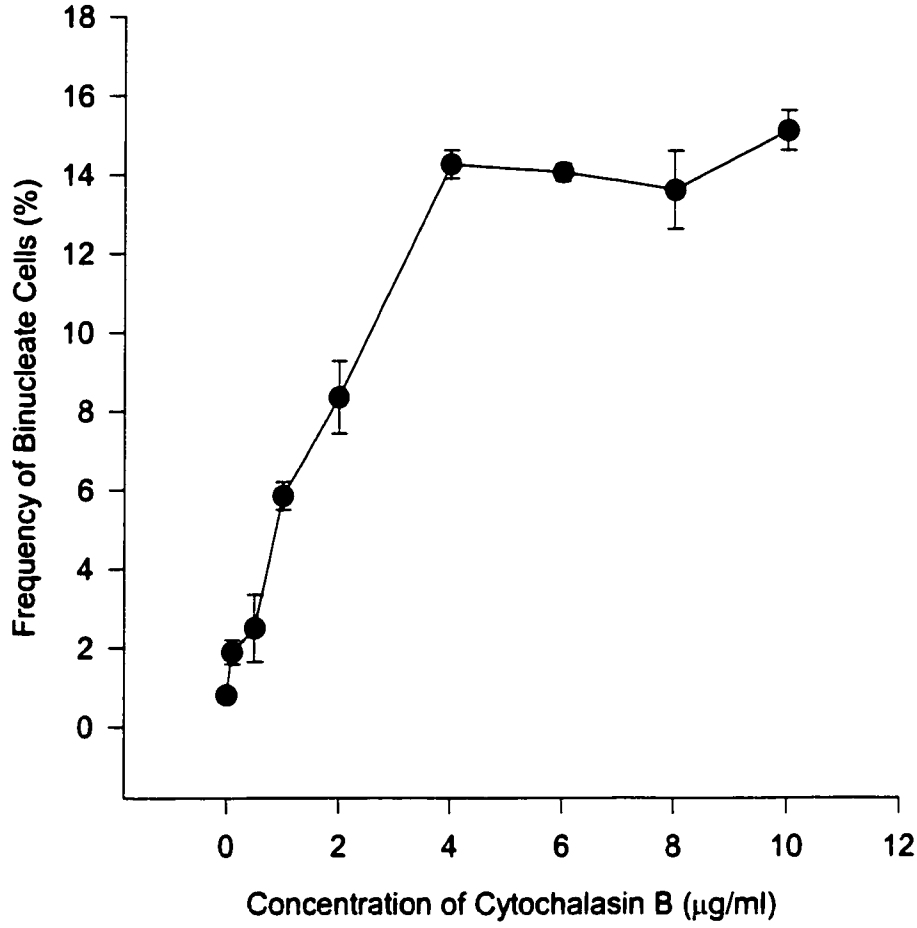

Frequency of Binucleate Cells (%)
18
16
14
12
10
8
6
4
2
0
0
2
4
6
8
10
12
Concentration of Cytochalasin B (µg/ml)

Figure 1.4 AG1522 fibroblast binucleate cell frequency induced by 5 μg/mL cytochalasin B and monitored as a function of time after seeding. Mean results are reported ±standard deviation, n=2.

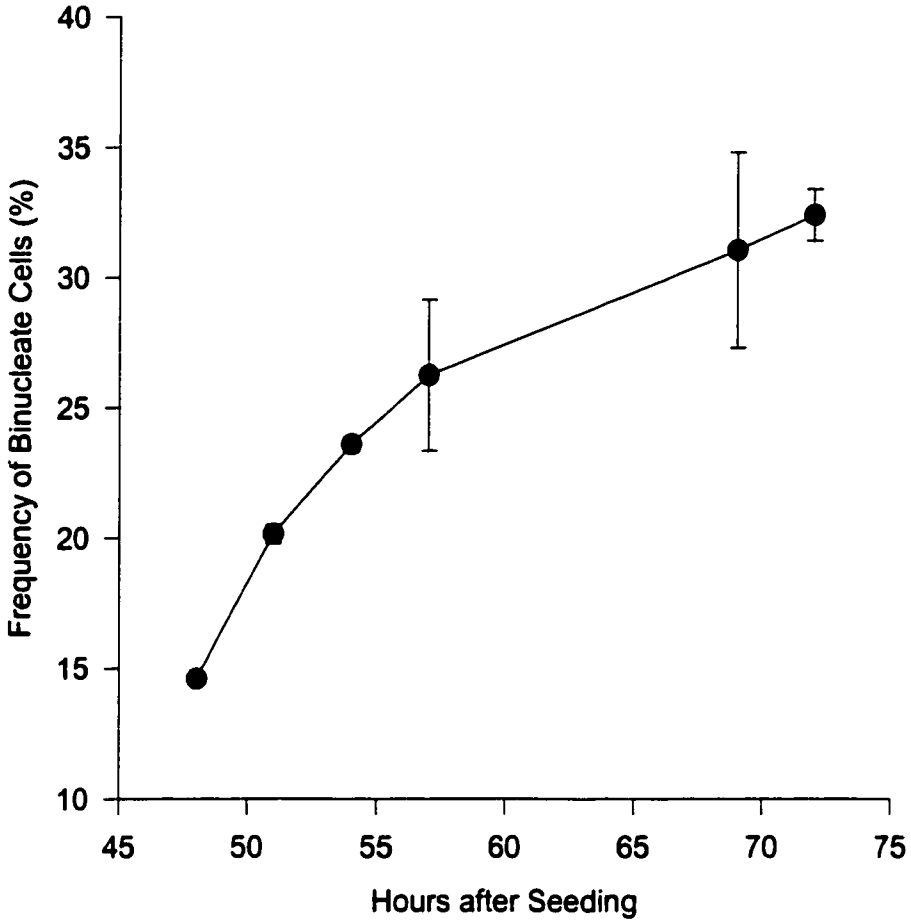

Frequency of Binucleate Cells (%)
40
35
30
25
20
15
10
45
50
55
60
65
70
75
Hours after Seeding

Figure 1.5 AG1522 fibroblasts fixed with 3:1 methanol:glacial acetic acid and stained with DAPI:propidium iodide. Arrow indicates a micronucleus contained within a binucleate cell. Image was captured under epifluorescence at 630x magnification with a Sony 3CCD Colour Video Camera, processed with Northern Eclipse Image Analysis Software (Version 2.0, Empix Imaging, Inc., Mississauga, ON, Canada). Bar, 10 μm.

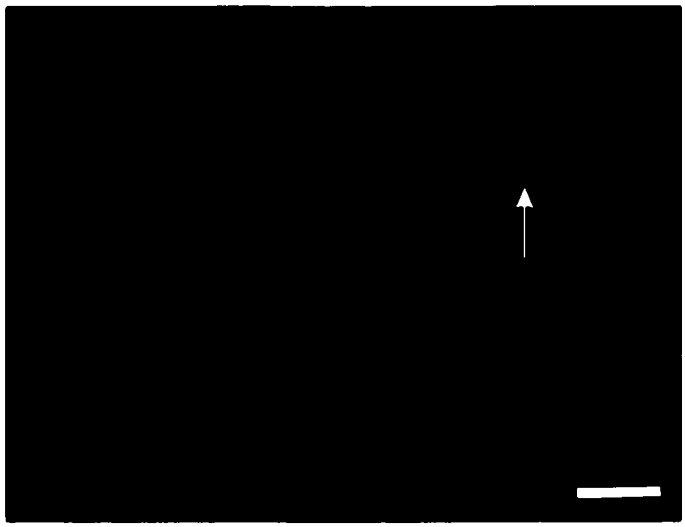


Figure 1.6 Level of adaption induced by ***^{60}Co γ-irradiation at 37 °C*** as indicated by micronucleus frequency within binucleate cells. All cells were incubated for 3 h at 37 °C after exposure to the adapting dose. Gray bar indicates control, unadapted cells exposed to the 4 Gy challenge dose. *Denotes adapted treatments significantly different than non-adapted, $P<0.05$. Mean results are reported ±standard deviation, n=3.

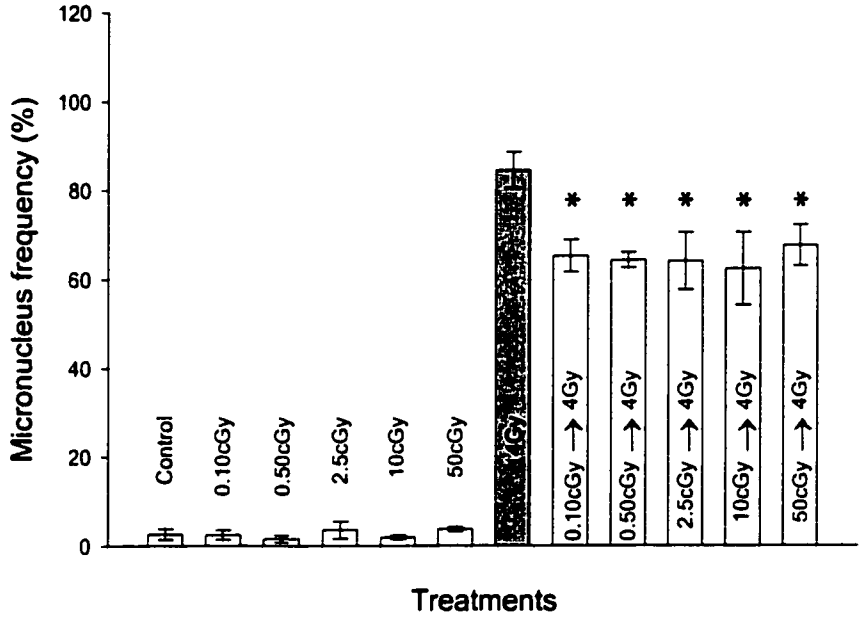

Micronucleus frequency (%)
120
100
80
60
40
20
0
Control
0.10cGy
0.50cGy
2.5cGy
10cGy
50cGy
4Gy
0.10cGy → 4Gy
0.50cGy → 4Gy
2.5cGy → 4Gy
10cGy → 4Gy
50cGy → 4Gy
*
*
*
*
*
Treatments

Figure 1.7 Level of adaption induced by ***tritium β-irradiation at 37 °C*** as indicated by micronucleus frequency within binucleate cells. All cells were incubated for 3h at 37 °C after exposure to the adapting dose. Gray bar indicates control, unadapted cells exposed to the 4 Gy challenge dose. *Denotes adapted treatments significantly different than non-adapted, $P<0.05$. Mean results are reported ±standard deviation, n=2.

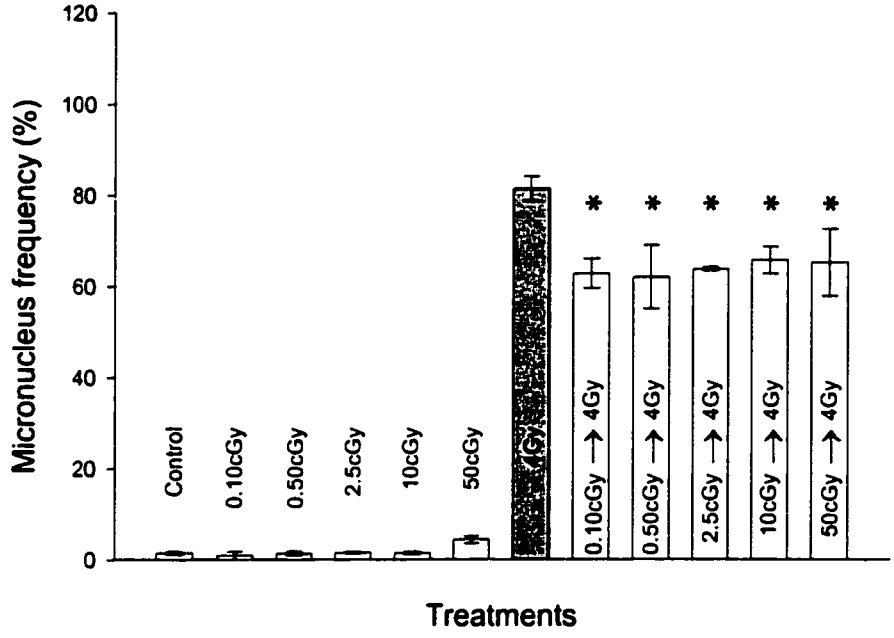

120
100
80
60
40
20
0
Micronucleus frequency (%)
Control
0.10cGy
0.50cGy
2.5cGy
10cGy
50cGy
4Gy
0.10cGy → 4Gy
0.50cGy → 4Gy
2.5cGy → 4Gy
10cGy → 4Gy
50cGy → 4Gy
*
*
*
*
*
Treatments

Figure 1.8 Level of adaption induced by ***^{60}Co γ-irradiation at 0 °C*** as indicated by micronucleus frequency within binucleate cells. All cells were incubated for 3h at 37 °C after exposure to the adapting dose. Gray bar indicates control, unadapted cells exposed to the 4 Gy challenge dose. *Denotes adapted treatments significantly different than non-adapted, $P<0.05$. Mean results are reported ±standard deviation, n=3.

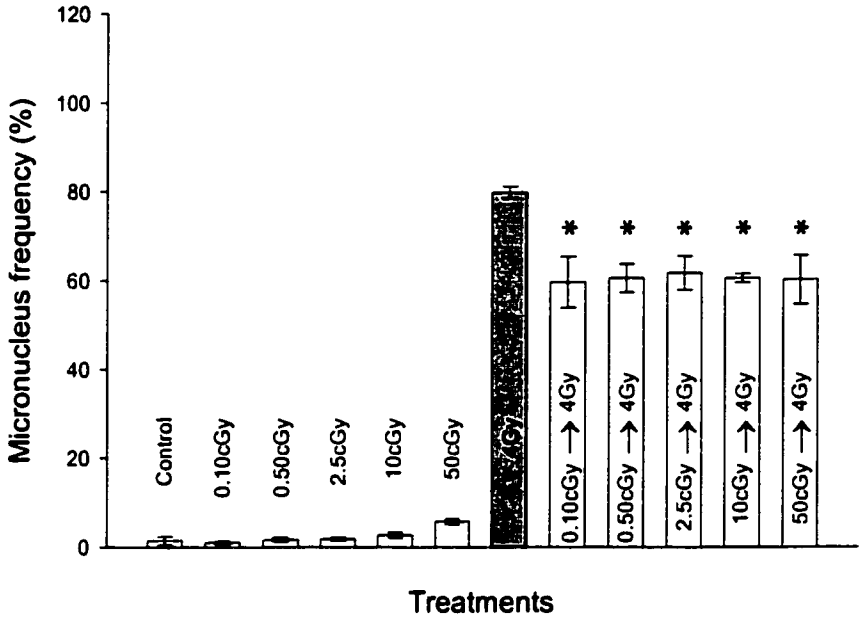

Micronucleus frequency (%)
120
100
80
60
40
20
0
Control
0.10cGy
0.50cGy
2.5cGy
10cGy
50cGy
4Gy
0.10cGy → 4Gy
0.50cGy → 4Gy
2.5cGy → 4Gy
10cGy → 4Gy
50cGy → 4Gy
*
*
*
*
*
Treatments

Figure 1.9 Level of adaption induced by ***tritium β-irradiation at 0 °C*** as indicated by micronucleus frequency within binucleate cells. All cells were incubated for 3h at 37 °C after exposure to the adapting dose. Gray bar indicates control, unadapted cells exposed to the 4 Gy challenge dose. *Denotes adapted treatments significantly different than non-adapted, $P<0.05$. Mean results are reported ±standard deviation, n=2.

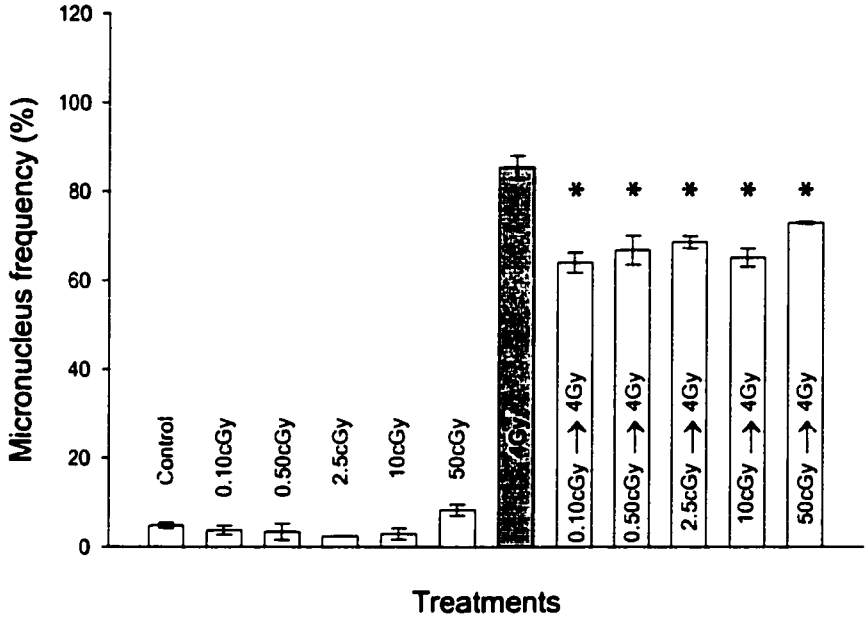

120
100
80
60
40
20
0
Micronucleus frequency (%)
Control
0.10cGy
0.50cGy
2.5cGy
10cGy
50cGy
4Gy
0.10cGy → 4Gy
0.50cGy → 4Gy
2.5cGy → 4Gy
10cGy → 4Gy
50cGy → 4Gy
*
*
*
*
*
Treatments

Table 1.2 Two factor ANOVA of tritium β-irradiation and ^{60}Co γ-irradiation on the ***level of adaption at 37 °C***. Five different adapting doses (0.10, 0.50, 2.5, 10 and 50 cGy) were compared. H_o is accepted in all cases.

Effect on level of adaption	Critical Value	$F_{0.05(1),4,15}^{*}$ $F_{0.05(1),1,15}^{\ddagger}$	*P*-value	
Adapting doses	0.00585	3.06*	>0.25	Do not reject H_o: There is no difference in level of adaption among the different adapting doses.
Type of radiation	1.01	4.54‡	>0.25	Do not reject H_o: There is no effect on level of adaption with respect to type of radiation used as adapting dose.
Interaction of radiation type and adapting dose	0.000835	3.06*	>0.25	Do not reject H_o: There is no interaction of radiation type and size of adapting dose with respect to level of adaption.

Tabie 1.3 Two factor ANOVA of five different adapting doses of tritium β-irradiation and ^{60}Co γ-irradiation on the ***level of adaption at 0 °C***. Five different adapting doses (0.10, 0.50, 2.5, 10, and 50 cGy) were compared. H_o is accepted in all cases.

Effect on level of adaption	Critical Value	$F_{0.05(1),4,15}^{*}$ $F_{0.05(1),1,15}^{\ddagger}$	*P*-value	
Adapting doses	0.000960	3.06*	>0.25	Do not reject H_o: There is no difference in level of adaption among the different adapting doses.
Type of radiation	1.01	4.54‡	>0.25	Do not reject H_o: There is no effect on level of adaption with respect to type of radiation used as adapting dose.
Interaction of radiation type and adapting dose	0.00115	3.06*	>0.25	Do not reject H_o: There is no interaction of radiation type and size of adapting dose with respect to level of adaption.

Figure 1.10 Kinetics of the adaptive response as indicated by micronucleus frequency within binucleate cells. (A) Adaption was induced by 10 cGy ^{60}Co γ-irradiation followed by a 3, 6, 12, or 24 h incubation prior to the 4 Gy challenge dose. (B) Following the 4 Gy challenge dose cells were held for an additional 24 h prior to release from confluence. Gray bars indicate 4 Gy challenge dose controls. *Denotes adapted treatment significantly different than non-adapted, $P<0.05$. Mean results ±standard deviation are reported, n=3.

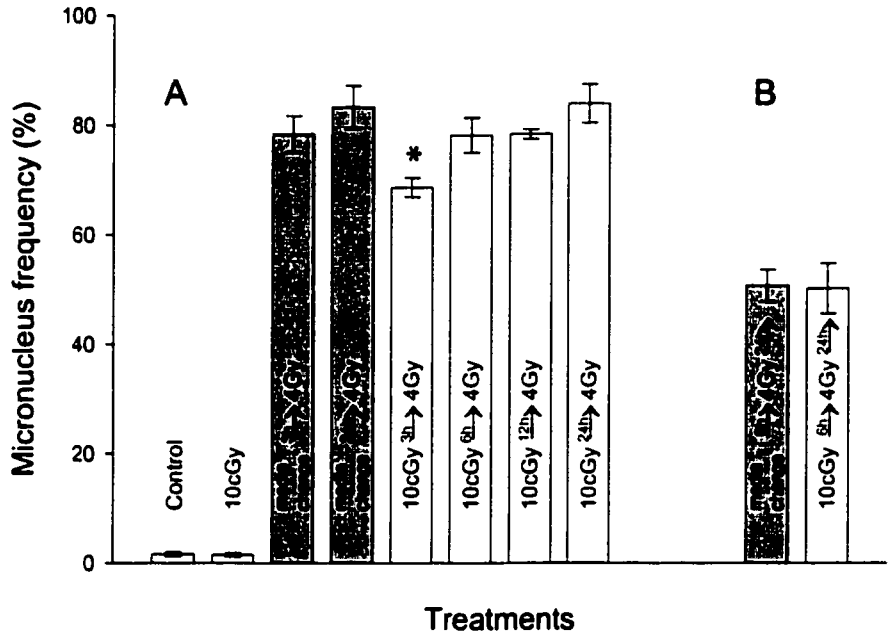
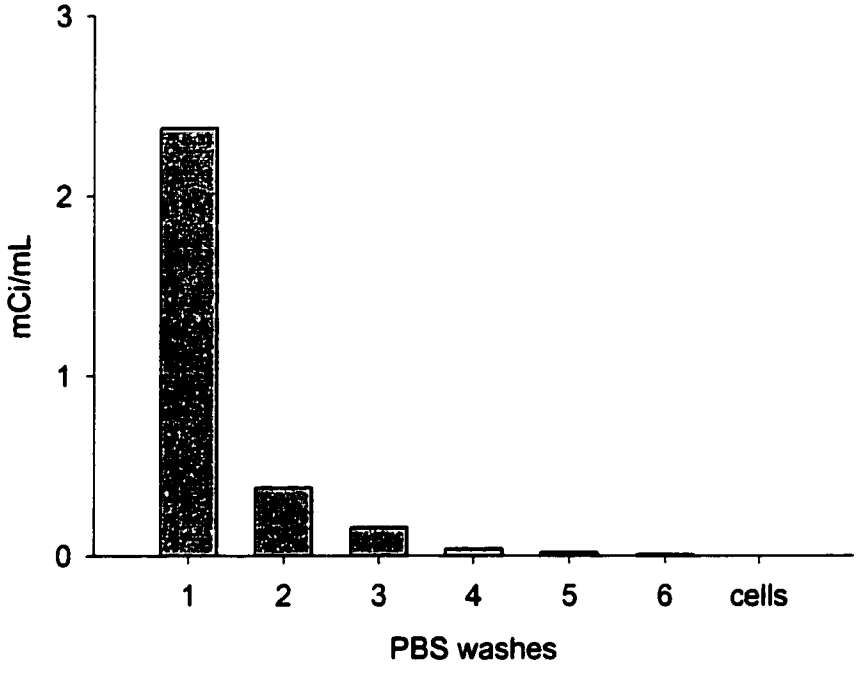

100
80
60
40
20
0
Micronucleus frequency (%)
A
B
*
Control
10cGy
10cGy 3h→ 4Gy
10cGy 6h→ 4Gy
10cGy 12h→ 4Gy
10cGy 24h→ 4Gy
10cGy 6h→ 4Gy 24h→
Treatments

Figure 1.11 Removal of tritium from cells. The figure shows the tritium activity in 6 consecutive PBS washes and in cells after the 6^{th} wash, as measured by a liquid scintillation counting.


3
2
1
0
mCi/mL
1
2
3
4
5
6
cells
PBS washes

1.4. Discussion

1.4.1. *Determination of the relative biological effectiveness of tritium*

Relative biological effectiveness (RBE) is defined as the ratio of absorbed dose of a reference radiation (i.e. gamma) to the absorbed dose of a test radiation (i.e. beta) required to produce the same biological effect (QCDR 1979). The level of adaption induced by exposure of cells to tritium β-particles (at 0 °C or 37 °C), was measured by the micronucleus frequency produced by a 4 Gy γ-radiation exposure within binucleate human fibroblast cells. The RBE of low doses of tritium, in the range of 0.10-50 cGy, delivered either chronically (37 °C) or acutely (equivalent dose and dose rate, but administered at 0 °C to preclude DNA repair) was shown to be 1. Other *in vitro* studies using endpoints such as transformation, mutagenesis, cell killing and various cytogenetic assays have reported a wide variety of RBE values for tritium. The values range from 1.0-5.9 with a standard deviation of 1.0 and a mean of 2.26, n=26 (references within Straume and Carsten 1993). It should be noted that the reference radiation was not the same for all studies; either 250 kVp X-rays, ^{60}Co γ-rays, or ^{137}Cs γ-rays were employed. In addition, the values were obtained using radiations at higher doses and dose rates than would be seen by either the public or atomic radiation workers. Dobson and Kwan (1976) and others (Straume and Carsten 1993; Ujeno 1982) have suggested that the RBE of tritium increases under chronic irradiation conditions. Clearly, such findings would have important implications with respect to establishing radiation protection guidelines. However, the data presented here show that doses administered chronically (0.003 Gy/min at 37 °C) or acutely show no increase in the RBE of tritium (Figure 1.6-1.9).

To the best of my knowledge this is the first study to examine the RBE of tritium at occupational exposure levels. Thus, additional study, using other endpoints sensitive to low doses, is required to further clarify the RBE of low doses of tritium. One technique which may

prove beneficial is the Dynamic Microscope Image Processing Scanner (DMIPS) cell analyzer assay (Palcic and Jaggi 1986; Spadinger and Palcic 1993). DMIPS is able to trace the reproductive history of individual living cells following low dose irradiation and, as a result, is able to generate survival curves at extremely low doses. In fact this technique has been recently utilized to examine the RBE of ^{60}Co γ-rays, 55 kVp X-rays, 250 kVp X-rays and 11 MeV electrons at low doses (Spadinger and Palcic 1992). It should be noted, however, that at the present time this technique's limit of detection is still two orders of magnitude greater than the lowest dose presented here, and that cell killing is not usually an important risk factor for occupational exposures.

1.4.2. *Insights into the adaptive response*

The underlying molecular mechanisms that elicit the adaptive response have, historically, been elusive. Recently, however, research has been advancing on this front, resulting in the identification of several necessary components (reviewed in the *Literature Review* chapter). One integral part of the process that has yet to be definitively determined in mammalian cells, however, is the identification of the trigger that initiates the phenomenon.

Ionizing radiation generates a spectrum of DNA lesions within the cell via direct ionization events within the DNA itself and indirect action through the production of highly reactive ion radicals and free radicals, typically via radiolysis of water (Hall 1978). Types of damage include DNA double strand breaks (dsb), DNA single strand breaks (ssb), DNA-protein cross-links, and various base and sugar modifications. Of these lesions, all, some or possibly none are potential candidates as the eliciting signal. It is reasonable to assume that for a trigger to be effective it must occur at a level, or with a spatial distribution, significantly greater than or different from the background endogenous damage, oxidative and otherwise, which occurs naturally as a result of everyday cellular processes (reviewed by Lindahl 1993). Billin

(1990) has estimated the spontaneous rate of DNA damage per cell per day to be as follows: 121,000 ssb, 30,000 base lesions, and 0.01 dsb. In terms of radiation induced DNA damage, Elkind and Redpath (1977) determined that the number of damage events per 0.10 cGy per cell to be 1 ssb, 0.95 base lesions and 0.04 dsb. Considering that the smallest adapting dose used in this study was 0.10 cGy and comparing the spontaneous rate to the radiation induced rate presented above, it is clear that the only lesion type that has a significant increase in incidence over the spontaneous level is DNA dsb (4 fold). In contrast however, the level of induction of the remaining lesions, ssb and base modifications, are negligible when compared with endogenous levels. It should be noted that the spontaneous rate of DNA-protein cross-links has not yet been determined, although the induction rate per 0.10 cGy per cell is known to be 0.015 (Ward 1988). Considering the relative levels of spontaneous to induced events within specific types of DNA damage and the demonstration that doses as low as 0.10 cGy are able to induce adaption (Figure 1.6-1.9), together they suggest that DNA dsb may be an integral component that signals the adaptive response in normal human fibroblasts. It should be noted that other possible eliciting signals exist, such as ionization of proteins, the plasma membrane, or the rapid production of free radicals *per se*. For the latter to be feasible it is likely the cellular process or processes which sense this rapid increase in ionization events must be functioning at the time of production of these very short lived highly reactive species. Figure 1.8 and 1.9, however, show that 0.10 cGy is able to initiate the adaptive response when given at 0 °C, a temperature at which such cellular processes would be nonfunctional. Thus, it seems that the lesion that signals the adaptive response must be stable, at least relative to free radicals. There are very few studies that have directly studied the eliciting signal for the adaptive response. One such investigation was carried out by Boreham and Mitchel (1991), who examined the level of adaption induced by low LET gamma radiation versus high LET neutrons in the yeast *Saccharomyces cerevisiae* (see appendix for LET definition). They found that for

equivalent doses of each radiation, gamma was more effective at adapting yeast than neutrons. As gamma radiation generates a higher ratio of DNA single strand breaks to double strand breaks Boreham and Mitchel (1991) concluded that DNA single strand breaks may be the important lesion that signals the induction of adaption.

It is interesting to note that if a 4 fold increase in DNA dsb is indeed sufficient to initiate the adaptive response, based on the numbers above, only 4 in every 100 cells would have received the eliciting signal. This further suggests that the adaptive response may be mediated via some form intercellular communication. Trosko (1994) was the first to speculate on the possible involvement of gap junctional intercellular communication in the adaptive response and it was recently demonstrated by Ishii and Watanabe (1996). Using human embryonic cells and application of the gap junctional inhibitor 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) during the adapting dose (13cGy X-rays) they were able to show that complete inhibition of intercellular communication corresponded with the elimination of the adaptive response as measured by the trypan blue dye-exclusion test.

It should be noted that other stresses such as heat are also able to induce radioadaption. Boreham and Mitchel (1994) found that a mild heat exposure of 40 °C for 15 min administered 12 h prior to a challenge dose of 4 Gy significantly reduced the micronucleus frequency within human fibroblasts. Such mild hyperthermia is unlikely to produce DNA damage or reactive radicals suggesting multiple induction mechanisms exist and that they share common pathways in the molecular chain of events that generate the adaptive response. Other adapting agent substitutions have also have also been demonstrated both *in vitro* and *in vivo* with hydrogen peroxide, UV B, and radiomimetic agents such as bleomycin (reviewed by Stecca and Gerber 1988).

Figures 1.6-1.9 demonstrate further that doses as low as (on average) one track per cell

followed by a 3 h incubation period were able initiate the same maximum rate of development of the adaptive response as did doses that produce many tracks per cell (ex. 50cGy). From this follows the question; do even smaller doses also have the capacity to initiate the adaptive response? Such studies are in progress.

Its seems clear from this and other studies that within mammalian cells multiple pathways may exist that are able to initiate the processes necessary to generate the adaptive response. Further, a single low dose, of approximately background or occupational exposure levels, is able to reduce the potential risk of DNA damage associated with a subsequent larger dose of ionizing radiation. This finding has important implications with respect to establishing low-level radiation protection guidelines, as it contradicts the currently accepted linear no-threshold model (for a review of this topic please refer to the *Literature Review* chapter).

Chapter II

Fluorescence *in situ* hybridization of micronuclei using directly labelled DNA probes: A protocol for cytoplasm preservation and its application

2.1. Introduction

Chromosomal damage can be assessed quantitatively using a variety of endpoints. Due to its relative simplicity and rapidity, micronucleus frequency is one measure that has been used extensively. Micronuclei are small spherical bodies found in the cytoplasm of interphase cells and contain acentric DNA fragments originating from unrepaired chromosomal breaks or whole chromosomes that lag because of a faulty spindle apparatus or damaged kinetochore. Such DNA fails to be incorporated in either daughter nucleus following nuclear division, and instead becomes encapsulated in its own nuclear envelope (Walker *et al.* 1996). To facilitate micronucleus scoring, the cytokinesis-block technique was developed (Fenech and Morley 1985; Fenech 1993). This approach employs cytochalasin B, a fungal metabolite that allows nuclear division while inhibiting cytokinesis. Consequently, cells that have undergone one division can be identified by the presence of two nuclei. The frequency of micronuclei found in these binucleate cells has been used to measure the aneugenic or clastogenic capabilities of chemical and physical agents in a wide variety of cell cultures (Tucker and Preston 1996).

Recently a number of fluorescence *in situ* hybridization (FISH) techniques have been used to investigate the chromosome content of radiation and chemically induced micronuclei in binucleate cells using various combinations of whole chromosome specific paints, centromeric probes and telomeric probes (Slavotinek *et al.* 1996; Walker *et al.* 1996; Fimognari *et al.* 1997; Vral *et al.* 1997; Wuttke *et al.* 1997). In such studies it is important to be able to identify, accurately and unambiguously, binucleate cells and any associated micronuclei within a population of mononucleate cells. Ideally this is achieved by preserving cell cytoplasm throughout the *in situ* hybridization process. However most studies have been unable to accomplish this and instead have based the identification of a binucleate cell on the close proximity of two nuclei each having roughly the same size and shape (Slavotinek *et al.* 1996). Without cytoplasm to distinguish one cell from another these scoring criteria are highly

subjective, not only with respect to the identification of binucleate cells, but also as to the origins of a given micronucleus. In addition, this difficulty necessitates low cell density as a means of reducing ambiguities in association, greatly reducing scoring efficiency and increasing costs. These concerns prompted the development of the protocol described here which allows *in situ* hybridization of a directly labelled DNA probe to interphase nuclei and micronuclei while maintaining binucleate cell cytoplasm. This protocol was then applied using a degenerate oligonucleotide primed PCR (DOP-PCR) generated directly labelled human centromeric DNA probe to examine the centromere content of radiation induced micronuclei in binucleate cells versus those micronuclei observed in non-irradiated control cells.

2.2. Materials and methods

2.2.1. *Cell culture and fixation*

The protocol was developed using the normal human male cell line, AG1522, (N.I.A. Aging Cell Culture Repository at the Coriell Institute for Medical Research, Camden, NJ, USA). Cells were grown at 37 °C in flasks containing D-MEM:F-12 culture medium (Gibco, Grand Island, NY, USA) supplemented with 15% fetal calf serum (Gibco), 2 mM glutamine and 25 μg/mL gentamicin sulfate (Gibco) and incubated in a humidified atmosphere containing 2% CO_2 in air. To form binucleate cells, fibroblasts were cultured in 5 μg/mL cytochalasin B (Sigma, St. Louis, MO, USA) for 72 h. The cells were then detached using 1x trypsin-EDTA solution (Sigma) and separated from the medium by centrifugation. Following one wash in phosphate buffered saline (PBS), the cells were resuspended in 1% sodium citrate (w/v) and incubated for 20 min at 37 °C. The cells were recovered by centrifugation, resuspended in 5% glacial acetic acid (v/v) and incubated for 10 min at room temperature. The suspension was centrifuged and the cells resuspended at room temperature in 3:1 methanol:glacial acetic acid fixative. Samples were held for 10 min at room temperature then stored at -20 °C for future use.

2.2.2. *Irradiation conditions*

Cells were subjected to 4 Gy ^{60}Co γ-irradiation (GammaCell 200, Atomic Energy of Canada Ltd., Chalk River, ON, Canada) in complete culture medium at 37 °C. Following irradiation cells were released by trypsinization and seeded in cytochalasin B, as above.

2.2.3. *Centromere probe production*

The original template used to generate the centromere probe was kindly provided by J.D. Tucker (Lawrence Livermore National Laboratory, Livermore, CA, USA). 1 μL of this template was amplified by DOP-PCR (Telenius *et al.* 1992) using 1 μL of the primer 5'-

GATCAAGCTTNNNNNNATGTGG-3'. Also included in the 50 μL reaction mixture was 0.25 μL of the fluorochrome, Fluorogreen (Amersham, UK), 0.25 μL dTTP, 3 μL d(AGC) (USB, Cleveland, OH, USA), 24 μL ddH_2O, 20 μL buffer (10 mM TRIS (pH 8.3), 50 mM KCl, 0.010% gelatin, and 2 mM $MgCl_2$) and 0.50 μL AmpliTaq (Perkin Elmer, Branchburg, NJ, USA). Initially the mixture was denatured at 95 °C for 2 min, then 35 cycles at 95 °C (1 min), 62 °C (1 min), 72 °C (1 min), with a final extension of 5 min at 72 °C on a PTC Programmable Thermal Controller, MJ Research Inc. The directly labelled centromere probe generated was concentrated and purified using a Centricon-30 centrifugal concentrator (Amicon, Oakville, ON, Canada) and stored at -20 °C until required for FISH.

2.2.4. *Slide preparation*

Premium microscope slides (Fisher, Pittsburgh, PA, USA) were first cleaned in a solution of 62.5% ethanol, 22.5% H_2O and 15% HCl for 24 h and then dried and stored in 100% ethanol at -20 °C until required. Fixed cells were recovered by centrifugation and resuspended in a minimum volume of fresh 3:1 methanol:glacial acetic acid fixative to yield the desired cell density. Slides were removed from the ethanol and dipped in H_2O (treated with Milli-Q ultrapure water systems, Millipore Corporation, Bedford, MA, USA) at 0 °C until the water sheeted cleanly from the surface. The concentrated cell suspension was then aliquoted (20-30 μL) directly into the sheeting water and the slide was flushed with 3:1 methanol:glacial acetic acid fixative, blotted and dried over a 60 °C water bath for 1-2 min. Phase contrast microscopy was used to confirm the presence of intact cytoplasm and desired cell density. Slides prepared in this manner could be used for FISH for up to one week, with optimal FISH achieved using 0-2 day old slides. Alternatively, slides could be stored for up to 1 month at -20 °C without significantly compromising the intensity of the FISH signal and cytoplasm preservation.

2.2.5. *Fluorescence in situ hybridization*

Cellular DNA was denatured by immersing the slides in a 73 °C, 70% formamide/2x SSC solution (pH 7.0) for 5 seconds. Slides older than 2 days required 2-3x longer denaturation times. The reaction was quenched by dipping the slides in ethanol at 0 °C (2 min sequentially, in each of 70%, 80% and 95%) and the slides were air dried. The hybridization mixture containing the centromere probe was prepared and applied according to the Vysis protocol for Whole Chromosome Paints (Downers Grove, IL, USA). The slides were then placed in a sealed box humidified with the 70% formamide/2x SSC denaturation solution and incubated at 37 °C for 18 h. Following hybridization, unbound probe was removed by sequential 5 min washes in 50% formamide/2x SSC solution (pH 7.0), and 2x SSC (pH 7.0), both at 46 °C. After a final 2.5 min wash with 2x SSC/0.10% NP-40 (pH 7.0) at 46 °C, the slides were removed and immediately counterstained with a 3:1 mixture of 4',6-diamidino-2-phenylindole (DAPI, 0.1 μg/mL):propidium iodide (2.5 μg/mL) (both Oncor, Gaithersburg, MD, USA).

2.3. Results

2.3.1. *Characterization of DOP-PCR generated all human centromere directly labelled DNA probe*

Slides of AG1522 fibroblast metaphase spreads were prepared and used as a means to assess probe specificity and efficiency. Twenty-eight separate metaphases were photographed using colour negative film. The slides were then projected and all 1288 chromosomes (28 spreads x 46 chromosomes) were traced, counted and the presence or absence of centromere signal was assessed. Probe efficiency was found to be 97.2% (Table 2.1). Four of the 28 metaphase spreads examined exhibited signal at all centromeres. The remaining 24 metaphase spreads had 1 or 2 chromosomes which lacked detectable signal. Based on chromosome morphology those chromosomes without signal appeared to be the same across metaphase spreads. Probe specificity is illustrated in Figure 2.1, demonstrating that only the centromeres exhibited probe hybridization.

2.3.2. *Development of protocol for simultaneous preservation of cytoplasm and FISH*

An example of a binucleate AG1522 fibroblast cell prepared and probed under these conditions is illustrated in Figure 2.2. The preserved cytoplasm is stained red by the addition of propidium iodide while within the interphase nuclei and micronucleus the centromere probe fluoresces green (appearing yellow in the red background). DAPI, a DNA specific counterstain which fluoresces blue, was also added to facilitate the accurate identification of micronuclei. An example of a binucleate fibroblast and associated micronuclei probed with a Whole Chromosome Paint (WCP) for chromosome 4 is shown in Figure 3.2. Slides were scored on a Zeiss Axiophot 2 epifluorescence microscope (Carl Zeiss Canada Ltd., Don Mills, ON, Canada) equipped with the appropriate single-bandpass filters at 630x.

2.3.3. *^{60}Co γ-Irradiation has an aneugenic component with respect to micronucleus formation*

Micronuclei may be composed of whole chromosomes or acentric chromosome fragments. One factor that distinguishes these two possibilities is the presence or absence of a centromere. Typically γ-rays are thought to induce micronuclei via the creation of DNA double strand breaks. This clastogenic action may result, assuming that that lesion is not repaired, in the generation of an acentric chromosome fragment which has the potential to manifest itself as a micronucleus following the next round of cellular division. Data presented in Table 2.2 indicate that while 91.95% of micronuclei within binucleate cells which have arisen from cells exposed to 4 Gy irradiation do indeed contain acentric fragments, 8.05% contain whole chromosomes. Standardizing the signal positive frequency for the control and 4 Gy treatment on a per binucleate cell basis and comparing the resultant frequencies demonstrates that not all centromere positive micronuclei appearing within the 4 Gy treated cells arise independently from the radiation exposure; that is, the normalized frequency in the 4 Gy exposed cells (6.80%) is greater than that observed in the unexposed control cells (1.71%). This suggests that ^{60}Co γ-irradiation, in addition its clastogenic action, has an aneugenic component to its mode of action with respect to micronucleus formation.

Figure 2.1 AG1522 fibroblast metaphase spread subjected to *in situ* hybridization using the DOP-PCR generated directly labelled human centromere DNA probe and counterstained with DAPI. The arrow and arrowhead indicate centromeres exhibiting and not exhibiting probe signal, respectively. The image was captured under epifluorescence at 630x magnification with a Sony 3CCD Colour Video Camera, processed with Northern Eclipse Image Analysis Software (Version 2.0, Empix Imaging, Inc., Mississauga, ON, Canada). Bar, 10 μm.

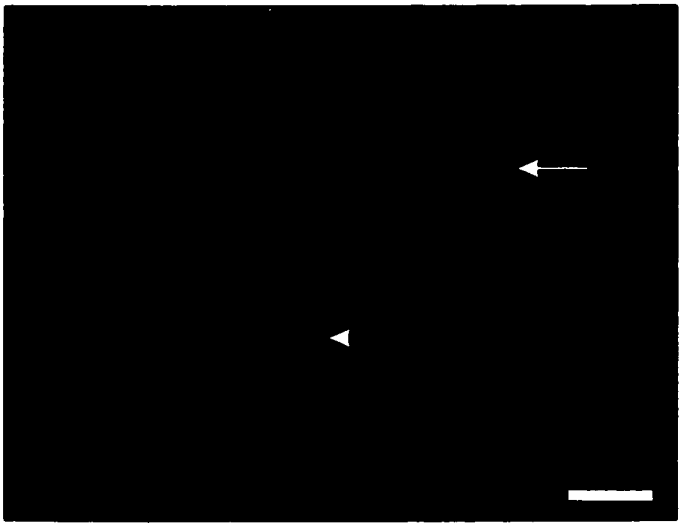


Figure 2.2 Fixed binucleate AG1522 fibroblasts subjected to *in situ* hybridization using the DOP-PCR generated directly labelled human centromere DNA probe and counterstained with DAPI:propidium iodide. The arrow indicates a centromere positive micronucleus. The image was captured under epifluorescence at 630x magnification with a Sony 3CCD Colour Video Camera, processed with Northern Eclipse Image Analysis Software (Version 2.0, Empix Imaging, Inc., Mississauga, ON, Canada). Bar 10 μm.

Table 2.1 Determination of hybridization efficiency of DOP-PCR generated directly labelled human centromere DNA probe.

Total Metaphase Spreads	Total Chromosomes	Chromosomes with Probe Signal	Hybridization Efficiency
28	1288	1252	(1252/1288)x100= **97.2%**

Table 2.2 Frequency of centromere positive micronuclei within binucleate, unexposed, control cells and cells exposed to 4 Gy ^{60}Co γ-ray treatments.

	Frequency of MN per BNC (%)	Frequency of Centromere Positive MN in BNC (%)	Frequency of Centromere Positive MN per BNC (%)
Control	2.53±1.22*	67.80±0.74‡	1.71§
4 Gy	84.44±4.06*	8.05±3.23*	6.80§

*Mean results ±standard deviation are reported, n=3 (calculated as per equation presented in chapter I, section 1.2.4.).

‡Mean results ± standard deviation are reported, n=2.

§Frequency determined by multiplying the frequency of MN per BNC by the frequency of centromere positive MN in BNC.

(MN) Micronuclei; (BNC) Binucleate Cell

2.4. Discussion

The micronucleus assay has been used to assess the mutagenic potential of a wide variety of agents, principally because is technically easy to master, and extremely rapid (Tucker and Preston 1996). Several methods have been employed to distinguish micronuclei induced by aneugens, agents that induce lagging chromosomes (i.e. mitotic spindle toxins) from clastogens, those which induce acentric fragments (i.e. DNA damaging agents). These include size determination (Yamamoto and Kikuchi 1980), measuring DNA content (Grawe *et al.* 1993, 1994; Nüsse *et al.* 1996) and the use of anti-kinetochore antibodies (reviewed by Schuler *et al.* 1997).

Recently fluorescence *in situ* hybridization has been employed to examine not only centromere content (Schriever-Schwemmer and Adler, 1994; Darroudi *et al.* 1996; Vral *et al.* 1997; Fimognari *et al.* 1997) but also chromosome content of radiation and chemically induced micronuclei in binucleate cells using various combinations of whole chromosome specific paints (Slavotinek *et al.* 1996; Walker *et al.* 1996; Wuttke *et al.* 1997). The methods used in these studies did not preserve the cellular cytoplasm throughout the *in situ* hybridization procedure, and the investigators relied on relative sizes and proximity of nuclei to differentiate binucleate cells from mononucleate cells. This study presents an alternative method which maximizes cytoplasm preservation while still allowing hybridization of DNA probes. This method therefore allows not only unambiguous scoring of binucleate cells but also correct assignment of micronuclei to their respective cells.

Several alternative fixation protocols were attempted with the aim of maximizing hybridization efficiency while allowing cytoplasm preservation throughout the *in situ* hybridization process. Maintenance of cytoplasm was also achieved by using low concentrations of a formaldehyde fixative (0.10-3% (w/v), in PBS). While this crosslinking agent

was found to be extremely effective at preserving cytoplasm it also prevented hybridization via extensive crosslinking of the proteins surrounding the genomic DNA target sequences. Permeabilization using Triton-X and/or Proteinase K following fixation improved, somewhat, the hybridization of the DNA probe. However, signal strength was still not sufficient for scoring purposes. However, methanol:acetic acid, a non-crosslinking fixative, with freshly prepared slides (0-2 days old), very short denaturation (5 sec) and reduced post-hybridization wash times (12.5 min total) was found to be extremely effective in both preserving cytoplasm and allowing efficient hybridization.

Once the protocol was established, the centromere probe was used to examine the aneugenic versus clastogenic action of ionizing radiation, specifically ^{60}Co γ-rays. It was found that in addition to a profound clastogenic action, there was an increase over control levels in the proportion of micronuclei which contained centromeres; this result indicated that whole chromosomes, and not just acentric chromosomal fragments are involved in micronuclei generated by a high dose radiation exposure. This mild aneugenic potential has been reported for X-rays in human hepatoma cells (Darroudi *et al.* 1996) and is likely a result of alterations to the kinetochore, or spindle apparatus. Recently, Tallon *et al.* (1998) have examined this aneugenic potential of X-rays in human lymphocytes. They observed that cells irradiated in G_1 exhibited a slightly higher proportion of centromere positive micronuclei as compared with those irradiated in G_2. In addition, centromere-positive micronuclei from G_1 irradiated cells exhibited a dose-response relationship while G_2 irradiated cells did not. They conclude that the cell cycle stage specificity may suggest the involvement of multiple targets.

It has been suggested that the ability to identify and subsequently eliminate spontaneous background micronuclei (i.e. those containing centromeres) from analysis will enhance the micronucleus assays sensitivity as a biological dosimetry tool. However if ionizing radiation is able to induce not only acentric fragments but also micronuclei containing whole chromosomes

then clearly more work is required before this becomes accepted practice. Lower doses should also be examined (i.e. lower than the 4 Gy studied here); it is possible that a large dose such as 4 Gy is required before this aneugenic potential of ionizing radiation is realized, and that at smaller doses it is in fact negligible. If this proved true then using centromere probes at lower doses as a means of increasing the micronucleus assays sensitivity may remain a valid approach.

Chapter III

Adaption of human fibroblasts to radiation alters biases in DNA repair at the chromosomal level

3.1. Introduction

The adaptive response is a phenomenon whereby cells exposed to a low dose of ionizing radiation acquire resistance to the effects of a subsequent higher dose (reviewed by Wojcik and Streffer 1994). This response to ionizing radiation has been reported in a variety of cell types, including: human cervical carcinoma cells (Kim *et al.* 1995), Chinese hamster cells (Ikushima 1987, 1989), human lymphocytes (Domínguez *et al.* 1993; Shadley 1994; Vijayalaxmi *et al.* 1995; Wojewódzka *et al.* 1996, 1997) and human fibroblasts (Azzam *et al.* 1992, 1994a,b). The first report in human cells (Olivieri *et al.* 1984) characterized chromatid aberrations from [^{3}H]thymidine incorporated into human lymphocytes.

The underlying mechanisms that produce the adaptive response have yet to be fully elucidated. However, based on several lines of evidence, it is generally held that a low adapting dose induces increased DNA repair capacity through specific gene activation and *de novo* protein synthesis, and is not due to a decreased susceptibility to the initial radiation damage (Azzam *et al.* 1994a; Ikushima *et al.* 1996; Rigaud and Moustacchi 1996; Le *et al.* 1998). This same response to low doses has also been reported to reduce the risk of both radiation induced (Azzam *et al.* 1994b) and spontaneous (Azzam *et al.* 1996) neoplastic transformation in rodent C3H 10T1/2 cells and of spontaneous neoplastic transformation in a human hybrid cell line (Redpath and Antoniono 1998).

The repair systems monitored during the adaptive response to radiation are frequently those that rejoin broken chromosomes, i.e., DNA double strand breaks, and the micronucleus assay has often been used as a measure of the cells' ability to repair such breaks (Azzam *et al.* 1992, 1994a,b). Detected as small spherical objects in the cytoplasm of interphase cells, radiation induced micronuclei are composed of acentric DNA fragments or lagging chromosomes which, following nuclear division, become encapsulated in a nuclear envelope

(Walker *et al.* 1996). Typically micronucleus frequency is enumerated using the cytokinesis-block technique developed by Fenech and Morley (1985). The approach employs cytochalasin B, a fungal metabolite that allows nuclear division while inhibiting cytokinesis. Consequently, all cells that have undergone one division can be identified by their binucleate state.

Utilizing fluorescence *in situ* hybridization (FISH) with whole chromosome DNA probes, it has been recently demonstrated in lymphoblastoid cell lines (Slavotinek *et al.* 1996) and human fibroblasts (Walker *et al.* 1996) that the frequency of inclusion of specific chromosomes in radiation-induced micronuclei deviates significantly from that expected based on relative DNA content. Such bias has also been observed for radiation-induced chromosomal aberrations such as translocations, dicentrics and deletions in **human fibroblasts** (Lee and Kamra 1981; Dutrillaux *et al.* 1983; Kano and Little 1986), **human lymphocytes** (San Roman and Bobrow 1973; Cooke *et al.* 1975; Buckton 1976, 1983; Dutrillaux *et al.* 1977, 1981; Dubos *et al.* 1978; Bauchinger and Götz 1979; Tanaka *et al.* 1983; Tawn 1988; Barrios *et al.* 1989; Lucas *et al.* 1992; Natarajan *et al.* 1992a,b; Sachs *et al.* 1993; Knehr *et al.* 1994, 1996; Finnon *et al.* 1995; Boei *et al.* 1996; Granath *et al.* 1996) and **Chinese hamster cells** (Slijepcevic and Natarajan 1994a,b; Domínguez *et al.* 1996) using cytogenetic banding and FISH methodologies. It should be noted however that not all studies have found such biases (Kovacs *et al.* 1994; Fimognari *et al.* 1997; Wuttke *et al.* 1997). Assuming however that a bias does exist and that the initial deposition of DNA damage by radiation is random, it appears that when cells suffer DNA damage after exposure to radiation they repair that damage in a manner that favours some chromosomes over others. Surrallés *et al.* (1997a) have also shown this apparent targeting of DNA repair to specific chromosomes. Using FISH they demonstrated that DNA repair is preferentially directed towards chromosomes with high gene density as indicated by the relative enrichment of CpG islands, CG-rich regions found at the 5'-end of most mammalian genes.

There are numerous factors that could contribute to this hierarchy of chromosomal

repair. It is generally accepted that heterochromatin and non-transcribed regions and strands are repaired less efficiently (Mullenders *et al.* 1991) not only due to inaccessibility of repair factors but also because of a lack of transcriptional-coupled repair (reviewed by Drapkin *et al.* 1994). Indeed, Surrallés *et al.* (1997b) have recently shown a relative deficiency of DNA excision repair within heterochromatin at human chromosome band 1q12, a region of the genome known to be prone to breakage and observed in numerous cancers. The effect of this hierarchy in chromosomal repair is the creation of a natural bias, whereby the probability of repair of radiation damage in genes that reside in the favoured chromosomes is increased and conversely the probability of repair of genes in less favoured chromosomes is decreased. Since this situation occurs normally, the observed bias may contribute to the natural difference in the risk of radiation induced cancer observed between various tissues, which is linked to the various genes responsive to oncogenic transformation by radiation in those tissues.

Using FISH with whole chromosome DNA probes, we have investigated the influence of adaption to radiation on the stability of this hierarchy in chromosomal repair, by examining the relative chromosomal content of micronuclei in radiation adapted and non-adapted human fibroblasts. If the induction of DNA repair by exposure to a low adapting dose of radiation changes the existing bias, this would manifest itself as an altered relative chromosomal content in micronuclei and perhaps imply a change in the natural differential risk of the various types of cancer that can appear either spontaneously or after a subsequent higher radiation exposure. Such changes could have important implications with respect to biological dosimetry and radiation risk assessment.

3.2. Materials and methods

3.2.1. *Cell culture, irradiation and fixation*

The normal human male cell strain AG1522 (N.I.A. Aging Cell Culture Repository, Coriell Institute for Medical Research, Camden, NJ, USA) was maintained at 37 °C in D-MEM:F-12 culture medium (Gibco, Grand Island, NY, USA) supplemented with 15% fetal calf serum (Gibco), 2 mM glutamine and 25 μg/mL gentamicin sulfate (Gibco) and incubated in a humidified 2% CO_2/98% air atmosphere. Initially, cells, in passage 9, were seeded at $2x10^5$ cells/25 cm^2 flask (Nunc, Naperville, IL, USA). Seven days after seeding, with one feeding on day 4, the now confluent cultures (91% G_0/G_1, 7% G_2/M and 2% S, as determined by flow cytometry) were exposed to 10 cGy ^{60}Co γ-irradiation (GammaCell 200, Atomic Energy of Canada Ltd., Chalk River, ON, Canada) or held in complete culture medium for an equivalent period of time. The medium was then replaced with fresh medium and the cells allowed to adapt during a 3 h, 37 °C incubation prior to a challenge dose of 4 Gy of ^{60}Co γ-rays. All irradiations were performed in complete culture medium at 37 °C with a dose rate of 1.12 cGy/sec. Immediately following the 4 Gy challenge dose the cells were detached using 1x trypsin-EDTA solution (Sigma, St. Louis, MO, USA), and seeded into 80cm^2 flasks (Nunc) containing 5 μg/mL cytochalasin B (Sigma).

Following 65 h incubation at 37 °C the cells were washed twice in phosphate-buffered saline (PBS), detached by trypsin treatment, collected in complete medium and separated by centrifugation. After two washes in PBS, the cells were resuspended in 1% sodium citrate (w/v) and incubated for 20 min at 37 °C. The cells were then collected by centrifugation, resuspended in 5% glacial acetic acid (v/v) and incubated for 10 min at room temperature. The suspension was again centrifuged and the cells resuspended at room temperature in the final fixative, 3:1 methanol:glacial acetic acid. Samples were held for 10 min at room temperature and stored at -20 °C for use in future FISH analysis.

For each treatment, replicate chambered slides (Nunc) were also established ($2.5x10^4$ cells/slide, 5 μg/mL cytochalasin B) as a means of assessing adaption by the conventional cytokinesis-block micronucleus assay. After 65 h incubation at 37 °C the cells were fixed by consecutive 5 min treatments of 1% sodium citrate (v/v), 1:1 sodium citrate:(3:1 methanol:glacial acetic acid), and 3:1 methanol:glacial acetic acid, all at room temperature. The slides were stained with 1:1 4',6-diamidino-2-phenylindole (DAPI):propidium iodide (both Oncor, Gaithersburg, MD, USA) and micronuclei in 500 binucleate cells/treatment were scored on an epifluorescence microscope equipped with the appropriate single-bandpass filters according to criteria previously described (Fenech 1993).

3.2.2. *Slide preparation*

Premium microscope slides (Fisher, Pittsburgh, PA, USA) were immersed in a solution of 62.5% ethanol, 22.5% H_2O and 15% HCl for 24 h, dried and stored in 100% ethanol at -20 °C until required. Fixed cells, recovered by centrifugation, were then resuspended in a volume of fresh 3:1 methanol:glacial acetic acid to yield the desired cell concentration. The precleaned slides were removed from the ethanol and repeatedly dipped in H_2O (treated with Milli-Q ultrapure water systems, Millipore Corp., Bedford, MA, USA) at 0 °C until the water sheeted cleanly from the surface. 20-30 μL of the concentrated cell suspension was then aliquoted directly into the sheeting water, the slide was then rinsed with 3:1 methanol:glacial acetic acid and suspended over a 60 °C water bath for 1-2 min to dry. Phase contrast microscopy was used to confirm the presence of intact cytoplasm and desired cell density. Slides for FISH analysis were used within one month of preparation (stored at -20 °C), but optimal FISH was achieved with 0-2 day old slides.

3.2.3. *Fluorescence in situ hybridization*

Cellular DNA was denatured with 70% formamide/2x SSC (pH 7.0) at 73 °C for 5 sec. Older slides required 2-3 fold greater denaturation times. The slides were then washed in sequential solutions of 70%, 80% and 95% ethanol at 0 °C for 2 min, air dried and the hybridization mixture containing a probe specific for either chromosome 2, 4, 7, 18, 19 (Whole Chromosome Paint (WCP) SpectrumGreen, Vysis, Downers Grove, IL, USA) was prepared and applied according to the manufacture's protocol. Chromosome 19 WCP has been reported to hybridize to the centromeres of chromosome 1 and 5 (Jalal *et al.* 1996). However, under our *in situ* conditions this was not observed (on metaphase spreads among interphase cells). Hybridization was carried out at 37 °C for 18 h in a sealed box humidified with the 70% formamide/2x SSC denaturation solution. Following consecutive 5 min post-hybridization washes in 50% formamide/2x SSC (pH 7.0), and 2x SSC (pH 7.0) both at 46 °C and a final 2.5 min wash with 2x SSC/0.10% NP-40 (pH 7.0) at 46 °C, the slides were removed and immediately counterstained with a 3:1 mixture of DAPI:propidium iodide (Oncor). Each fluorophore was visualized using the appropriate single-bandpass filter placed in a Zeiss Axiophot 2 epifluorescence microscope (Carl Zeiss Canada Ltd., Don Mills, ON, Canada).

The use of DAPI ($excitation_{max}$ 367 nm, $emission_{max}$ 452 nm), a DNA specific fluorophore that fluoresces blue, ensured accurate identification of micronuclei. Propidium iodide ($excitation_{max}$ 543 nm, $emission_{max}$ 614 nm) was used to identify the preserved cytoplasm, staining it and the nucleus red/orange. Micronuclei containing DNA originating from the chromosome specific for the WCP being used fluoresced green under the Vysis SpectrumGreen single-bandpass filter due to excitation of the SpectrumGreen fluorophore ($excitation_{max}$ 509 nm, $emission_{max}$ 538 nm).

3.2.4. *Statistical analysis*

Differences between adapted and non-adapted treatments were assessed using one and two tailed Student's *t*-test analysis of arcsine transformed data for micronucleus frequency and chromosomal content of micronuclei, respectively. Observed versus expected frequency of micronucleus content was based on chi-square analysis of pooled data determined to be homogeneous. In all cases significance was assessed at the 5% level (i.e. α=0.05).

3.3. Results

As expected (Azzam *et al.* 1992) exposure of these human fibroblasts to a 10 cGy ^{60}Co γ-ray adapting dose 3 h prior to a 4 Gy ^{60}Co γ-ray challenge dose resulted in a significantly lower micronucleus frequency than that observed in cells exposed only to the 4 Gy challenge dose (Figure 3.1). Replicate slides from these experiments were probed for micronucleus content.

A protocol was developed that maximized hybridization efficiency while allowing cytoplasm preservation throughout the *in situ* hybridization process. This protocol enabled accurate identification of signal positive micronuclei, binucleate cells, mononucleate cells and the origin of any associated micronuclei (refer to Chapter 2). Figure 3.2 illustrates the resultant ability to easily identify signal positive micronuclei (chromosome 4 in this case) from those lacking probe signal. The cell contains two micronuclei. One micronucleus exhibits probe hybridization, and therefore contains DNA originating from chromosome 4, while the other is devoid of any appreciable probe signal, indicating that DNA originated from a chromosome other than chromosome 4. Also evident in the figure are chromosomal domains within the interphase nuclei. Hybridization efficiency within these domains was used as a positive control to identify areas that should be excluded from scoring.

Micronuclei in both binucleate and mononucleate cells were assessed as signal positive or negative. Subsequently, colour CCD images of these micronuclei were captured, converted to an 8 bit gray scale, and scanned for average signal intensity using SigmaScan/Image (Version 1.20.09, Jandel Scientific, San Rafael, CA, USA). The micronuclei were found to fall into two distinct populations (Figure 3.3). Those scored as signal positive were found to have an average signal intensity ≥110 gray level units (values range from 0, black to 255, white) while the signal negative micronuclei fell in the range of 40-100 gray level units. With only a few exceptions hybridization of the WCP encompassed the entire micronucleus. In those infrequent

cases of partial hybridization the signal positive area was clearly delineated and interpreted to be the additional inclusion of DNA originating from another chromosome. For analysis, only the signal positive areas of these rare micronuclei were included in calculating average signal intensity.

A total of 28,744 micronuclei were scored for the presence or absence of probe signal for chromosome 2 (4222 micronuclei), 4 (6852 micronuclei), 7 (6292 micronuclei), 18 (5415 micronuclei) or 19 (5993 micronuclei) for both adapted (10 cGy, 3 h prior to 4 Gy) and non-adapted (4 Gy) treatments in 3 independent experiments (Figure 3.4). In terms of proportional representation based on DNA content of individual chromosomes, only DNA from chromosomes 4 and 7 within micronuclei associated with mononucleate cells was found to not deviate significantly from expected values (Figure 3.4B). However when micronuclei from either binucleate cells alone or binucleate and mononucleate cells combined were considered (Figure 3.4C) all chromosomes investigated exhibited a bias of incorporation into radiation-induced micronuclei. For chromosome 2 (Figure 3.4A,C) and 18 (Figure 3.4A,B,C) this bias of incorporation significantly decreased when a 10 cGy adapting dose was administered 3 h prior to the 4 Gy challenge dose. Conversely the appearance of DNA from chromosome 19 in micronuclei significantly increased (Figure 3.4A,C) while the frequency of micronuclei containing DNA from chromosome 4 or 7 remained unchanged (Figure 3.4A,B,C). The percentage change of incorporation for each chromosome is illustrated in Figure 3.5, where positive and negative values represent an increase or decrease in bias of incorporation, respectively. Table 3.1 reviews the results of similar studies in a variety of cell types.

Figure 3.1 Frequency of micronucleus formation in binucleate fibroblast cells pre-exposed to 10 cGy 3 h prior to a 4 Gy challenge dose. *Indicates that the difference between adapted and non-adapted treatments is significant, $P<0.01$. Mean results ±standard deviation are reported, n=3.

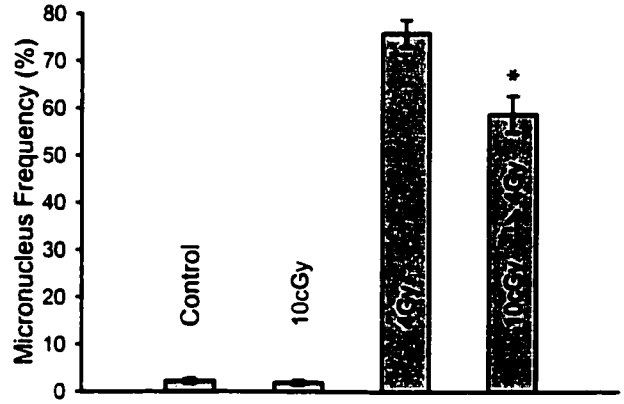

Micronucleus Frequency (%)
80
70
60
50
40
30
20
10
0
Control
10cGy
*

Figure 3.2 Fixed binucleate fibroblast containing 2 micronuclei probed with SpectrumGreen Whole Chromosome Paint (WCP) specific for chromosome 4 (C) and counterstained with DAPI (A) and propidium iodide (B). (D) composite image of WCP and DAPI. Arrow indicates a micronucleus containing a portion of chromosome 4. Arrowhead indicates a micronucleus lacking signal. Images were captured under epifluorescence at 630x magnification with a Sony 3CCD Colour Video Camera, processed with Northern Eclipse Image Analysis Software (Version 2.0, Empix Imaging, Inc., Mississauga, ON, Canada). Bar, 10 μm.

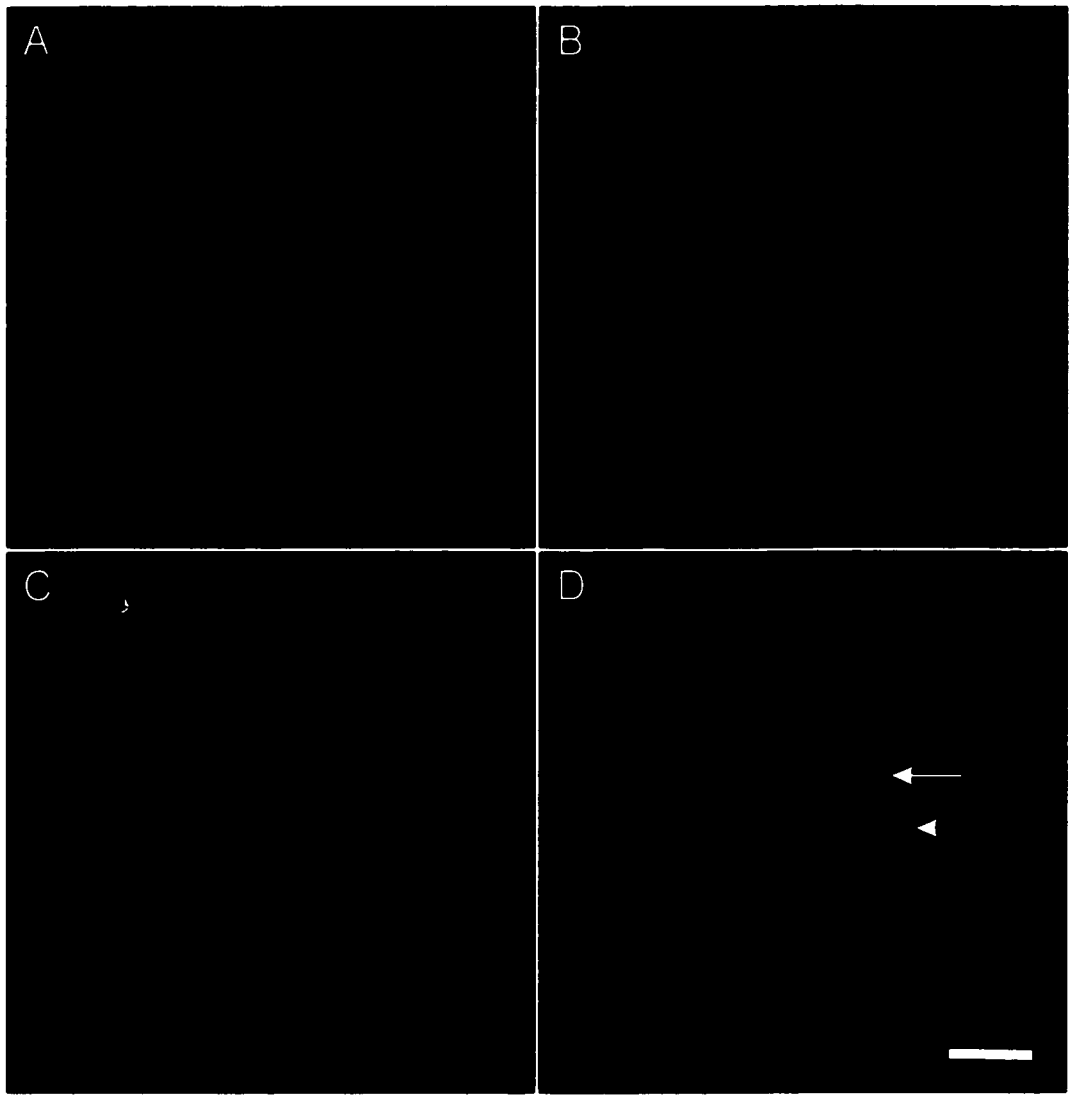

A
B
C
D

Figure 3.3 Histogram of the average signal intensity of signal positive (black bars) and signal negative (gray bars) micronuclei probed with SpectrumGreen Whole Chromosome Paint for chromosome 4. Images of micronuclei were captured under epifluorescence with a Sony 3CCD Colour Video Camera using a fixed number of integrations, converted to 8 bit gray scale images and scanned for average intensity, n=210.

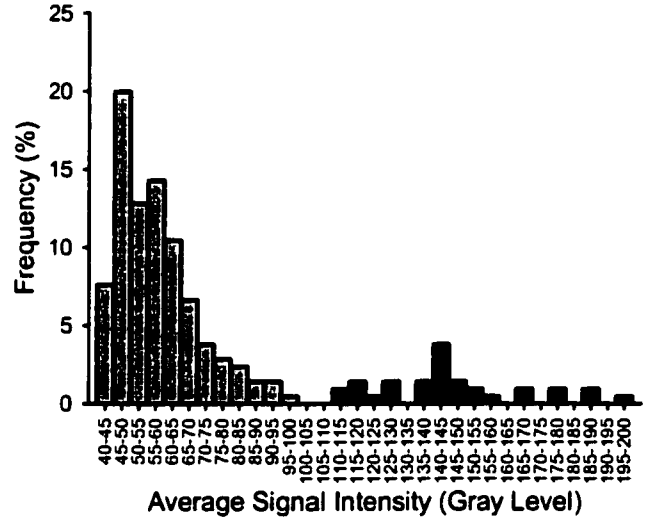

25
20
15
10
5
0
Frequency (%)
40-45
45-50
50-55
55-60
60-65
65-70
70-75
75-80
80-85
85-90
90-95
95-100
100-105
105-110
110-115
115-120
120-125
125-130
130-135
135-140
140-145
145-150
150-155
155-160
160-165
165-170
170-175
175-180
180-185
185-190
190-195
195-200
Average Signal Intensity (Gray Level)

Figure 3.4 Frequency of micronuclei containing probe signal for chromosome 2, 4, 7, 18 or 19 in fibroblasts either irradiated with 4 Gy (black bars) or adapted by exposure to 10 cGy 3 h prior to the 4 Gy challenge (gray bars) as a function of: (A) total micronuclei within binucleate cells exhibiting ≥1 micronucleus, (B) total micronuclei in mononucleate cells exhibiting ≥1 micronucleus, (C) combined data for (A) and (B). The expected frequency (white bars) indicates the proportion of genome encompassed by each chromosome (Morton 1991). *Denotes adapted treatments significantly different than non-adapted, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. •Indicates 4 Gy treatments not significantly different than expected, $0.05<P<0.75$. Mean results ±standard deviation are reported, n=3.

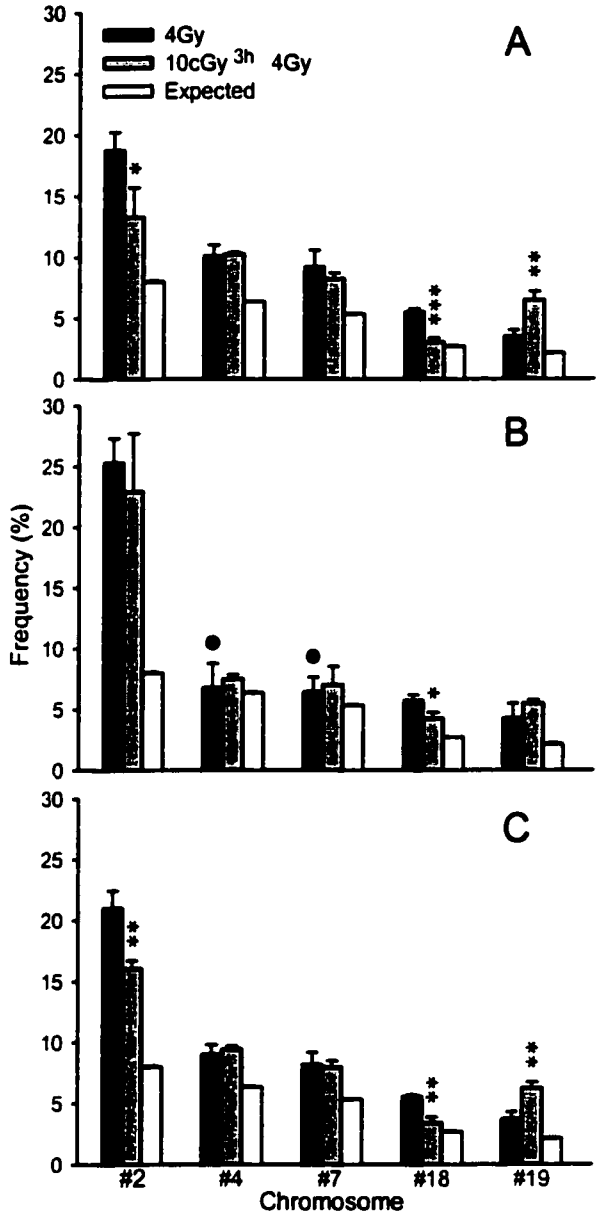

4Gy
10cGy 3h 4Gy
Expected
A
B
C
Frequency (%)
30
25
20
15
10
5
0
#2
#4
#7
#18
#19
Chromosome

Figure 3.5 Percent change of micronuclei containing probe signal for chromosome 2, 4, 7, 18 or 19 in adapted fibroblasts, both binucleate and mononucleate, with ≥1 micronucleus. The percent change was generated by normalizing the data to the frequency of signal-positive micronuclei observed in the non-adapted 4 Gy treatment. Mean results ±standard deviation are reported, n=3.

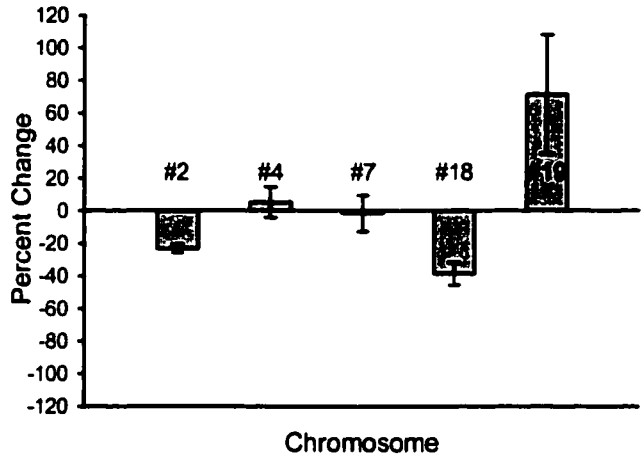

120
100
80
60
40
20
0
-20
-40
-60
-80
-100
-120
Percent Change
#2
#4
#7
#18
Chromosome

Table 3.1 Literature review of chromosome specific involvement in various aberrations based on proportional representation of DNA content. Unless otherwise stated all cell types are human and all aberrations were induced by exposure to ionizing radiation. (O) Over represented; (U) Under represented; (e) Involved at expected frequency; (CHL) Chinese hamster lung fibroblasts; (CHS) Chinese hamster splenocytes; (CHE) Chinese hamster embryonic cells (MN) micronuclei; (†) Proportional representation in all chromosomes studied. (‡) Observed in non-irradiated individuals. Significance determined at $P<0.05$.

				Chromosome																							
Reference	**Cell type**	**Aberration scored**	**Conditions**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**X**	**Y**
Walker *et al.* 1996	fibroblasts	micronuclei	*in vitro*		O					O				e					e								
†Kovacs *et al.* 1994	fibroblasts	dicentrics & translocations	*in vitro*	e			e				e					e											
Kano and Little 1986	fibroblasts	translocations	*in vitro*	O	e	e	e	e	e	e	U	e	e	e	e	U	e	e	e	e	e	e	e	e	e	e	e
		breaks & deletions		e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e
Dutrillaux *et al.* 1983	fibroblasts	translocations	*in vitro*	e	e	e	U	e	e	e	e	e	e	O	e	e	e	e	e	e	e	e	e	e	e	e	e
Lee and Kamra 1981	fibroblasts	translocations	*in vitro*	O	e	e	e	e	e	O	e	e	e	e	O	e	e	e	e	e	e	e	e	e	e	e	e
Barquinero *et al.* 1998	lymphocytes	All aberrations	*In vitro*	e	U	U	e	e	U	e	e	e	e	e	e	e	e	e	O	O	e	e	O	e	e	e	
Boei *et al.* 1997	lymphocytes	translocations	*in vitro*	e			e																				
		dicentrics		e			O																				
†Fimognari *et al.* 1997	lymphocytes	micronuclei	*in vitro*	e						e				e			e			e				e			
Stephan and Pressl 1997	lymphocytes	translocations	*in vitro*		U		O				e																
		dicentrics	*in vitro*		U		O				U																
		translocations	*in vivo*		U		O				O																
Wuttke *et al.* 1997	lymphocytes	micronuclei			e					e																	
		Colchicine induced MN			e					O																	
Natarajan *et al.* 1996	lymphocytes	dicentrics & translocations	*in vitro*				O																				
Granath *et al.* 1996	lymphocytes	translocations	*in vitro*	e	e		e											O				e				e	
		dicentrics		e	e		e											e				e				e	
Knehr *et al.* 1996	lymphocytes	translocations	*in vitro*	e	U	U	O		e	e	e	O	e		e		e										O	
		dicentrics		e	e	e	e		e	e	e	O	O		e		e										e	
Finnon *et al.* 1995	lymphocytes	dicentrics & translocations	*in vitro*		e	e		O																				
Knehr *et al.* 1994	lymphocytes	translocations	*in vitro*	e	U	e	e		e	e	e	e	e		e		e										e	
		dicentrics		e	e	e	e		e	e	e	O	O		O		e										e	
Sachs *et al.* 1993	lymphocytes	translocations	*in vivo*	O	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e
		pericentric inversions		e	e	e	e	e	e	O	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e
Lucas *et al.* 1992	lymphocytes	G-banding translocations	*in vivo*	O	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e		
		FISH translocations	*in vivo*	e	e	e	e										e										

Table 3.1 Continued

Reference	Cell type	Aberration scored	Conditions	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
Lucas *et al.* 1992, continued	lymphocytes	FISH translocations	*in vitro*	e	e		e																				
Barrios *et al.* 1989	lymphocytes	translocations	*in vivo*	O	e	O	e	e	e	O	e	e	e	e	e	U	e	U	e	e	e	e	e	U	U	e	U
‡Tawn 1988	lymphocytes	All aberrations	*in vivo*							O							O										
Buckton 1983	lymphocytes	translocations	*in vivo*	e	e	U	e	U	e	e	e	O	O	e	e	e	O	O	e	e	e	U	e	e	e	e	e
Tanaka *et al.* 1983	lymphocytes	translocations	*in vivo*	U	U	e	e	e	U	e	O	U	e	U	U	e	e	O	e	e	O	e	e	e	O	U	e
Dutrillaux *et al.* 1981	lymphocytes	translocations	*in vitro*	e	e	e	e	e	e	e	e	e	e	e	e	e	e	O	e	e	e	e	e	e	e	e	e
Bauchinger and Götz 1979	lymphocytes	dicentrics	*in vitro*	O	e	e	e	O	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e
Buckton 1976	lymphocytes	Chromosome aberrations	*in vitro*	e	e	e	e	e	e	e	e	e	e	e	O	e	e	O	e	O	e	e	e	e	e		
San Roman and Bobrow 1973	lymphocytes	dicentrics & translocations		e	e	O	e	e	e	e	e	e	e	e	e	e	e	e	U	e	e	e	e	e	e	e	e
Slavotinek *et al.* 1996	lymphoblastoid	micronuclei	*in vitro*	e						U				e			e										
Dominguez *et al.* 1996	CHL/CHS	dicentrics & translocations	*in vitro*		U	e		O			O															e	e
Slijepcevic and Natarajan 1994a	CHE	Chromosome aberrations-G_1	*in vitro*	e	e	e	e	O	e	e	O	e	e													U	
Slijepcevic and Natarajan 1994b	CHE	Chromosome aberrations-G_2	*in vitro*	O	O	e	U	O	U	U	e	U	U													U	U

3.4. Discussion

Using fluorescence *in situ* hybridization with whole chromosome paints for chromosomes 2, 4, 7, 18 and 19 we have examined whether the induction of increased DNA repair capacity (Azzam *et al.* 1994a) by a low dose of ionizing radiation, a phenomenon termed the adaptive response, alters the natural hierarchy observed in human fibroblast chromosomal repair (Mullenders *et al.* 1991). Specifically, we compared the frequency of incorporation of these chromosomes into micronuclei arising from a radiation exposure of radiation-adapted and non-adapted human fibroblasts.

These five chromosomes were selected because they are representative of the spectrum of chromosome sizes, and we have previously observed a bias of incorporation for chromosomes 2 and 7 in micronuclei formed after radiation exposure (Walker *et al.* 1996). In addition, chromosomes 18 and 19, while being similar in size, are reported to vary considerably in gene density; chromosome 19 is relatively gene rich while chromosome 18 is gene poor (Surrallés *et al.* 1997a).

Using the same cell strain of fibroblasts (AG1522) as Azzam *et al.* (1992) and Walker *et al.* (1996) cultured under similar conditions, (with the exception that Walker *et al.* used exponentially growing cells rather than confluent cells), we obtained similar results. Like Azzam *et al.* (1992), a low dose of radiation induced an adaptive response and protected the cells against micronucleus formation resulting from a second higher dose (Figure 3.1). It should be noted that the low adapting dose in this case was delivered at a high dose rate and still resulted in adaption. We have previously described the ability of human fibroblasts to adapt when exposed to low doses at high dose rate (Mitchel *et al.* 1997). This ability of fibroblasts is unlike the response observed in lymphocytes (Shadley and Wiencke 1989) where no adaptive response is seen after high dose rate exposure.

Like Walker *et al.* (1996), after a single challenge dose of 4 Gy (Figure 3.4), chromosomes 2 and 7 (as well as 4, 18 and 19 in the present study) were incorporated into micronuclei more frequently than would be expected based on proportional DNA content, in binucleate cells (Figure 3.4A) and total cells (mononucleate plus binucleate cells, Figure 3.4C). However, within mononucleate cells alone (Figure 3.4B), chromosomes 7 and 4 were incorporated at the frequency expected. The remaining chromosomes, 2, 18 and 19 were incorporated more frequently than would be expected.

Most studies, including the one presented here, have reported, albeit with some inter-study variability with respect to specific chromosomes, that there are biases in the distribution of chromosomal alterations following repair of ionizing radiation exposed cells (reviewed in Table 3.1). We investigated the influence of adaption to radiation on the stability of this natural hierarchy in chromosomal repair and its resulting natural differential risk. We found that when human fibroblasts were exposed to 10 cGy of ^{60}Co γ-irradiation 3 h prior to a challenge dose of 4 Gy, the probability of inclusion of DNA originating from chromosome 2 or 18 in a micronucleus decreased as compared with those cells receiving only the 4 Gy treatment. Conversely, micronuclei were more likely to contain DNA from chromosome 19 while there was no observed change in the existing bias for chromosomes 4 and 7 (Figure 3.4). It appears that 10 cGy, a dose which induced the adaptive response in this cell system and increased the overall cellular repair capacity (Figure 3.1), effectively altered the natural hierarchy in chromosomal repair such that chromosome 2 and 18 were preferentially repaired, while repair of chromosome 19 was reduced. Interestingly, In terms of gene density, chromosome 19, a relatively gene rich chromosome, was actually incorporated into micronuclei (unrepaired) more frequently as a result of a prior adapting dose than chromosome 18, a gene poor chromosome of similar size. This result is the opposite of what might be expected based on Surrallés *et al.* (1997a) findings that DNA repair is preferentially targeted to gene rich chromosomes. This suggests that gene

density may not be the sole indicator for targeting of DNA repair and that other transient factors, such as transcriptional state, may play as important a role.

We have previously shown that adaption to radiation in the lower eukaryote *S. cerevisiae* specifically involves induction of an error free homologous recombinational DNA repair process (Mitchel and Morrison 1982, 1984b, 1987; Boreham *et al.* 1991). Since radiation adaption in human and rodent cells results in an increased ability to repair broken chromosomes, i.e. DNA double strand breaks (Azzam *et al.* 1992, 1994a), it is very likely also to involve induction of a recombinational repair process. Additionally, since this radiation induced adaption reduces the probability of radiation generated (Azzam *et al.* 1994b), and spontaneous neoplastic transformation (Azzam *et al.* 1996; Redpath and Antoniono 1998), the repair process is also likely to be error free; i.e. the induced repair process closely resembles that seen in *S. cerevisiae* and is therefore likely to be evolutionarily conserved (Mitchel *et al.* 1997). The results reported here demonstrate that induction of error free double strand break repair, which results from adaption to radiation, also results in a change in the natural hierarchy of chromosomal repair that occurs in response to a large radiation exposure. If the probability of neoplastic transformation which results from either spontaneous DNA damage or from a large radiation exposure represents some balance between correct repair of DNA double strand breaks (i.e. error free homologous recombinational repair) and incorrect repair (possibly some form of error prone non-homologous repair) then our data suggest that adaption to radiation (induction of error free recombinational repair) not only changes the overall balance of error free and error prone repair, but that the induced repair is preferentially directed toward specific chromosomal breaks which would otherwise lead to transformation.

Such biases in repair could also have implications for tissue differences in survival responses to radiation. For example, adapting doses of radiation increase the clonogenic survival of human fibroblasts subsequently exposed to higher doses of radiation (Azzam *et al.*

1992). In contrast, adapting doses of radiation sensitize human lymphocytes to death by apoptosis from subsequent higher doses (Cregan *et al.* 1994). Both the transformation and the survival considerations suggest, therefore, that when induction of the adaptive response alters existing natural repair biases it might affect the natural tissue differential risk of cancer. Such considerations would have implications for radiation risk estimates and for the tissue weighting factors used in those estimates.

These results may also have implications for biological dosimetry estimates of radiation exposure. One assumption in biological dosimetry is that radiation induced cytogenetic damage is random; that is, the probability of damage actually observed in a specific chromosome (by micronucleus formation, chromosomal translocations, etc.) is proportional to dose and to its DNA content relative to the entire genome. However the results presented here support the conclusion that the assumption is not valid and that chromosomal biases normally exist. Further, the biases that exist naturally were influenced by a prior exposure and therefore biological dosimetry results from high exposures could be influenced by a prior low exposure.

Summary

This thesis sought to address two main questions:

(1) What is the RBE of low doses of tritium beta radiation?

(2) Does an adapting dose of radiation alter the risk of inclusion of a specific chromosome in a micronucleus?

Using level of adaption, as indicated by the reduction in micronucleus frequency, the RBE of low doses of tritium beta radiation was determined to be one. This is in agreement with previous studies at high doses, which have also shown an RBE of one or greater and suggests that the W_r of tritium need not be altered for low dose exposures.

The second question was addressed by examining the frequencies of specific chromosomes in radiation induced micronuclei from adapted and non-adapted cells. Alterations in such frequencies between the two groups suggested that repair associated with the adaptive response is biased such that some chromosomes are preferentially repaired at the expense of others. These results indicate that a prior low dose exposure can influence the risk of residual damage within specific chromosomes. This could ultimately influence the risk of radiation induced carcinogenesis, since lesions residing in genes such as tumour suppressor genes or proto-oncogenes, may be either preferentially repaired or discriminated against if they reside on favoured or non-favoured chromosomes, respectively.

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Appendix

Units of measure

Bq	Becquerel, SI unit of activity (1 Bq=1 disintegration/sec)
°C	degrees centigrade
cGy	centiGray
Ci	Curie, non-SI unit of activity (1 Bq=2.707x10^{-11} Ci)
erg	unit of energy
g	gram
Gy	Gray, SI unit of absorbed dose (1 Gy=1 J/kg=100 rad)
h	hour
mCi	milliCurie
mGy	milliGray
MeV	megaelectron volt
min	minute
mL	milliliter
mM	millimolar
mW	milliwatt
nm	nanometer
rad	radiation absorbed dose, non-SI unit of absorbed dose (1 rad=100 erg/g)
rem	radiation equivalent man, non-SI unit of dose equivalent
sec	second
Sv	Sievert, SI unit of dose equivalent, absorbed dose (Gy) multiplied by a radiation weighting factor (W_r) (1 Sv = 100 rem)
v/v	volume to volume
w/v	weight to volume
W	watt
W_r	radiation weighting factor, see Sv above.
µg	microgram
µL	microliter
µm	micrometer

Miscellaneous

CO_2	carbon dioxide
dTTP	deoxythymidine triphosphate
d(AGC)	1:1:1 deoxyadenine, deoxyguanine, deoxycytosine triphosphate
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
G_0	quiescent cells
G_1	gap 1 of the cell cycle prior to S phase
G_2	gap 2 of the cell cycle post S phase prior to M
HTO	tritium oxide
KCl	potassium chloride
LET	linear energy transfer, a quantity to express radiation quality for radiobiological purposes; defined as the quotient of dE by dl, where dE is

	the average energy lost in collisions, and dl is the distance transversed by the particle. Units are typically keV/μm.
M	mitosis
$MgCl_2$	magnesium chloride
MMC	mitomycin C
MN	micronucle(us/i)
NaCl	sodium chloride
PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
RBE	relative biological effectiveness
RNase	ribonuclease
S	synthesis of DNA phase of the cell cycle
SSC	saline sodium citrate
UV	ultraviolet
WCP	Whole Chromosome Paint

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