

RADIATION-INDUCED DNA SINGLE-STRAND BREAK  
INDUCTION AND REPAIR IN MURINE TISSUES  
MEASURED BY THE COMET ASSAY

by

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## ABSTRACT

DNA damage produced in C3H/HeN murine tissues by ionizing radiation was characterized at the level of the individual cell with the goal of defining tissue dependent differences in DNA single-strand break (SSB) induction and repair. Subsequently, nicotinamide, was examined as a modifier of initial DNA damage and SSB rejoining following irradiation. The alkaline comet assay, a single-cell gel electrophoresis method, was used to examine cells from SCCVII tumours, spleen, bone marrow, liver, jejunum, testis, thymus and cerebellum. Cells from all tissues irradiated *in vitro* showed similar radiosensitivity. However, for *in vivo* irradiation, rapid SSB rejoining which occurs in cells during irradiation led to differences between tissues. Also, tumour and testis showed less damage *in vivo* than other normal tissues. Consistent with previous studies, these two tissues were found to contain radiobiologically hypoxic cells. Efficiency of SSB rejoining was cell type-dependent; cells from SCCVII tumors rejoined breaks about 5 times more rapidly than cells from cerebellum. Heterogeneity in speed of rejoining was minimal among cells of a tissue, and no significant damage remained 4 hours following 15 Gy. However, extensive DNA degradation was observed in all tissues except brain 48 hours after 15 Gy. DNA ladder patterns in agarose gels, typical of apoptosis, were observed 4 hours after 2-10 Gy in spleen and thymus.

The vitamin B analogue, nicotinamide, was shown to improve testis and tumour oxygenation, in agreement with other studies. A new observation was that nicotinamide (500 mg/kg or more) given before irradiation inhibited SSB rejoining in cells of all tissues except brain. Furthermore, radiation-induced DNA degradation was found to be greatly accelerated by nicotinamide. Both effects are likely to involve poly(ADP-ribose) polymerase inhibition. However, while nicotinamide significantly retarded radiation-induced SSB rejoining in tumors, the biological significance of this effect is questionable since nicotinamide did not enhance oxygen-independent killing of irradiated tumor cells.

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## 1. INTRODUCTION

### 1.1 Questions and hypotheses

X-rays were discovered exactly one century ago, and since that time, radiotherapy has developed into one of the most effective modalities for the treatment of solid tumours. Since radiotherapy is more successful in treating some tumour types than others, research has been concerned with understanding the mechanisms of radiation-induced cell killing and identifying factors that determine tumour response in relation to normal tissue damage. The early appearance of radiation-induced damage to skin made radiotherapists appreciate the problem of normal tissue damage, and radiotherapy can be fatal when the dose to a vital organ exceeds the tolerance of that organ. Therefore, the effectiveness of radiotherapy in treating cancer is limited by the reaction or sensitivity of normal tissues unavoidably included in the radiation field. The prescribed dose of radiation must be determined by normal tissue tolerance rather than by any factors related to the specific tumour.

Considerable research has concentrated on both tumour cell and normal tissue cell response *in vitro* (e.g. texts by Elkind 1967, Hall 1978, Alper 1979, Hendry 1983, Denekamp 1982, Ono and Okada 1974; Vexler et al. 1982; Midander et al. 1986; and reviews by Frankenberg-Schwager 1990). In these studies, models for describing cell survival after irradiation were developed and tested. However, it has been known that there is a difference in radiosensitivity between tissues irradiated *in vivo* and cultured cells exposed *in vitro* (Ono and Okada 1974), and because methods for evaluating damage to tissues were limited, there have been few molecular studies describing the *in vivo* radiosensitivity of normal tissues. Since mechanistic studies are not easily conducted on clinical material, animal models provide a useful alternative to obtain basic knowledge on tissue-dependent differences in response to radiation. The aims of this study were to clarify the following questions using inbred C3H/HeN mice:

- (1) Does radiosensitivity in different normal tissues and tumours vary in terms of initial levels of DNA damage?
- (2) Do cells from normal tissues irradiated *in vivo* show a similar relationship between DNA damage and radiation dose as cells irradiated *in vitro*, and do cells from all tissues repair damage at a similar rate?

(3) Do any tissues contain cells incapable of recovering from radiation-induced damage or which die shortly after irradiation by apoptosis or necrosis?

(4) Are there any radioresistant cells or hypoxic cells in normal tissues as suggested in some studies?

(5) What is the effect of a putative DNA repair inhibitor, nicotinamide, on repair of radiation damage in cells from tumours and a variety of normal tissues?

Since normal tissues demonstrate both early and late responses to radiation damage (Hopewell 1983), different endpoints have been used by different research workers to describe damage. For example, during the earliest time immediately after irradiation, DNA damage, especially strand breaks and chromosome aberrations, have been the major focus of study (e.g. review by George and Cramp 1988). At the molecular level, radiation induced gene expression has recently been evaluated (Fuks et al 1994). Cell survival is also a critical endpoint in detecting the level of unrepaired damage which is expressed in the progeny cells. At longer times after irradiation (weeks to months), histological evaluation has been valuable for assessing damage to some tissues like liver (Geraci and Mariano 1993), lung (Franco and Sharplin 1993), central nervous system and other organs (Hopewell 1983, Van der Kogel 1991).

In this study, DNA strand breakage was chosen as the endpoint for measuring response to X-rays. A major incentive for this study was the recent development of a sensitive method that allows detection of DNA damage in individual cells taken from virtually any tissue (Fairbairn et al., 1995). The development of the "comet assay" provides an opportunity to re-examine controversial issues and to develop new concepts relevant to normal tissue repair. Measurement of strand breaks allows detection of DNA damage at early times, before any histological phenomena would be observable. This project was therefore mainly concerned with characterizing the radiosensitivity of different normal tissues at the level of DNA in order to provide a basis for studies to measure enhancement or protection against radiation damage. The working hypotheses of this study were:

(1) In the absence of hypoxic cells or DNA repair, the induction of single-strand breaks in a cell is independent of the tissue of origin,

(2) *In vivo*, heterogeneity in DNA strand break induction may indicate the existence of

hypoxic cells,

3) *In vivo*, rejoining of DNA strand breaks in a cell is influenced by the tissue of origin, and,

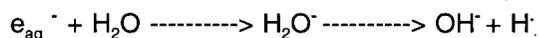
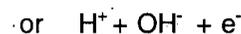
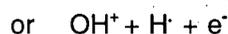
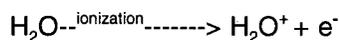
(4) The DNA repair inhibitor and blood flow modulator, nicotinamide, may inhibit repair of radiation damage differentially in normal tissues.

## 1.2 DNA as the target of radiation damage

### 1.2.1 Mechanisms of radiation-induced damage to cells

X-rays consist of photons which carry energy. To produce a biological effect when they pass through a substance, they must deposit their energy within the molecules in their path which causes ionizations and excitations. Chemical dissociations and rearrangements follow. The resultant chemical effects are of two types: the "direct effect" occurs when a molecule is ionized or excited directly by the incident radiation, while the "indirect effect" is a result of transfer of energy from the incident photon beam to the abundant water molecules and then to the biologically relevant molecule (DNA).

In biological tissues, water is the major component (cells are 80% water). The effects of radiation on mammalian cells are primarily the result of the indirect effect from ionization of water molecules and the formation of three major reactive species: OH $\cdot$ , H $\cdot$  and aqueous form of electrons e $_{aq}^-$ . These species are capable of reacting with water and other molecules (Wardman 1983):



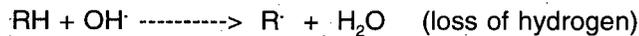
The subsequent reactions of H $_2\text{O}^+$ , H $_3\text{O}^+$  and e $^-$  can also form hydrogen and hydroxyl free radicals:



and formation of hydrogen peroxide, a reactive molecule as well:



All of these short lived reactive species are able to modify and may inactivate biological molecules by chemical reactions with them. For any biological molecule RH,



If a biological macromolecule is in an unstable reactive state such as R<sup>·</sup>, it can be restored by rapid chemical repair. If a molecule in the reactive state is trapped in this state by another molecule (like oxygen), only biological repair can restore the original function (e.g., DNA damage is repaired by specific repair pathways).

### 1.2.2 DNA is the primary target of ionizing radiation

Very high doses of X-rays (10<sup>3</sup> to 10<sup>6</sup> Gy) are needed to directly disrupt cell enzyme activity, ATP production and protein synthesis, while doses as low as 2 Gy can easily delay cell division and induce chromosome damage (Tubiana et al. 1990). A considerable amount of evidence indicates that the primary target of radiation damage is the disruption of vital structures and functions of DNA molecules, the genetic material responsible for cell functions and reproduction (Berger and Altman 1970). The impaired DNA may cause cell death, disfunction and mutation. Sometimes the effect of radiation may not be observed for several cell generations.

As the DNA in a cell is intimately associated with a substantial quantity of water, it is said to be hydrated. About five water molecules are tightly bound to each phosphate group and a further fifteen water molecules per nucleotide are associated with the DNA base to help assemble the DNA molecule in the B (hydrated) form (Fielden and O'Neill 1989). This hydration enables the production of abundant reactive species

around DNA molecules when radiation energy is deposited. About 75% of the radiation damage to DNA molecules is mediated via hydroxyl radicals, which have a relatively long lifetime. Less frequent is the direct attack of DNA molecules by electrons. Whatever the initial event, the chemical nature of the DNA damage is similar, although the distribution of damage can differ. The site on DNA most vulnerable to attack is the sugar moiety of the phosphodiester backbone. This results in either the complete elimination of the sugar and associated base with phosphoryl groups at both 5' and 3' termini, or a break with a phosphoryl group at the 5' end, but an open ring structure of the sugar at the 3' end (Fielden and O'Neill 1991).

### 1.2.3. Nature of DNA Damage by Ionizing Radiation:

Several types of lesions of DNA molecules have been identified (from Wartens and Childers 1982; Wlodek and Hittelman 1987) (Table 1).

TABLE 1: NATURE AND AMOUNTS OF DNA DAMAGE PRODUCED IN MAMMALIAN CELLS BY IONIZING RADIATION

Types of damage	Number of lesions per diploid cell per Gy
Double strand breaks (DSB)	40
Single strand breaks (SSB)	500-1,000
Base damage	1,000-2,000
Sugar damage	800-1,600
DNA-DNA crosslinks	30
DNA-protein crosslinks	150

DNA double strand breaks can be formed by the passage of one electron through the duplex, or from two events produced on the opposite sides of the duplex but in reasonable proximity. There is evidence from yeast mutants indicating that one double strand break can be lethal if unrepaired (Frankenberg et al. 1981). Therefore, measurement of DNA double strand breaks should provide information relevant to cell survival and radiosensitivity of cells.

### 1.2.4 Factors affecting DNA damage production

#### 1.2.4.1. Oxygen

Since various chemical reactions are involved in DNA damage production, there are also several molecules

known to affect the process. For low LET (linear energy transfer) ionizing radiation, oxygen is an important molecule involved in chemically modifying the damage produced by free radicals or reactive molecules. These modifications can otherwise be restored by chemical repair (e.g., hydrogen addition by glutathione, GSH). But oxygen makes this process irreversible by rapidly forming adducts with the ionized macromolecules, a process often called "fixation".

As early as 1909, Schwartz recognized that oxygen could modify radiosensitivity. Later, Mottram (1935) identified an effect of oxygen on tumours from rats. However, it was not until the 1950s when Gray and others clearly showed that in biological systems, oxygen can sensitize cells to ionizing irradiation (Thomlinson and Gray 1955). It was found that oxygen can enhance the degree of cell killing by a factor of 3 compared with irradiation under anoxic conditions (Alper and Howard-Flanders 1956). Hypoxia was subsequently shown to influence tumour response to fractionated radiation treatments in a number of clinical studies (Bush 1978, Overgaard 1979). The concern here is that hypoxic cells which survive radiation exposure will be largely responsible for tumour recurrence (Denekamp 1983).

A class of chemicals called bioreductive agents have been used to detect cellular hypoxic state by the availability of oxygen molecules in cells. RSU 1069 (1(2-nitro-imidazolyl)-3-aziridino-2-propanol) is one chosen for examination in this thesis. Each molecule of RSU 1069 has both an aziridine group and a nitro group; the latter can be metabolized differently depending on the oxygenation state. Under hypoxic conditions, the nitro group is reduced through a series of intermediates and the molecule can bind to DNA. The aziridine group at the other end of the molecule can then attack a nearby DNA base producing DNA interstrand crosslinks. The presence of these crosslinks inhibits DNA denaturation in alkali, thus reducing the fraction of damaged DNA able to migrate in an electric field. Under oxic conditions the aziridine group can capture electrons from the DNA backbone, producing DNA strand breaks in aerobic cells (Olive 1995b,c). However, no interstrand crosslinks are produced under oxic conditions. Consequently, the proportion of crosslinks and strand breaks in a DNA molecule will determine its ability to migrate during gel electrophoresis, and the least migration will be seen in hypoxic cells. Therefore, subpopulations of hypoxic cells can be detected in tumours by examining the patterns of DNA damage induced in individual cells by RSU 1069 (Olive 1995b).

#### 1.2.4.2. Thiols

Cellular thiols influence the initial degree of DNA damage. GSH is the major non-protein sulfhydryl in mammalian cells and is believed to play a significant role in the protection of hypoxic cells from ionizing radiation (DenBoer et al. 1990). The hydrogen atoms from GSH act as free radical scavengers and can chemically repair DNA molecules by hydrogen donation. Depletion of thiols prior to irradiation (Held and Hopcia 1993) or irradiation of glutathione synthetase-deficient cells (Midander et al. 1986) decreases cell survival. Conversely, adding thiols before irradiation enhances cell survival (Held 1988).

#### 1.2.4.3. Higher order chromatin organization

Some evidence indicates that chromatin organization can influence the way in which cells respond to ionizing radiation, and DNA-bound proteins can have significant radioprotective effects (Ljungman et al. 1991, Olive 1992). Chromosome associated proteins like histones control the packaging and condensation of chromatin into higher-order structures, thereby limiting the accessibility of DNA to free radicals. Nuclei stripped of nuclear proteins show 18 to 50-fold more double strand breaks (Xue and Oleinick 1994; Olive and Banath 1995a).

DNA conformation may alter during the process of cell differentiation. An obvious example is the differentiation of mouse spermatocytes. The more differentiated spermatids yield less DNA SSB than spermatogonia (Van Loon et al. 1991). Cells are not only sensitive to radiation during mitosis, changes in radiosensitivity are also seen in cells progressing through the cell cycle (Tolmach 1961).

#### 1.2.4.4. Quality of ionizing radiation

Finally, radiation quality itself is important. Radiation energy which is deposited more densely (high LET radiation) yields more free radicals along the path, giving less opportunity for chemical repair. For low LET x-rays, ionization events are more sparse which enables restoration of excited molecules. Based on the considerations of the nature of ionizing radiation, Ward (1981) proposed a mechanism to explain radiation-induced complex damage of intracellular DNA, which he called local multiply damaged sites (LMDS). LMDS are caused by non-uniform energy deposition events in DNA. The reactive species that attack DNA arise within a volume around the DNA which is limited by the presence of radical scavengers. LMDS are

considered to be more biologically significant than single events since the cells are challenged to cope with multiple damaged sites in a small volume. This theory also explains why cell mutation is a more likely consequence of the presence of radiation damage within a gene than the presence of damage by agents which produce only single events (Ward 1994).

#### 1.2.5 Enzymatic repair of DNA damage

It has been known for many years that cells can eliminate DNA lesions by enzyme-mediated processes (Rupert et al. 1958). Later Setlow and Carrier (1964) described a mechanism of DNA repair called excision repair which is universal to all eukaryotes. During the past several decades the enzyme repair systems have been extensively studied. Now it is known that there are several pathways in DNA repair (Sancar 1994): base excision repair, nucleotide excision repair, single-strand break repair, DSB repair, recombinational and post-replication repair, and in lower eukaryotes, photoreactivation. Each repair pathway has its own specific substrates and enzymes, but may overlap to some extent with others and with transcription complexes within the cell.

##### 1.2.5.1 DNA repair pathways

As shown previously in Table 1, base and sugar damage are abundant lesions produced by ionizing radiation. More than 100 kinds of lesions have been identified in these categories alone and they are repaired by the same pathway, base excision repair (Wallace 1994). The enzyme system responsible for the repair has been characterized in *E. coli*. The damaged base is first recognized and removed by a DNA glycosylase, leaving the abasic sugar (AP site). An incision is made by AP lyase and the abasic sugar is released by 5' apurinic (AP) endonuclease. Then the single base gap is filled in by DNA polymerase I and sealed by DNA ligase.

In nucleotide excision repair, the enzyme system involved is called an excinuclease which consists of at least 17 polypeptides in humans (Sancar 1994). Patients with the disease xeroderma pigmentosum (XP) have been found to be defective in excision repair. Seven genes are responsible for the mutation, XPA through XPG. The damaged nucleotide is recognized by XPA, which binds to the XPF-ERCC1 (ERCC1, excision repair cross complementing gene1) heterodimer and to the replication protein HSSB (human

single-strand binding protein); the latter binds to the site of the lesion. The repair factor TFIIH (transcription factor IIH) which contains XPB, XPD and another six subunits is recruited to the damaged site by XPA. XPC and XPG are also recruited via the action of TFIIH. The two proteins with nuclease activities XPF and XPG make incisions at each side of the damaged site. It is suggested that XPG makes the 3' incision and XPF the 5' incision. Usually a 12-13 base nucleotide in procaryotes or 27-29 base nucleotide oligomer in eucaryotes is cut (Sancar 1995). Then protein RPA which particularly binds single stranded DNA displaces the damaged oligonucleotide and unwinds that region. The catalytic enzymes are released from DNA by proliferating cell nuclear antigen (PCNA). The excision gap is filled in by polymerase d and e and ligated (Wood 1995).

DNA SSB rejoining involves several repair enzymes. It has been suggested that the first activated enzyme is poly(ADP-ribose) polymerase (PARP). This enzyme can "recognize" strand breaks and bind to them (Gradwohl et al.1990), and may stimulate the rejoining process by "recruiting" repair enzymes to damaged sites (Boulikas 1993, Satoh and Lindahl 1994). Thus it is a damage recognition protein which may cooperate with protein XPA in DNA repair. Next, "excision repair" takes place, a similar process to nucleotide excision repair (review by Powell and McMillan 1990). Although many studies showed that SSB may not be lethal to cells, some authors have claimed that under some particular conditions, SSB can be lethal (Churchill et al. 1991).

A sub-pathway in nucleotide excision repair has also been identified called transcription-coupled DNA repair (TCR). This pathway seems to be operative in all kinds of cells including post-mitotic-neurons, and it does not vary in efficiency through the cell cycle. Even p53 deficient cells, which have a significantly reduced capacity for global excision repair, still retain TCR (Hanawalt 1994). This type of repair seems to be important in maintaining basic functions of the cell.

In DSB rejoining, different mechanisms have been proposed over the years (eg. review by Weaver 1995). It was initially the study of V(D)J (variable diversity joining) recombination, an important mechanism involved in the formation of genes responsible for antigen-recognition molecules and TCR, that led people to realize that protein *Ku* specifically recognizes the ends of DSB. The *Ku* 70/80 heterodimer can bind to each side

of the double strand break, relocating DNA ends for repair. Furthermore, a molecule called DNA-dependent protein kinase (DNA-PK) is also activated by DNA fragments, resulting in phosphorylation of many transcription factors (and other DNA-binding proteins) to modulate their functions (Anderson 1993). Actually the heterodimer of *Ku* itself is the component of DNA-PK; its function is probably to target the catalytic subunit of DNA-PK to DNA (Siple et al. 1995). The fact that diploid yeast can only repair DSB in G<sub>2</sub> but not G<sub>1</sub> phase suggests that recombination is required for repair in this organism (Brunborg et al. 1980). However, if the nucleotides on both strands are lost, the lesion is hardly likely to be restored, and is likely to lead to cell mutation or cell death. The precise mechanism of DSB repair is still in question, and studies from mutant mammalian cells indicate that the ability to repair these lesions is an important determinant for cell radiosensitivity (Zaffaroni et al. 1994, Lee et al. 1995). In some studies, cell survival has been shown to be related to the amount of unrepaired double strand breaks following irradiation (Kellend et al. 1988; Kysela et al. 1993), supporting the idea that rejoining of the DSB is critical for cell viability. However, other studies do not support the theory that cells varying in radiosensitivity also vary in amount of residual DNA damage (Olive et al. 1995).

Cells from severe combined immune deficiency (SCID) mice are known to be deficient in their ability to rejoin DNA DSB (Fulop and Philips 1990, Beiderman et al. 1991). Recently the SCID gene has been cloned, and shown to be identical to p450 or so called DNA-PKcs, the catalytic subunit of DNA-dependent protein kinase which was originally called p350 (Kirchgessner et al. 1995). The study also showed that both p450 and a gene complementing the SCID defect co-localize to human chromosome 8q11. Furthermore, the HYRC1 locus on human chromosome 8q11 was found to encode the SCID factor involving all V(D)J recombination joint formation and in 30-40% of DSB repair (Komatsu et al. 1995). Mouse SCID cells are found to have lost the p450 subunit. Therefore, the SCID defect seems to impair the DNA-PK holoenzyme, the critical enzyme in DNA DSB repair. Interestingly, all SCID cell lines only exhibit hypersensitivity to agents causing DSBs (X-rays and bleomycin) but not to chemicals causing SSBs or crosslinks (Hendrickson et al. 1991). This suggests that DNA DSB repair involves a different pathway than SSB repair.

The most important example of a mutation in human resulting in radiosensitivity is the disease ataxia-

telangiectasia, a genetic disorder first described by Syllaba and Henner in 1926. In addition to the principal features of neuromotor dysfunction, extreme immunodeficiency, a high frequency of lymphoreticular and other types of neoplasia, cells from these patients are hypersensitive to radiation and bleomycin (Taylor 1975). Cells are incapable of repairing radiation-induced potentially lethal DNA damage and lack normal cell cycle checkpoints, with the consequence that these cells continue cycling in spite of extensive DNA damage. Chromosome aberrations are high in these cells. The genetic mapping of the A-T gene localizes to an 850 kb region on chromosome 11q23.1 (Gatti et al. 1994). Recently the AT gene was identified and cloned, and was found to encode a protein of considerable size with similarity to phosphatidylinositol 3-kinase, an enzyme involved in signal transduction that controls signals for cellular proliferation (Savitsky et al., 1995). Earlier studies have suggested that A-T cells have an altered chromosome structure so that DNA damage is more readily converted into DSBs, and DSBs into chromosome breaks. (Hittelman and Pandita 1994). Without enough time for repair, cells are consequently more prone to death. It seems that the A-T gene product is likely to involve a complex signal transduction pathway that ultimately leads to the vital maintenance of chromosome integrity.

#### 1.2.5.2. Factors affecting DNA repair

A variety of factors can affect DNA repair and cell survival after irradiation. The degree of chromatin condensation may affect the accessibility of DNA to repair enzymes (Wheeler et al. 1983). Cell differentiation stage and proliferation state have been shown to influence the process of DNA repair as seen in both rodent cells (Sinclair et al. 1972, Rasko et al. 1993) and human cells (Kaminskas and Li 1992). Even for the same DNA molecule, a transcriptionally active gene is preferentially repaired over an inactive gene (Ho and Hanawalt 1991), supporting the importance of DNA conformation in repair kinetics.

Inhibitors of DNA repair include poly(ADP)ribose polymerase inhibitors (e.g., 3-aminobenzamide, nicotinamide), ATP inhibitors which reduce the energy level of the cell (2,4-dinitrophenol), and DNA polymerase and ligase inhibitors (ara-C). Not all of these repair inhibitors are effective *in vivo*. Some studies have indicated that nicotinamide, an inhibitor of poly(ADP-ribose)polymerase is effective not only *in vitro* (Ben-Hur et al. 1985) but also *in vivo* (Kelleher and Vaupel 1994a). Due to the low toxicity of this drug and the excellent sensitization obtained in mouse tumour models (see Section 1.5), it appears to be

a promising radiosensitizer for the clinic. However, nicotinamide appears to be acting primarily as a blood flow modulator *in vivo*, and the importance of the DNA repair modifying properties of this vitamin analogue have not been well defined.

#### 1.2.5.3. Radiation-induced DNA damage to normal tissues: *in vitro* studies

The earliest discovery of radiation-induced damage to normal tissue may be traced back almost one hundred years when radiation workers were first observed to develop skin lesions following exposure. Since the development of molecular methods for the detection of DNA strand breaks by McGrath and Williams in 1966, DNA damage and repair have been extensively investigated in cultured mammalian cells (Ono and Okada 1974, Vexler et al. 1982, Sakai 1984, Churchill 1991 and reviews by George and Cramp 1988, Powell and McMillan 1990). In these studies, the degree of DNA strand breaks and rejoining kinetics were examined, but since different methods were used, the initial degree of DNA damage could not be readily determined for different tissues. However, using a variety of cultured cell lines, some investigators found that the number of DNA strand breaks is linearly related to radiation dose, and the yield of DNA strand breaks per dose was independent of cell type. Although some conflicting data also exist which showed variations in yield of single-strand breaks (Meyn et al. 1980) and double strand breaks from one cell type to another (reviewed by Ward 1990), these differences have been attributed largely to methodology and to the influence of chromatin structure on the ability to detect DNA strand breaks.

Since the pioneering work of Len Tolmach (1961), it has been known that cells in different phases of the cell cycle have different intrinsic radiosensitivities. Cell survival analysis indicates that cells at the end of G<sub>1</sub> phase are generally the most radiosensitive, but cells then become progressively more resistant as they move through S phase. As cells pass into G<sub>2</sub>, they become sensitive again and are most radiosensitive during mitosis (Sinclair 1972). It was also found that sensitivity varies with the state of cell differentiation. For testicular cells, regardless of whether cells are irradiated *in vitro* (Coogan and Rosenblum 1988) or *in vivo* (VanLoon et al. 1991), the terminally differentiated, elongated spermatids always demonstrate less DNA damage than the proliferating spermatocytes. Therefore, DNA conformation appears to influence cellular radiosensitivity.

Changing the intracellular thiol levels (Held 1988) influences the intrinsic cellular radiosensitivity. Some authors have even suggested that protein thiols (non-GSH) may participate in the chemical repair process (Held 1988). Furthermore, by changing cellular oxygenation, cell surviving fraction is three times lower in aerobic cells than in cells irradiated under nitrogen (Cerutti 1974). Assays from initial strand break induction also agreed with this value (Frankenberg-Schwager 1990) which is called the oxygen enhancement ratio (OER). Using the alkaline sucrose gradient technique, Chapman et al. (1974) showed that the relationship between concentration of oxygen and cell killing was identical to the relationship between oxygen concentration and number of DNA single-strand breaks. More recently, Zhang et al. (1995), using the more sensitive alkaline elution method, have also confirmed that these "k curves" are identical for cell killing and strand breakage. This result is important since it means that strand breaks can be used to differentiate between aerobic and radiobiologically hypoxic cells (Olive et al., 1993b; Zhang and Wheeler, 1993). On the curve describing radiosensitivity as a function of oxygen partial pressure (k curve), the OER increases from 1 to about 3 as oxygen tension increases from 0 to 30 mmHg, then the plateau is reached. Therefore, a tissue with oxygen tension lower than 30 mmHg approaches radiobiological hypoxia. The k value is defined as the oxygen partial pressure which produces half of the maximum OER (2.0) and is approximately equal to 3 mmHg (Hall, 1978).

Many studies indicate that the rate of SSB rejoining is much faster than rejoining of DNA DSB. All repair studies show that SSB are rejoined rapidly with time during postirradiation incubation of cells under optimum growth conditions. The rate of rejoining can often be divided into two or more phases. For DNA SSB, up to 95% of all breaks are rejoined independently of dose with a half-time,  $t_{1/2}=2-5$  min during the fast phase. A slower phase with  $t_{1/2}=50-70$  min is said to be dose-dependent. One explanation is that there exist sites less accessible for DNA repair enzymes (Wheeler et al. 1983). Alternatively, new SSB may form with time due to incisions at base damage sites. In some studies examining the kinetics of SSB rejoining, a third phase with half time of rejoining longer than 1 hour has been observed, which is explained as the period during which DSB are rejoined (Frankenberg et al. 1981). For DSB rejoining, the same general pattern is observed but the half time for each phase is longer (about 10-25 min, and 50-120 min for the fast and slow phases respectively) (Vanankeren 1988), again perhaps indicating that a different repair mechanism operates to rejoin SSB and DSB.

In reviews by Fertil and Malaise (1981), Ward (1990), McMillan and Peacock (1994), differences in intrinsic cellular radiosensitivity have been ascribed to several reasons: cellular repair capacity, cellular oxygenation, thiol levels, and differences in DNA conformation which can affect both damage and repair. Some authors also suggest that DNA-bound proteins are important for DNA conformation and cell radiosensitivity (Ljungman et al. 1991). During the development of male germ cells, there is a dramatic change in chromatin components which significantly affects radiosensitivity (Balhorn 1982). For somatic cells, early studies using microbeams indicated the DNA segments close to the nuclear lamina are found to be most radiosensitive (Cole et al. 1980).

### 1.3. *In vivo* studies of normal tissue damage

Early studies by Karran and Ormerod (1973) found that many of the nonreplicating mammalian cells are capable of rejoining x-ray induced DNA SSB. They showed that cells from rat thymus and spleen possess repair enzyme(s) although these cells do not synthesize DNA regularly. The same ability was also seen in muscle cells from 1-day-old rats but not from one week or older rats. Furthermore, it is interesting that newly formed nucleated chicken erythrocytes cannot rejoin strand breaks suggesting that repair enzymes may be lacking in these cells. Terminally differentiated cells *in vivo*, with this one possible exception, appear able to repair the majority of DNA single-strand breaks. This is perhaps not surprising since oxidative DNA lesions, a necessary by-product of metabolism, must be repaired in order for transcription to continue.

In a more in-depth study by Ono and Okada (1974), DNA strand break induction and rejoining differed for cells irradiated *in vitro* compared to *in vivo*. They found that thymocytes irradiated *in vitro* always demonstrated more SSB per Gy than those from whole-body-irradiated mice, ascribing this to the different milieu including oxygenation of cells, although this needs to be confirmed in light of the rapid apoptosis induced in these cells upon *in vitro* cultivation. They also found that thymocytes rejoin SSB faster *in vitro* than that *in vivo*, with a half time for rejoining 35 min and 45 min respectively after 100 Gy of gamma-rays. Hepatocytes rejoined breaks more slowly *in vivo* compared with thymocytes, with a half time of 70 min. This result was taken to mean that non-cycling ( $G_0$ ) cells are less efficient in strand break rejoining. At the same time, rapidly proliferating intestinal epithelium was studied. Rydberg and Johanson (1975) found that

SSB rejoining was relatively fast in these cells. Most of the rejoining was completed within 15 min, and at the end of 30 min after 3-20 Gy gamma-rays almost all the damage was repaired. Using the radiolabelling method they found that the repair process for crypt and villous cells were quite similar. But when these cells were examined histologically at specific times, significant cell death (pyknotic cells) were found in 80% of crypt cells but not in villous cells. In agreement with the report of Devik (1971) and Tsubouchi et al.(1974), such cell death occurs 3-6 hrs after 7-20 Gy. The incidence of cell death has a reverse relationship with mitoses; the number of mitoses increases after the peak of cell death. Therefore, these authors called this form of cell death "interphase cell death" which is now understood to be apoptosis. But for some reason, DNA degradation in these cells was not observed when crypt cells were examined for DNA rejoining.

Wang and Wheeler (1978) compared the response of cerebellar neurons with intracerebral 9L tumour cells, and found the same number of DNA strand breaks after 50 Gy x-rays. Neurons could fully repair the damage but required 1.5 hour while 9L tumour cells only required 30 min. Considering that both types of cells are well oxygenated (the 9L tumour has less than 1% hypoxic cells), the authors suggested the slower repair rate of neurons may be due to a biological property of the nuclei. In a later study by Wheeler et al. (1983), they showed that 9L tumour cells rejoin 95% of SSB in the fast phase ( $t_{1/2} < 5$  min) but only 87% of cerebellar neuron SSB are rejoined in this phase with  $t_{1/2} = 5.5$  min. Both of the cell types had a slower repair phase with  $t_{1/2} > 1$  hour. It was postulated that tumour cells have more repair capacity per unit of DNA and their chromatin is more accessible for repair than that of neurons.

A problem with comparing results from different studies is that a variety of methods have been applied which have different sensitivities to DNA damage and which may be affected to different degrees by the presence of degrading/dying cells. Only Meyn and colleagues have made a concerted effort to examine several tissues in the same laboratory with the same technique - alkaline filter elution. Meyn et al. (1980, 1983) found that differences not only exist between cells irradiated *in vitro* and *in vivo*, but tissues from the same mice have different degrees of strand break induction. They found that bone marrow cells are 2-4 times more sensitive than fibrosarcoma cells for SSB induction after 10 Gy gamma-rays, no matter whether irradiation occurred *in vivo* or *in vitro*. Among the normal tissues they studied, bone marrow, spleen and

brain cells were most sensitive, while cells from testes, liver and gut were less sensitive in terms of SSB production. Fibrosarcoma cells were said to be radioresistant due to their large portion of hypoxic cells *in vivo*, but these authors also postulated that the variation of normal tissues may be due to radiobiological hypoxia in some normal tissues.

Murray et al (1984) found that two fibrosarcoma tumour lines rejoined SSB completely within two hours after 12.5 Gy, but 7-16% of unrepaired lesions were still present in normal tissues. Among all the normal tissues in their study, liver repaired damage fastest. Reduced glutathione (GSH) was also assayed. Liver was found to have the highest level and fibrosarcoma had the lowest. However, these authors concluded that overall GSH level was not the major determinant of DNA repair *in vivo*, but cell differentiation seemed to be more important. Murray and Meyn reiterated this finding in a subsequent study (1987). By radiolabeling proliferating cells in jejunum and bone marrow, they showed that rapidly proliferating cells rejoined SSB faster than the overall cell population. This ability decreased as cells matured. The authors suggested this may be due to the decreased accessibility of damaged chromatin to repair enzymes or decreases in nucleotide pools essential for repair synthesis. Imai et al (1987) studied the radiosensitivity of haematopoietic precursor stem cells. Cell survival after 3 Gy whole-body x-ray irradiation indicated that erythroid precursor cells were more sensitive to radiation than rest of the cell population.

Clearly the issue of normal tissue hypoxia is an important one in relation to normal tissue damage by radiation. It has been suggested that some normal tissues are nearly hypoxic *in vivo* (Hendry 1979, Meyn et al. 1983, Zhang and Wheeler 1993). Some tumours have been carefully evaluated for hypoxia and classified as diffusion-limited or perfusion-limited. Malignant tumours generally show a rapid rate of proliferation. Tumour blood vessels may not be able to develop rapidly enough so that areas of hypoxia and high interstitial fluid pressure can develop. As seen on the diagram of Fig. 1, some tumour cells distant from the blood supply are termed to be oxygen diffusion-limited (chronic) hypoxia. Conversely, a transient stop of blood flow in tumours causes acute or oxygen perfusion-limited hypoxia (Brown 1979, Chaplin et al. 1989). The high interstitial fluid pressure is a likely cause of intermittent blood flow and therefore acute hypoxia in cells close to blood vessels (Trotter et al. 1989). Increased plasma viscosity and vessel clogging by blood cells may also contribute (Price et al. 1995).

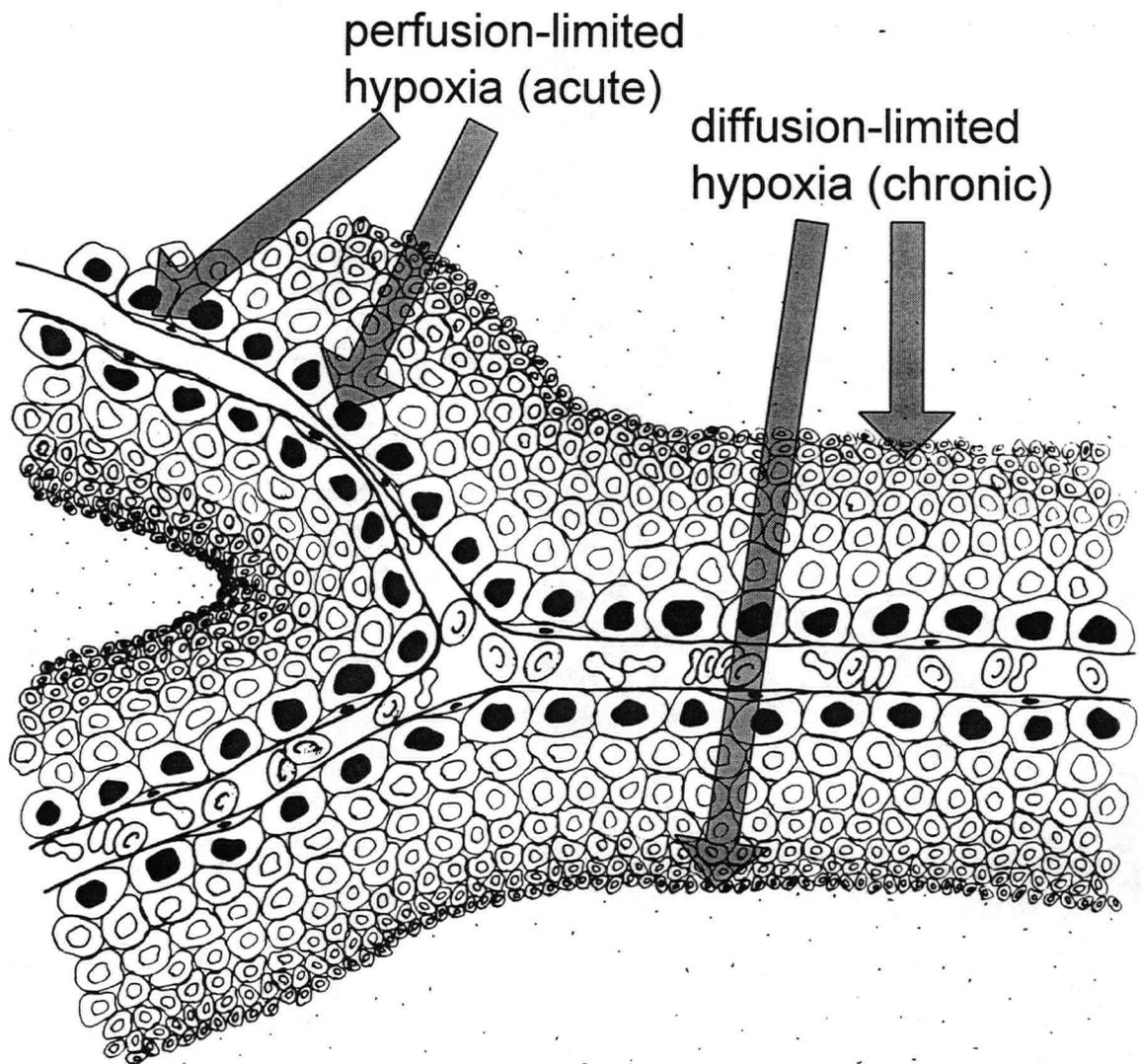


Fig.1: Diagram illustrating the patterns of hypoxia which can develop in solid tumors. Inadequate tumor vasculature leads to areas far from blood vessels which contain chronically hypoxic cells, and areas adjacent to blood vessels undergoing transient changes in perfusion.

For normal tissues, hypoxia is more often due to a diffusion limitation as suggested by Setchell (1978) and Max (1992). They found the oxygen tension can be as low as 2 mm Hg in seminiferous tubules while it is 80-90 mm Hg in arterioles supplying the tissue. Areas of the bone marrow are also suggested to be hypoxic (Olive et al.1994). Allalunis et al. (1983) found a group of granulocyte precursor cells residing in compact bone to be resistant to ionizing radiation, but the same type of cells in medullary cavity were sensitive; they did not study the situation of overall heterogeneity in the bone marrow cavity.

Testis is a tissue which has been studied extensively because of its importance in germ cell production and its special characteristics in cell development. An early study by Ono and Okada (1976) showed some evidence that testicular cells are radiobiologically hypoxic since the overall degree of damage in this tissue is less than that of other tissues. Many studies have established that the degree of DNA strand break induction and rejoining rate decreases with stage of differentiation, i.e. from spermatogonia to spermatids (Coogan and Rosenblum 1988, Joshi et al.1990, VanLoon et al.1991, 1993 and VanderMeer et al.1992). Although the spermatogonia are believed to be most radiosensitive, a previous study showed that a hypoxic cell radiosensitizer misonidazole increased stem cell killing induced by radiation, suggesting at least part of the spermatogenic stem cells are not well oxygenated and therefore relatively radioresistant.

In conclusion, *in vivo* studies have shown differences in the degree of DNA damage induction and rate of rejoining for various tissues. Variation is also seen among different cells of the same tissue when animals are whole-body irradiated. The explanations may vary for different tissues, but could involve DNA conformation, cellular thiol levels, tissue oxygenation status, the ability of cells to repair DNA damage, and specific mechanisms resulting in cell loss (eg. apoptosis versus necrosis). Further effort is needed to improve our knowledge of the mechanisms of radioresistance at the cellular and molecular levels, and to attempt to resolve some of the conflicting results in the literature.

#### 1.4. Radiation-induced apoptosis in different tissues

##### 1.4.1. Morphology, biology and genetics of apoptosis

Since apoptosis was first named by Kerr, Wyllie and Currie (1972) this special mode of cell death has been studied intensively. It is characterized by cell shrinkage and loss of specialized surface elements such as

microvilli and cell-cell junctions. Inside the apoptotic cell, chromatin condenses towards the nuclear periphery and chromatin fragments become surrounded by nuclear membranes. This can be followed by cell surface protrusion and budding to form many membrane-enclosed apoptotic bodies. The formation of these bodies is believed to be the result of a change in the microfilament network (Naora 1995). Nuclear fragments may or may not be included in each apoptotic body. *In vivo*, these apoptotic bodies will generally be phagocytosed and digested by nearby cells. There is no associated inflammation with the exudation of specialized phagocytes as is evoked by necrosis, and apoptotic bodies may be phagocytosed by epithelial and neoplastic cells as well as by tissue macrophages (Kerr and Searle 1980). The early events of apoptosis are finished quickly, with only a few minutes elapsing between onset of the process and the formation of apoptotic bodies (Kerr and Winterford 1994). Therefore, it is rare to see budding and convoluted cells in tissue sections. After phagocytosis, digestion is complete within hours. Apoptosis characteristically affects individual scattered single cells in tissues while necrosis involves a larger group of adjoining cells.

A characteristic biochemical feature of the apoptotic process is double-strand cleavage of nuclear DNA at the linker regions between nucleosomes leading to the production of 180-200 nucleotide-long oligonucleosomal fragments. These fragments are detected readily by agarose gel electrophoresis which often shows a characteristic ladder pattern. Each band of a ladder is a multiple of units containing 180-200 base pairs. But degradation occurs for DNA from necrotic cells, and only a smear of randomly-sized DNA fragments is seen following electrophoresis (Kerr and Winterford 1994).

Apoptosis plays an essential role in normal tissue development and in maintenance of the steady state in continuously renewing tissues. It is seen in deletion of T and B cell lineages during negative selection in the immune response (review by Wyllie 1993), in organogenesis in the developing embryo, and in cerebral cortex and future subcortical white matter development during the first few days after birth (Ferrer et al. 1992). Apoptosis also occurs spontaneously in some tumours although it is not the only mode of cell death in neoplastic tissues (Sarraf and Bowen 1991). Furthermore, it can be induced by radiation, chemotherapy, mild hyperthermia, hormone ablation and/or supplement (e.g. glucocorticoids, retinoic acid and thyroxin), virus infection, cytotoxins or some special antibodies against Fas/APO-1 antigen in many tumours as well

as in some normal tissues (Yamada and Ohyama 1988, Gorczyca et al.1993a, Bursch et al.1990, Garvy et al. 1993, Alison and Sarraf 1995).

The signals for apoptosis can be initiated by cell injury or cytokines. Several receptors are responsible for the apoptotic pathway: two common examples are tumour necrosis factor TNF-alpha receptor in most cells which pass signals by activating ceramide; and the Fas/APO-1 receptor which is seen in cytotoxic T-lymphocytes and hepatocytes. A third receptor Nur77 signals death in thymocytes pre-stimulated by the CD3/T-cell receptor (Wyllie 1995). The regulation of apoptosis is very complex and the interactions of the regulators, signal transduction pathways, and effector molecules are not clearly understood. Most of the relevant genes belong to the family of oncogenes and oncosuppressor genes. Apoptosis is associated with c-myc gene activation and rb-1/growth factor deprivation in many cells. The oncosuppressor gene p53 can initiate apoptosis by causing G<sub>1</sub>/S arrest in cells expressing c-myc. It is thought that the p53 gene product protects cells against the accumulation of mutations and subsequent conversion to a cancerous state by deleting damaged cells via apoptosis (Levine et al.1994). p53 may trigger apoptosis when DNA repair fails. But p53-induced apoptosis is not the only pathway, since apoptosis also occurs when this protein is mutant or absent as seen in p53-null animals (reviewed by Wyllie 1995). The proto-oncogenes ras and bcl-2 rescue cells from susceptibility to apoptosis and convert a high-turnover state into one of rapid cell expansion. By forming a heterotetramer with bcl-2, a related protein bax combats bcl-2 to promote apoptosis depending on the ratio of the two proteins (Korsmeyer 1995). Finally, interleukin-1B-converting enzyme (ICE) is suggested to cleave a precursor cytotoxic protein into its active form, the apex of a cascade of proteolytic events in the common pathway (Alison and Sarraf 1995). DNA is first cleaved to large fragments (50-300 kb) and only later to the nucleosomal "ladders". There are several more genes related to the process at different stages and circumstances although their effects are not yet clear. Some growth hormones may be important in different cell populations by acting as protectors in sparing cells from apoptosis, as has been shown in the haematopoietic system (Yu et al. 1993). At the cellular level, a series of biochemical events are also seen. The signal transduction pathways (which may include Ca<sup>2+</sup>, cAMP, protein kinase C, phosphatases and other kinases) and molecular details of the transcriptional response of cells to receptor stimulation may vary from one cell/receptor type to another (Fesus 1993). The cytosol level of Zn<sup>2+</sup> (Sunderman 1995), changes in the Ca<sup>2+</sup> ionophore, and even oxidants produced from

metabolism (Slater et al.1995) may all play important roles in inducing apoptosis.

#### 1.4.2 Ionizing radiation-induced apoptosis

Ionizing radiation can trigger apoptosis via DNA damage-activated protein kinase (DNA-PK) and other kinase activation, leading to the initiation of a signal transduction system (Sutherland et al. 1992, Anderson 1993). It has also been postulated that the same type of DNA damage can be translated into different cellular processes leading to either apoptosis or another kind of cell death, depending on the opening of an avenue to the signalling pathways and/or the availability of the specific receptor that initiates the pathways in different cells (Szumiel 1993). DNA strand breakage activates extensive formation of ADP-ribose polymers, causing exhaustion of NAD<sup>+</sup> pools and depletion of ATP for resynthesis of NAD<sup>+</sup>. Since some cell types cannot cope with the stress of ATP depletion, they die by apoptosis. This may be the situation observed by Radford (1994), who found that lymphoid ST4 cells can repair 60% of double strand breaks induced by 45 Gy x-rays before they undergo apoptosis. ATP depletion is not always the cause for initiating apoptosis; Wells et al. (1990) found that heavily damaged cells still maintain a normal level of ATP.

Among all the cells from different murine tissues studied, cells from the hemopoietic system, especially thymic lymphocytes (Yamada et al.1988) and bone marrow B-lymphocytes (Garvy et al. 1993) are the most radiosensitive cells and undergo apoptosis within hours after a low dose of radiation *in vitro*. Cultured human B lymphoblast TK6 cells are also observed to undergo apoptosis after 0.5-15 Gy X-rays. In these cells, the largest increase in apoptosis is observed between 8-10 hours after irradiation and is dose-independent (Olive. et al.1993a). But radiation-induced apoptosis is also seen *in vivo* in other tissues. Monkey lacrimal gland (Gazda et al.1992), young rat cerebellar cells from external granular layer (Fritsch et al. 1994) and oligodendrocytes (Vrdoljak et al. 1992), endothelial cell lining of the pulmonary microvasculature (Fuks et al.1994), cells from spleen and thymus (Nomura et al.1992), small intestinal cells (Merrit et al.1994) and many tumour cells (Stephens et al.1991) all display apoptosis following irradiation.

Nomura et al. (1992) found that as low as 2.5 Gy dose of whole-body irradiation can induce apoptosis in sensitive murine tissues such as thymus, spleen and small intestine. The amount of apoptosis is dependent on the strain of mouse, with C57BL/6J and AKR/J mice highly susceptible while C3H mice show a relatively low rate of apoptosis. Radiation-induced apoptosis always occurs several hours after treatment

and only lasts for a few hours *in vivo*, probably due to phagocytosis by nearby cells. Often the more rapidly growing cells (with the exception of thymocytes) undergo radiation-induced apoptosis, while apoptosis in terminally differentiated cells such as hepatocytes can be difficult to induce by radiation but can be demonstrated after treatment with some chemicals such as cycloheximide (Ledda-Columbano et al.1992) or following withdrawal of growth stimulants (Garcea et al.1989, Bursch et al.1990). Even in the small intestine, only certain crypt cells undergo apoptosis after irradiation. This indicates that apoptosis is induced via different pathways in different cells and for cells in different stage of proliferation. Merritt et al. (1994) found that in small intestine, p53 was mostly expressed in stem cells, i.e., cells at positions 1-6 in the crypts, but it decreased sharply thereafter. This pattern correlated with the incidence of radiation-induced apoptosis in the hierarchy of cells. However, it was concluded that cells above position 7 can undergo apoptosis by other mechanisms especially induced by some chemotherapeutic drugs (Potten et al.1994). Radiation-induced apoptosis happens primarily in rapidly proliferating cells probably due to differences in signal transduction pathways or receptors for these signals.

Apoptosis is readily induced by radiation in radiosensitive tumours such as the murine ovarian carcinoma but was not observed in a radioresistant hepatocellular carcinoma (Stephens et al.1991). Lymphoma F9 stem cells display more apoptosis when irradiated compared to their differentiated counterparts (Langley 1993). This observation is consistent with the *in vivo* results indicating that the stage of cell differentiation is important in apoptosis. Furthermore, some tumour cells may retain some features from the parental cells, or they may develop different pathways leading to death. For example, a mutation in the p53 oncosuppressor gene is a common late occurrence in the evolution of a tumour, and loss of functional p53 will mean loss of the rapid apoptotic pathway (Vogelstein and Kinzler 1992). Although many avenues may lead to apoptosis following damage by radiation, it has been shown that calcium and signal transduction pathways are important factors in regulating radiation-induced apoptosis (Meyn et al.1993).

#### 1.5. Nicotinamide: an agent with many effects *in vivo*

Since the first description of nicotinamide as a radiosensitizer by Calcutt et al (1970), this amide form of vitamin B<sub>3</sub> has been studied for more than twenty years due to its many functions in biological systems. This chemical is an inhibitor of poly(ADP-ribose)polymerase (PARP), a multifunctional enzyme that appears

to be involved in repair of DNA strand breaks (Durkacz et al.1980, Creissen et al.1982, Mathis and Aothaus 1990, Gradwohl et al.1990), and in many other biological processes related to alterations of chromatin architecture such as cell differentiation, cell cycle control, transformation and transcription (Boulikas 1993, Banasik and Ueda 1994). PARP affects several important enzymes including DNA polymerase, topoisomerase, ligase, RNA polymerase, histones and many other nuclear proteins. Although the effects of this modification are still unclear, it has been shown that PARP can be activated and induced at the mRNA level by ionizing radiation and other DNA damaging agents (Ben-Hur and Elkind 1984, Bhatia et al. 1990). It is believed that the polymer helps to unwind the tightly packed nucleosomal structure of chromatin (Berger 1985, Gradwohl et al.1987). Furthermore, one of the two zinc fingers of PARP specifically recognizes DNA strand breaks (Durkacz et al.1980). Recently, a cysteine protease, apopain (prICE/ CPP32) has been found to be responsible for the proteolytic breakdown of poly(ADP)ribose polymerase (PARP) which occurs at the onset of apoptosis. Proteolytic cleavage of PARP separates the two zinc-finger DNA binding motifs, which presumably prevents PARP from coordinating DNA repair (Nicholson et al. 1995).

Based on these results, inhibitors of PARP were examined as sensitizers of murine tumours. Four classes of inhibitors are known: aromatic amides such as 3-aminobenzamide (3-AB), nicotinamide, thymidine and methyl xanthenes (e.g. caffeine). Previous studies on cultured cells indicated that 3-AB was more effective than nicotinamide in enhancing cell killing by ionizing radiation (Brown et al.1984, Ben-Hur et al.1985). Nicotinamide did not seem to be effective on all the cultured cells but was more effective in sensitizing tumours *in vivo* and much less toxic than 3-AB (Horsman et al. 1989a). Later this drug was shown to sensitize tumours by a mechanism other than inhibition of DNA repair. It was shown that regional tumour oxygenation was improved after administration of nicotinamide (Lee and Song 1992, Price et al.1995), explaining its ability to sensitize tumours to ionizing radiation. Many tumours have hypoxic cells due to a limitation of blood perfusion and oxygen diffusion. Nicotinamide may reoxygenate these perfusion-limited hypoxic cells by increasing the blood supply and decreasing tumour interstitial fluid pressure (Lee et al. 1992, Hill and Chaplin 1995, Hirst et al.1995); the latter is considered to be one cause of transient shortage of blood supply in tumours (Trotter et al. 1991). The improvement in perfusion was also seen in normal tissues, but as they are better oxygenated than tumours, sensitization is confined largely to the tumour.

It was found that the enhancement ratio (ER) for skin and bone marrow is 1.05-1.10 while >1.39 for rodent adenocarcinomas (Kjellen et al.1991). When combined with carbogen (95% O<sub>2</sub> +5% CO<sub>2</sub>) which can increase oxygen supply to tumour cords, the radiosensitization effect is increased even further, with ERs of 1.8-1.9 for SCCVII tumours (Ono et al.1993). All of these findings are consistent with the belief that radiosensitization by nicotinamide is mainly due to reoxygenation of tumours instead of inhibition of DNA repair. However, cell survival is the endpoint for most of these studies, and events occurring at earlier times after irradiation have received less attention.

Pharmacological studies have shown that nicotinamide is involved in the synthesis of nicotinamide adenine dinucleotide (NAD) which is depleted rapidly after extensive DNA strand breaks (Stratford et al.1994). An increased concentration of NAD was found in rodent sarcomas, liver and kidney after nicotinamide treatment (Kelleher and Vaupel 1994a). This increase in NAD seems to be contradictory to the actions of nicotinamide as a PARP inhibitor since one might think that increased NAD should accelerate DNA repair, not inhibit repair. NAD also acts as a coenzyme for more than 100 dehydrogenases, and changes in metabolic processes would be expected. An increase in ATP/Pi and ATP/ADP+AMP ratios is also observed (Horsman et al.1992). Furthermore, one of its metabolites, cyclic ADP-ribose, is an active substance involved in a calcium signalling pathway (Galion 1994), a pathway that leads to many changes in cellular functions and metabolism. It would seem that further understanding is needed to interpret all of these diverse effects of nicotinamide.

The use of nicotinamide in this thesis was initially based on reports by Dr. David Chaplin concerning studies from a group in Sweden who had observed DNA damage in tissues of mice following exposure to nicotinamide alone. Another group had already suggested that some factor in addition to tumour reoxygenation must be acting to sensitize the tumours, since tumour control data were better than could be expected on the basis of tumour reoxygenation alone (Rojas, 1992). Experiments were therefore designed to determine whether nicotinamide would improve tumour oxygenation as measured using the comet assay, and whether other potentially hypoxic normal tissues would be affected by this drug. In addition, by analyzing early SSB rejoining kinetics in the presence and absence of nicotinamide, it was hoped that effects of nicotinamide on DNA rejoining might be observed.

## 1.6. Methods to measure DNA damage: the comet assay

The comet assay, also called the single cell gel assay (SCG) or microgel electrophoresis method (MGE) was first introduced by Ostling and Johanson (1984) to directly visualize DNA damage in individual cells. Subsequent modifications to this gel electrophoresis method allowed independent detection of SSB and DSB (see Fairbairn et al., 1995 for review). Perhaps most important was the implementation of image analysis to provide objective and sensitive quantification of DNA damage (Olive et al., 1990). In experiments described in this thesis, the alkaline version of the comet assay was used exclusively since this method is approximately 20 times more sensitive to detecting radiation-induced DNA damage than the neutral version (Olive, 1995a). The current limit of sensitivity for detecting SSB is about 5-10 cGy in non-proliferating cells (e.g., lymphocytes). In cells replicating their DNA, each replication fork appears as a single-strand break in this assay, decreasing the sensitivity for detecting DNA damage. The comet assay is equally if not more sensitive than any other single-strand break assay which uses radiolabelled cells for analysis (e.g., alkali filter elution, alkali unwinding), and much more reproducible than fluorometric detection using these same methods. Moreover, detection at the level of the individual cell is of critical importance in determining heterogeneity of response to radiation within a mixed population of cells. Since cells, not homogenates, are being examined, the contribution of dead or dying cells can be easily eliminated from DNA repair studies so that kinetics represent the true response of the intact cells. The fact that dying cells can be excluded from analysis is of critical importance. It seems quite likely that the presence of degrading DNA acted as a confounding variable in earlier experiments which used population-based methods to analyze DNA damage *in vivo*.

To perform the comet assay, cells are lysed after being embedded in agarose gel on a microscope slide, electrophoresed and stained with a fluorescent DNA binding dye (propidium iodide). The negatively-charged damaged DNA fragments are pulled away from nucleus by the application of an electric field. When observed with a fluorescent microscope, images from each nucleus look like comets (Fig. 2), thus explaining the name given to the assay (Olive 1989). Since DNA damage can be determined for individual cells, histograms can be generated to demonstrate the presence of sub-populations which differ in initial damage or rate of strand-break rejoining. Populations varying in damage by a factor of 2 or more can be detected as evidenced by the ability of this method to detect hypoxic cells (Olive and Durand, 1992).

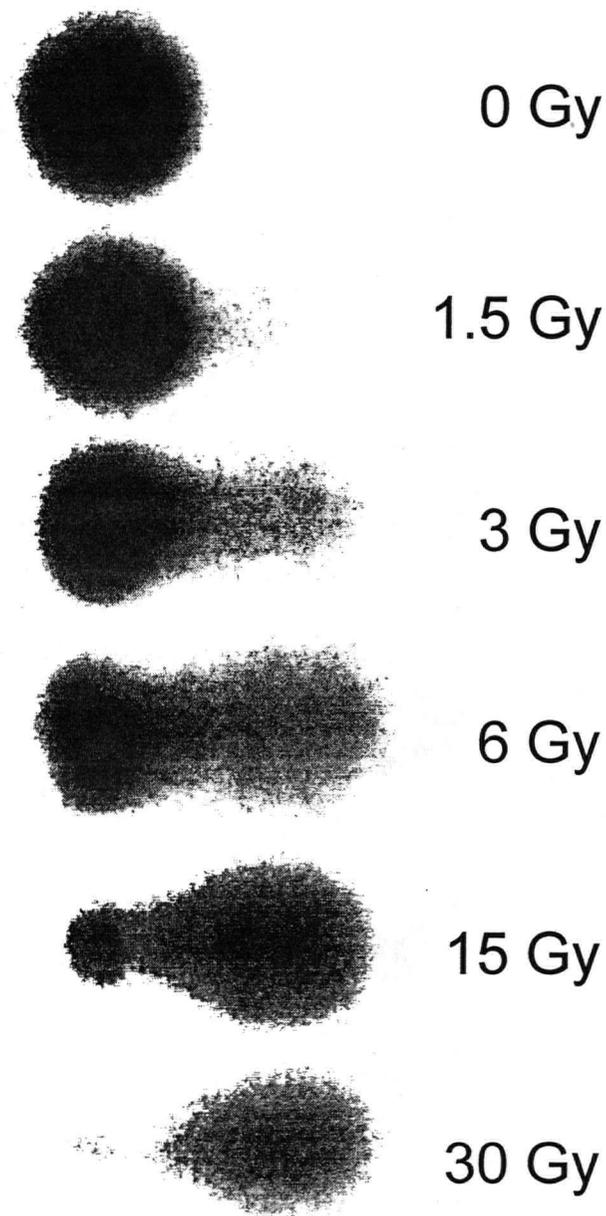


Fig. 2: Representative digitized images of comets from individual cells exposed on ice to the radiation dose shown. Each image shows the response of *one* cell analyzed for DNA damage using the alkaline comet assay. The direction of DNA migration during electrophoresis is to the right, and the longest comet image is approximately 100 microns. Note that as the number of DNA breaks increases, the amount of DNA able to migrate also increases.

### 1.6.1 The principles of detection of DNA damage

Various methods for detecting DNA strand breaks depend on the principle that DNA breakage results in smaller fragments from DNA molecules. Once the fragment size is smaller than about 10 Mbp, DNA is able to migrate in an electric field (Blocher et al. 1989). To allow DNA to migrate freely, cells are first lysed in detergent and high salt to remove proteins (including histones) and membranes. While denaturation is rapid at pH > 12.3, highly packaged and entangled DNA requires time to unwind.

Different sized DNA fragments can be separated according to gravity (sedimentation and precipitation assays) or size (alkaline or neutral elution assays). The comet assay utilizes a low voltage electric field to separate DNA fragments. Negatively charged DNA is pulled away from nuclei toward the anode during electrophoresis, and for the short electrophoresis time used, both large and small fragments migrate together in what is called a compression band. Most other methods require radiolabeling DNA and only provide an indication of the average number of strand breaks per cell within a population. The pH of the lysing solution governs the measurement of different types of DNA strand breaks. Neutral lysing and electrophoresis measures DNA DSB while alkaline lysis and electrophoresis allow denaturation and measurement of SSB. The comet assay detects DNA damage as well as total DNA content of a cell by using stoichiometric staining by a fluorescent DNA dye. The ability to measure DNA content provides an important control for the imaging system because regardless of the amount of DNA damage, the DNA content of a cell should not change. Any change in DNA content is an indication of non-linearity of the video camera or field of illumination.

The fragments which migrate away from the original cell nucleus or comet head form the "comet tail". The amount of DNA that migrates is dependent on the number of broken ends. Therefore the size of the tail increases as a function of radiation dose while the size of the comet head decreases (Fig. 2). The length of the comet tail initially increases with damage but reaches a maximum that is defined by electrophoresis conditions rather than size of fragments (Fairbairn et al. 1995). Usually the amount of DNA in the tail indicates the degree of DNA damage, and in cells undergoing apoptosis, almost all DNA fragments are pulled away, forming a large tail with only a trace of DNA remaining in the head. Using a video image analysis system consisting of a fluorescence microscope, CCD or integrating camera, and frame-grabber

board with computer, the image can be quantified in terms of degree of damage and DNA content (Olive et al.1990). The former is determined by analysis of several comet features, most useful of which is tail moment. The tail moment is defined as the percentage of DNA in the comet tail multiplied by the distance between the means of the head and tail fluorescence distributions. DNA content is the total (relative) image fluorescence which will vary according to camera gain. Camera gain is adjusted for each experiment to avoid saturation of the comet image and keep the signal proportional to intensity. However, the camera gain is not adjusted once collection of images begins. The percent DNA in the tail is not as sensitive an indicator of DNA damage as tail moment, but it is less affected by unavoidable variations in electrophoresis conditions (minor changes in electric current will occur when several times of electrophoresis is run).

#### 1.6.2 Cell sensitivity to radiation detected using a comet assay

Since the comet assay is able to detect damage with very high sensitivity and requires only a small number of cells, it has rapidly been adopted for studies in many scientific fields (for review, see Fairbairn et al. 1995). Most important, however, is the ability of this method to detect heterogeneity in DNA damage or rejoining of breaks within a population. Since comet analysis includes a measure of DNA content (total cell fluorescence), DNA damage to cells in different phases of the cell cycle or cells with different ploidy can be measured independently. This ability was responsible for revealing that G<sub>1</sub> cells repair radiation-induced SSB faster than S phase cells (Olive and Banath 1993). Subpopulations of radioresistant cells which vary in damage by even a small amount (e.g. hypoxic cells in tumours) can also be distinguished based on their different rates of strand breaks induction (Olive et al. 1993a).

Since this project was mainly focused on the *in vivo* radiosensitivity of cells in different tissues, examining heterogeneity was one of the most important and novel aspects of this study. Normal tissues are composed of a variety of cell types, and the comet assay provides an easy way to observe the presence of subpopulations which differ in damage. Furthermore, only three hours are needed to prepare a slide for analysis, and less than thirty minutes is needed to collect data from two hundred comets using the fluorescent image processing system (FIPS). It is therefore quite feasible to prepare and analyze many samples at the same time, reducing experimental variability.

## 2. MATERIALS AND METHODS

### 2.1. Animals

#### 2.1.1 C3H mice

Inbred male C3H/HeN mice, 12-16 week old and approximately 30g in weight, were obtained from a breeding colony at the B. C. Cancer Research Centre. They were kept in polycarbonate cages at a temperature of 21°C in holding rooms of the Animal Facility. Food (Picolab mouse diet 20-5058, PMI Feeds Inc., St. Louis, MO.) and water were constantly supplied. Cages with bedding were changed weekly. Lights were controlled automatically to be on from 7am to 7pm.

#### 2.1.2 SCID mice

Adult C.B17-SCID mice (12-16 week old and 30g in body weight) were also bred in house and fed in the same way except they were housed in a separate room in microisolators. Cages, covers and food pellets were autoclaved. Whenever mice were needed for experiment, they were transferred in the same room in a flow hood and the working area cleaned with ethanol. Masks and antiseptic solution-treated gloves were used when handling animals.

### 2.2. SCCVII tumours

The SCCVII squamous cell carcinoma was originally obtained from Dr. Michael Horseman at Stanford University, CA. Tumour cells were transplanted into syngeneic C3H/HeN mice of 7-10 weeks old. Cells were obtained by excising the tumour from the maintenance mouse, mincing them with scalpels and digesting using an enzyme mixture containing 0.15% (w/v) trypsin (Difco Laboratories, Detroit, MI), 0.024% (w/v) collagenase and 0.06% (w/v) DNase I (both from Sigma Chemical Co., St. Louis, MO). After 30 minutes digestion at 37°C, cells were centrifuged and the pellet was resuspended in medium, then filtered through a 50 µm nylon mesh. The cell number in the suspension was counted using a Coulter counter. The yield of tumor was about 10<sup>8</sup> cells/g (10<sup>7</sup> cells/g without enzyme digestion). About 10<sup>7</sup> cells in 0.5ml of final cell suspension were implanted subcutaneously in each mouse over the sacral region with a 26 gauge needle. Before the implanting operation, mice were anaesthetized by inhaling Metofane (Janssen Pharmaceutical, Mississauga, ON) and the area for implantation was shaved and sterilized using 70% ethanol. Mice were returned to the room in Animal Facilities after they had recovered from anesthesia.

A tumour of 450-600mg in weight was obtained after two weeks.

A maintenance tumour was always propagated at the same time. This was done by using brei obtained from the tumour from the maintenance mouse. After mincing the tumour, the brei was forced through a 20G needle 10 to 15 times until the mass was smooth. The volume of the tumour mass was measured in the syringe and transferred into a 15 cc disposable tube. PBS at five times the tumour volume was added and mixed with tumour cells. After shaving and cleaning the hind legs of an anaesthetized mouse with 70% ethanol, 0.1 ml of the cell suspension was injected intramuscularly. In order to maintain consistent tumour growth characteristics, a tumour was only passaged to ten generations before a new frozen stock was used.

### 2.3. Cell cultures

#### 2.3.1 TK6 cells

This cell line from human TK6 lymphoblasts was obtained from Dr. Helen Evans (Case Western Reserve University) and maintained in RPMI medium containing 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD) and antibiotics (Sigma Chemical Co., St. Louis, MO). Cells were grown in 70ml plastic Falcon flasks (Becton Dickinson Labware, Bedford, MA) and diluted bi-weekly. TK6 cells do not attach to tissue culture plastic and can be handled without using trypsin. They were kept along with other cell cultures in a CO<sub>2</sub> (5%) incubator.

#### 2.3.2 V79 cells

Chinese hamster V79 lung fibroblasts were originally obtained from Dr. W.K. Sinclair and routinely maintained in the lab. In this study, cells were grown exponentially in monolayer culture on Falcon plates, in Eagle's Minimal Essential Medium (Gibco BRL) containing 10% fetal bovine serum and antibiotics. Cells were subcultivated bi-weekly and single a cell suspension was prepared by trypsinizing cultures using 0.1% trypsin (Gibco BRL) in phosphate-buffered saline (PBS).

#### 2.3.3 EMT6 cells

EMT6 mouse mammary carcinoma cells were originally obtained from Dr. Sara Rockwell (Rockwell, 1973) and maintained as monolayers in minimal essential medium containing 10% fetal bovine serum.

## 2.4. Treatments

### 2.4.1 Drugs

#### 2.4.1.1. Nicotinamide

Nicotinamide (Sigma Chemical Co.) solution was freshly prepared by dissolving the drug in PBS immediately before use. Different doses of drug were given to mice by intraperitoneal injection from stock solutions containing 50-1,000 mg/kg, 30-90 minutes before irradiation. The volume was restricted to 0.3 ml for each mouse by altering the stock solution of nicotinamide.

#### 2.4.1.2. RSU 1069

RSU 1069 (1(2-nitro-1-imidazolyl)-3-aziridino-2-propanol) was supplied by Drs. T. Jenkins, I. Stratford and G. Adams of the MRC Radiobiology Unit at Harwell. Drug was freshly prepared in PBS at 5 mg/kg. Mice were injected intraperitoneally 90 min before sacrifice. Cells obtained from tissues were, in some cases, exposed on ice to 10 Gy X-rays to reveal the presence of DNA interstrand crosslinks.

### 2.4.2 Irradiation and dosimetry

Mice were whole body irradiated in a plexiglass jig using a 250 kVp X-ray unit (Phillips type RT-250, HVL 1.5 mm Cu) at the B.C. Cancer Research Centre. Dosimetry measurements were made using a Victoreen Model 500 electrometer with a 0.6 cm<sup>3</sup> ionization chamber. Measurements were taken with the probe at the site for irradiating sample(s) in the jig. The dosimetry calculations - the dose to the medium at the position of the probe placed,  $D_{med}$ , was calculated as:

$$D_{med} = MN_x(0.00873 \text{ J/kg R})(m_{ab}/\rho)^{medair}$$

where M is the electrometer reading in roentgens (R),  $N_x$  is a calibration factor and  $(m_{ab}/\rho)^{medair}$  is the mass-energy absorption coefficient ratio for the medium air. Electrometer readings were taken for at least five time settings for each dose rate. Different jigs were used for irradiating mice, single cells in test tubes or cultured cells on plates, so the dosimetry was measured in the same way for each jig.

During irradiation, mice breathed air or other gas mixtures supplied to the jig from compressed gas cylinders (flow rate was 2-4 l/minute). The dose rate according to the described calculation was 3.3 Gy/minute. When the *in vitro* dose response was measured, cells were kept in test tubes and irradiated

on ice in a plexiglass jig with dose rate of 7.3 Gy/minute. Cultured cells were irradiated in their flasks or plates on a flat jig with dose rate 7.94 Gy/minute.

In some experiments gamma rays from a  $^{137}\text{Cs}$  unit at the B.C. Cancer Research Centre were used for whole-body irradiation of mice. A plexiglass jig for irradiating a single mouse was used. The  $^{137}\text{Cs}$  radiation source was unshielded, and dosimetry was measured in the same way as described above by repeating the measurement five times. The average reading was obtained and the dose calculated according to the above formula. The resultant dose rate was 9.33 Gy/minute.

#### 2.4.3. Gas breathing conditions

In some experiments, mice were allowed to breathe either 10% oxygen in nitrogen to reduce tissue or tumour oxygenation, or carbogen (95% oxygen, 5% carbon dioxide) to increase tissue or tumour oxygenation. Both gas mixtures were obtained from Praxair Inc., Delta, B.C. Mice were placed in plexiglass chambers fitted with a gassing port and allowed to breathe the gas mixtures for 20-25 minutes before and during irradiation. The flow rate was 2-4 l/minute. In some experiments, mice were asphyxiated 15 minutes prior to irradiation by breathing carbon dioxide, or were killed by cervical dislocation.

### 2.5. Methods for detecting DNA damage and cell damage

#### 2.5.1 The comet assay

##### 2.5.1.1 Preparation of cell suspension

Organs were removed from mice killed by cervical dislocation either immediately or at various times after irradiation. The tissues were immersed in ice-cold PBS to prevent further rejoining of DNA strand breaks. When DNA damage at zero time was examined, an additional 30 seconds was needed after irradiation for removal of the tissue to ice-cold PBS. Bone marrow was obtained by cutting a section of femur and/or tibia; cells were flushed out with PBS using a 26 gauge needle and 1ml syringe. Entire SCCVII tumours, spleen, liver, cerebellum, thymus and about 3 cm-long sample of jejunum were examined. Tumour, spleen, cerebellum and thymus were chopped finely with scalpels in ice-cold PBS. Jejunum was cut open and the contents flushed out, then the tissue was transferred to a Petri dish with ice-cold PBS containing 2 mM ethylenediaminetetra-acetic acid (EDTA; BDH Chemicals Inc., Darmstadt, Germany) and 2% dimethyl

sulfoxide (DMSO; Sigma Chemical Co.). The mucosal surface was gently scraped by a scalpel into PBS and dispersed by a syringe. Liver was chopped in PBS/EDTA/DMSO in order to reduce DNA degradation by endogenous enzymes. The chopped brei was filtered through 30  $\mu\text{m}$  nylon mesh. Cells were centrifuged on ice, and the pellets resuspended in ice-cold PBS to final dilution of  $3 \times 10^4$  cells/ml. Cell viability was determined by dye exclusion after incubation with an equal volume of cell suspension in 0.25% (w/v) trypan blue (MCB Manufacturing Chemists, Morwood, OH) in PBS on ice. Viable cells (non-stained) were usually 90-95% of the population.

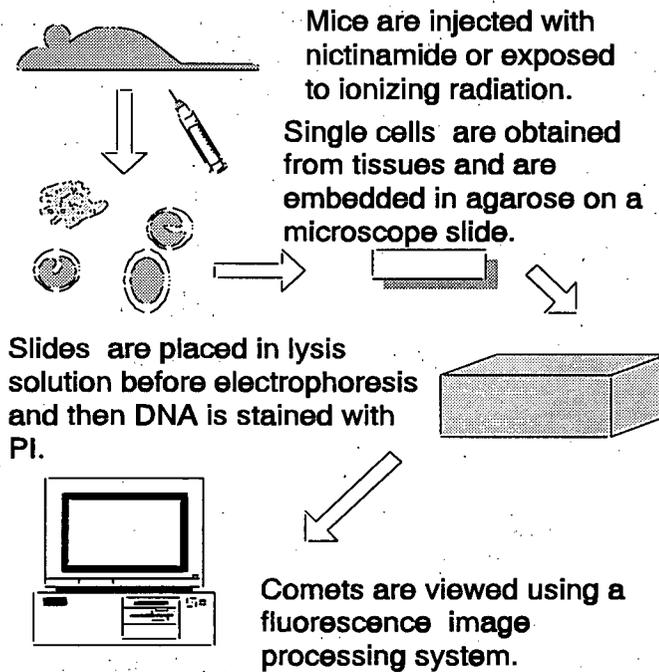
#### 2.5.1.2 Procedures for "comet" preparation

A diagram illustrating the general procedures used to measure DNA damage is shown in Fig. 3. Cell suspensions (0.5 ml containing about  $10^4$  cells) were mixed with 1.5ml 1% (w/v) low gelling temperature agarose (type II, Sigma Chemical Co.) which had been prepared in double-distilled water and equilibrated to 40°C. The final concentration of agarose was 0.75%. Then 1.5ml of the mixture was quickly pipetted onto a half-frosted microscope slide and allowed to gel for 1-2 minutes. Slides were submersed in alkaline lysis solution containing 0.03 M NaOH (BDH Inc., Darmstadt, Germany), 1M NaCl (BDH Inc.) and 1% sarcosyl (Sigma Chemical Co.) for one hour followed by a one hour rinse in 0.03 M NaOH, 2 mM EDTA (BDH Inc.) with one change of fresh solution after 30 minutes. All procedures were carried out in the dark at room temperature to prevent DNA damage produced by light. Following the rinse, slides were subjected to electrophoresis at 0.6 volt/cm in freshly made buffer containing 0.03M NaOH and 2 mM EDTA for 25 minutes. After a 15 minute rinse in distilled water, slides were submersed in 2.5  $\mu\text{g/ml}$  propidium iodide (Sigma Chemical Co.) for 20 minutes. After staining, slides were rinsed in distilled water and placed in a humidified air-and light-tight container. Slides could be viewed immediately or within several days.

#### 2.5.1.3 Comet collection and analysis

Typically 200 comets were collected and analysed for each sample. Comets were selected away from the four edges of the slide using a back and forth collection pattern to cover the full slide but minimize possible re-analysis of the same comet. Cell debris and overlapping comets were not collected. Samples were viewed using a 40x long working distance objective with a Zeiss epifluorescence microscope attached to

**Fig. 3: Use of the comet assay to detect DNA damage in tissues of mice exposed to nicotinamide or ionizing radiation.**



an intensified solid state charge-coupled device (CCD) camera and image analysis system (Olive et al. 1990). Cells were illuminated with 546 nm light excitation from a 100-w mercury light. Emission was monitored using a 580-nm reflector and 590 bandpass filter. The camera was set at a constant gain for each experiment, and optimised to avoid saturation of the comet image. The image from each comet (nucleus) was displayed on the video screen, and information from the digitized image was collected and analyzed by a program written by Dr. Ralph E. Durand. For each comet, the total fluorescence intensity was taken as a measure of total DNA content of that cell by adding the light intensity from all the pixels occupying the area of comet minus background. The position and intensity of the comet "head" was determined by assuming symmetry of the head region; all pixels in excess of the calculated value of the head region were consequently defined to be part of the comet tail. The percentage DNA in the head and tail regions was determined by the ratios of fluorescence in the head/tail to the total DNA content. The lengths of comet head and tail, and the distance between the means of the head and tail distributions were also determined by the program. The latter multiplied by percentage DNA in the tail was defined as the comet "tail moment". Individual images of comets were not stored, but instead, analysis of 10 features

of each image, performed in less than a millisecond, was saved on a disk. Approximately 600-800 comet images could be analyzed per hour.

Data were analyzed using SigmaPlot 5.0. Comet analysis data were imported into the worksheet, and histograms describing the distributions of tail moment and DNA content were generated. At least three independent experiments were performed for each data point.

### 2.5.2 In situ detection of DNA strand breaks by terminal deoxynucleotidyl transferase (TdT)

This method followed that of Gorczyca et al. (1993b) with some modifications. Single cell suspensions of spleen and testis were prepared from mice pretreated with radiation and /or nicotinamide. Approximately  $2 \times 10^6$  cells from each sample were fixed in 0.5% paraformaldehyde for 10 minutes, centrifuged and resuspended in 70% ice-cold ethanol overnight. After washing with PBS, cells were resuspended in 50  $\mu$ l of TdT buffer containing 0.1 M potassium cacodylate (pH 7; Sigma Chemical Inc.), 1 mM  $\text{CoCl}_2$  (Sigma Chemical Inc.), 12.5 mM Tris-HCl (Bio-Rad Laboratories, Richmond, CA), 0.1 mM dithiothreitol (Sigma Chemical Inc.), 0.125 mg/ml bovine serum albumin (Sigma Chemical Inc.), 10 units of TdT (Gibco BRL, Gaithersburg, MD) and 0.5 nM biotin-16-dUTP (Gibco BRL). Cells were incubated in this buffer at 37°C for 45 minutes then rinsed in PBS containing 4% fetal bovine serum and 0.1% Triton X-100 (Sigma Chemical Inc.). After washing, cells were stained with a 1/300 dilution of fluorescein isothiocyanate (FITC)-avidin (Sigma Chemical Inc.), incubated for 2 hours in the dark. To label DNA, cells were stained with 1  $\mu$ g/ml DAPI (4,6-diamidino-2-phenylindole, Sigma) for 30 minutes. Cells were analyzed using a Coulter Elite dual laser cell sorter using UV light to excite DAPI and blue light excitation for FITC.

### 2.5.3 DNA gel electrophoresis for detecting apoptotic cells

The nucleosomal ladder pattern associated with apoptosis was detected following the method described by Warters (1992) and Olive (1993). A single cell suspension was prepared as previously described from tissues of irradiated mice. Then  $1.5 - 2 \times 10^6$  cells from each tissue were resuspended in 50  $\mu$ l of ice-cold lysis buffer containing 100 mM  $\text{Na}_2\text{-EDTA}$  (BDH Chemical Inc., Darmstadt, Germany), 1 mg/ml proteinase K (BDH Inc.), 20 mM Tris (Bio-Rad Inc., Richmond, CA) and 20 mM NaCl (pH 7.8). Cells were immediately mixed with 50  $\mu$ l of 1% agarose, and pipetted into 100  $\mu$ m plug formers. After gelling for 30 minutes at

4°C, plugs were placed into microtest tubes each containing 0.7 ml of lysis buffer and sarcosyl (Sigma Chemical Inc.) added to a final concentration of 0.2%. After incubation at 37°C for two hours for proteolysis, the agarose plugs were rinsed for one hour in 0.5x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8). Then the plugs were transferred into wells of a 1.5% agarose (Sigma type II) slab and the gel was submerged in 0.5x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8). Lambda/Hind III DNA (Stratagene Co., La Jolla, CA) was used as size markers. Constant field gel electrophoresis was performed for 3.5 hours at 2.5 V/cm. The gel was stained with 1 µg/ml propidium iodide for 30 minutes, and transilluminated with UV light for photography using type 53 Polaroid film.

#### 2.5.4 Histological studies of tissue sections

Entire spleen and testis were excised from mice after various treatments. Organs were fixed in 10% buffered formalin (pH 7.0, Sigma Chemical Co.) at room temperature for 4-6 hours before dehydration and paraffin embedding. Sections 5-6 µm thick were fixed onto slides with 1% celloidin (Sigma Chemical Co.) and stained with haematoxylin and eosin Y solution. Slides were viewed using a Zeiss photomicroscope at 40x magnification using an oil immersion objective.

#### 2.5.5 Size measurement of DNA fragments

A method for measuring the size of DNA fragments in individual apoptotic or necrotic cells was described by Olive and Banath (1995b). Cells with damaged DNA obtained from mouse tissues were prepared at  $2.5 \times 10^4$  cells/ml in essential medium supplemented with 10% fetal bovine serum, then incubated with 0 to 15 µM mechlorethamine (Sigma Chemical Co.). After 30 minutes incubation at 37°C, cells were pelleted and washed with ice-cold PBS in preparation for the alkaline comet assay. The tail moment for the 20% most damaged cells was measured for each sample treated with different concentrations of mechlorethamine. The response of the 10% least damaged cells served as a control. The concentration of mechlorethamine required to reduce tail moment to 10 was calculated, and this was compared with a standard curve made from TK6 cells which had been irradiated with different radiation doses. The corresponding radiation dose was determined by extrapolation from the standard curve, and the approximate number of SSB was estimated by assuming that 1000 SSBs are produced by 1 Gy (Powell and McMillan, 1990). Then the size of the fragments was estimated by dividing the total number of DNA

base pairs for a mammalian cell ( $3 \times 10^9$ ) by the number of fragments.

#### 2.5.6 Hoechst 33342 cell sorting and clonogenicity study of SCCVII tumour cells

To examine the radiosensitivity of tumour cells as a function of distance from functional blood vessels, mice were injected intravenously with 0.1 ml of the fluorescent perfusion stain Hoechst 33342 (Sigma Chemical Co.) dissolved in PBS as 8 mg/ml. After 20 minutes, mice were killed directly or after exposure to radiation. Tumours were then removed and disaggregated using an enzyme mixture containing 0.15 mg/ml trypsin (Difco laboratories), 0.24 mg/ml collagenase and 0.6 mg/ml (both from Sigma Chemical Co.) for a 20 minute digestion period at 37°C. The resulting single cells were incubated with 1:100 dilution of FITC-conjugated goat anti-mouse IgG (Sigma Chemical Co.) for 5 minutes on ice, to stain macrophages which comprise the majority of normal cells in this tumour (Olive 1989). IgG(-) cells were further sorted by the flow cytometry on the basis of Hoechst 33342 gradient of cells from 10% most dimly to 10% most bright fluorescent populations. Each of the ten fractions of sorted cells were plated onto a 100-mm Falcon plate for 7-10 days until a clone containing at least 50 cells was formed. The plates were stained with malachite green (Sigma Chemical Co.) and counted. The clonogenic fraction was calculated by dividing the number of colonies by the number of cells plated.

#### 2.6. Study design

Different tissues from same animal were used whenever possible, i.e., when fast DNA repair was not a concern.

**TABLE 2: EXPERIMENTAL PLAN**

##### A. Initial DNA Damage In Vitro and In Vivo

Tissues	Number of mice per dose, in vitro	Number of radiation doses, in vitro	Number of mice per dose, in vivo	Number of radiation doses, in vivo
Tumor	3	7	3	4
Spleen	3	7	3	4
Marrow	3	7	3	4
Testis	3	7	3	4
Cerebellum	3	7	3	4
Jejunum	3	7	3	4
Liver	3	7	3	4

## B. Rejoining of DNA Strand Breaks In Vivo Following 15 Gy or Nicotinamide and 15 Gy

Tissues	Number of mice per time point: radiation only	Number of time points: radiation only	Number of mice per time point: nicotinamide + radiation	Number of time points: Nicotinamide + radiation	Number of nicotinamide doses
Tumor	3-4	10	3-4	5	3
Spleen	3-4	8	3-4	5	3
Marrow	3-4	8	3-4	5	3
Testis	3-4	8	3-4	5	3
Cerebellum	3-4	8	3-4	5	3
Jejunum	3-4	8	ND*	-	-
Liver	3-4	8	ND*	-	-

\* Not done.

## C. Other Experiments

Type of experiment	Number of dose/time points	Number of mice per point
Oxygen tension effects	3	6
Dose rate effects	1	9
RSU-1069	5	3
Nicotinamide - DNA degradation	20	3
Apoptosis	4	2-3
Cell survival	10	3

Total number of mice (approximately)

520

## 2.7. Statistics

Errors bars shown are standard errors or standard deviations of the mean for at least 3 independent experiments. Student's t-test was used to determine the statistical significance of the differences between data from two experimental samples. 95% confidence limits were used to show the deviation for the regression curves of dose response and the fast component of DNA repair for all the tissues. ANOVA was used to analyze the variances among a group of data derived from experimental samples.

### 3. RESULTS

#### 3.1. Radiation dose response for normal tissues

##### 3.1.1 *In vitro* dose response

Single-strand break induction measured using the alkaline comet assay increased linearly as a function of radiation dose for cells irradiated on ice (Fig. 4). Although there was some variation in the slopes for the regression lines (Table 3), no significant difference was seen in the dose-response relationship among the different normal tissues. An exception was the response of elongated spermatids. These haploid cells were not adequately lysed by the specific lysing conditions used in these experiments. Pre-treatment with 2-mercaptoethanol or dithiothreitol promoted lysis, but also gave rise to unacceptably high levels of background DNA damage (data not shown). Therefore it was not possible to analyze radiation-induced DNA damage in elongated spermatids. Elongated spermatids can be seen in Fig. 5 as cells with a haploid DNA content and a tail moment close to zero. Note that haploid cells showed the highest level of single-strand breaks followed by diploid and tetraploid cells. The different stages of male germ cell differentiation are shown in Fig. 32.

**TABLE 3. SLOPES OF RADIATION DOSE-RESPONSE CURVES FOR TISSUES OF C3H MICE**

Tissue	In vitro *	In vivo *	Ratio
Bone marrow	2.91 ± 0.40	1.72 ± 0.30	1.69
Spleen	3.08 ± 0.11	1.81 ± 0.21	1.71
Jejunum	3.02 ± 0.21	1.77 ± 0.34	1.70
Liver	2.89 ± 0.29	1.74 ± 0.39	1.66
Cerebellum	2.99 ± 0.33	1.87 ± 0.31	1.63
Testis	2.84 ± 0.35	1.14 ± 0.32	2.42
Tumour	2.69 ± 0.16	1.02 ± 0.35	2.64

\* Slope (Gy<sup>-1</sup>) and 95% confidence limits for tissues determined from data in Fig. 4.

##### 3.1.2 *In vivo* dose response

Since DNA repair occurs during irradiation, all tissues irradiated *in vivo* showed less DNA damage than cells from the same tissues irradiated *in vitro*. Liver, spleen, jejunum and bone marrow exposed to 10 Gy showed only about 60% of the amount of damage produced in cells irradiated *in vitro* (Fig. 4). The relative

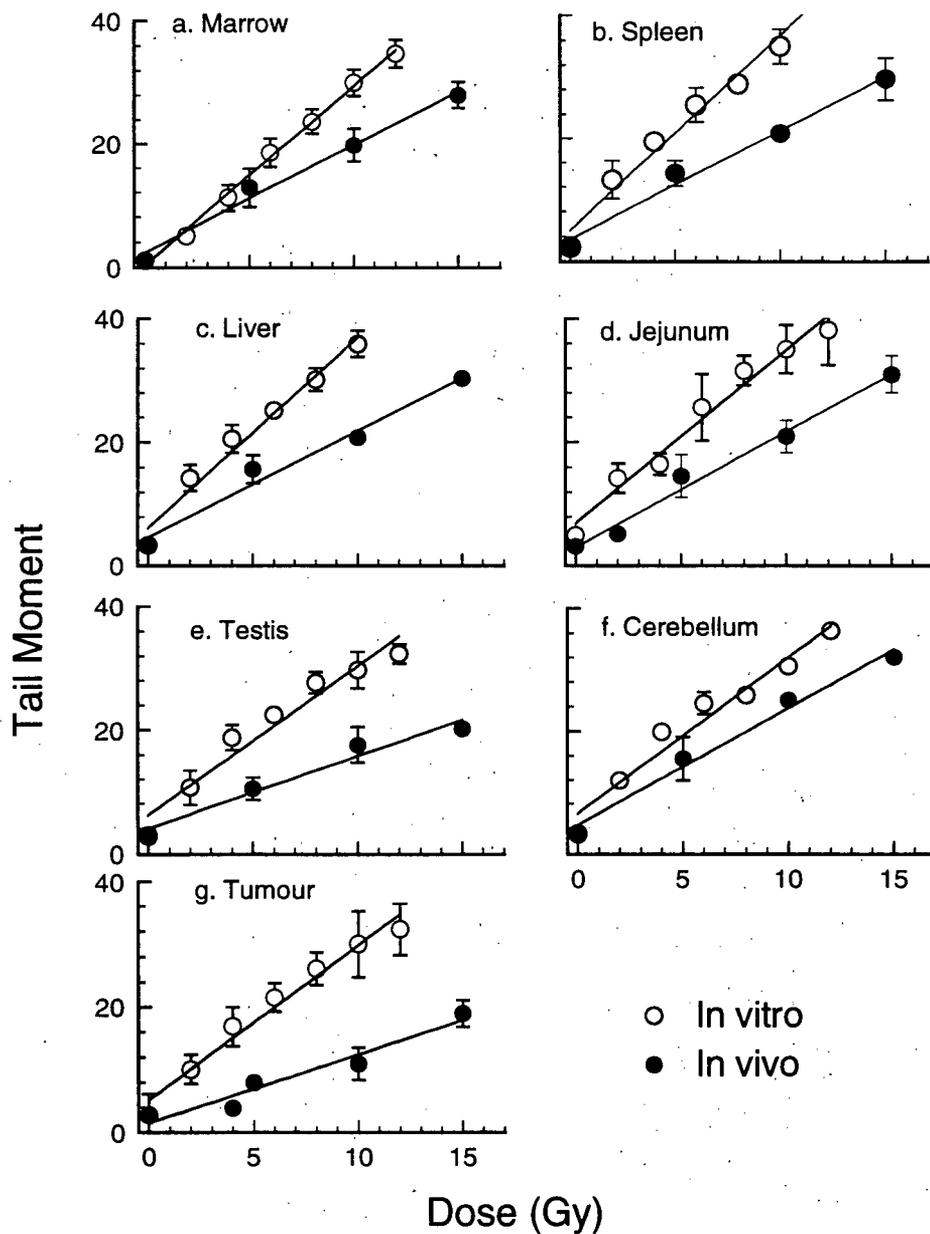


Fig. 4: Dose response relationships for tissues of C3H mice. Cells were irradiated on ice (  $\circ$  ) or mice received whole-body irradiation prior to excision of tissues and preparation of a single cell suspension (  $\bullet$  ). Each point is the mean for three experiments. The standard errors are shown.

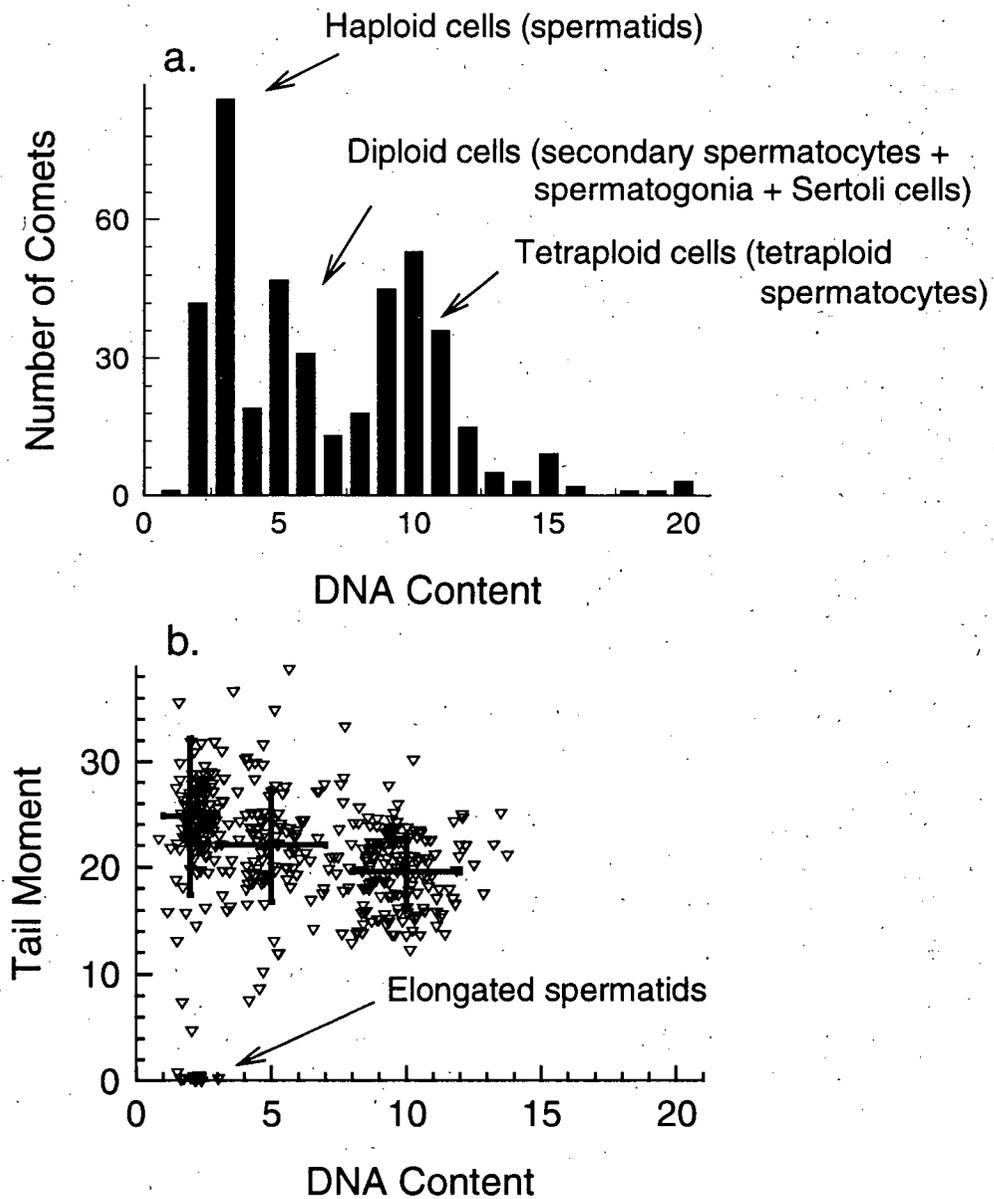


Fig. 5: DNA SSB induction by 15 Gy in testis irradiated in vivo. Three cell populations can be differentiated by DNA content which is determined by total PI fluorescence of each comet. Panel (a) shows the histogram of DNA content of cells seen in panel (b), a bivariate plot showing both DNA content and tail moment. Each symbol represents the data from one comet. Data were divided into 3 ranges and the mean and standard deviations were determined for each range.

difference was smaller for cerebellar cells, and larger for testis and SCCVII tumour cells. The difference between the *in vitro* and *in vivo* responses are also given in Table 2. These differences reflect variations in the rate of DNA strand break rejoining, and presence of hypoxic cells in some tissues.

An important advantage of the comet assay is the ability to analyze DNA damage according to DNA content. In Figure 5b, it is obvious from the bivariate plot that cells from testis show small differences in degree of damage according to their DNA content. Damage is least for tetraploid cells (tetraploid spermatocytes), intermediate for diploid cells (spermatogonia + secondary spermatocytes + Sertoli cells) and greatest in haploid cells (spermatids). This difference, while subtle, was observed for testis irradiated with 5, 10 or 15 Gy *in vivo*, but was not seen when cells were irradiated *in vitro* (Fig. 6).

### 3.1.3 Heterogeneity in DNA strand break induction

Heterogeneity in DNA SSB induction after irradiation was small in all the tissues with the exception of elongated spermatids indicating that in spite of heterogeneity in cell types within tissues, most cells demonstrate similar numbers of strand breaks when exposed to a given dose of ionizing radiation. This can be observed from the histograms showing tail moment measured immediately after irradiation *in vivo* (Fig. 9, upper row). In view of this lack of heterogeneity in initial DNA damage, no further effort was made to select or measure the response of specific subpopulations within these tissues.

## 3.2. Single-strand break rejoining kinetics in normal tissues and tumour *in vivo*

### 3.2.1 The general repair process

DNA strand break rejoining was studied in mice exposed whole-body to 15 Gy. Immediately after irradiation, all tissues with the exception of testis and SCCVII tumour demonstrated a tail moment of about 30. In tumour and testis, this value was closer to 20. All DNA rejoining curves showed two components, a fast component operating within the first 30 minutes, and a slow component operating over the next several hours.

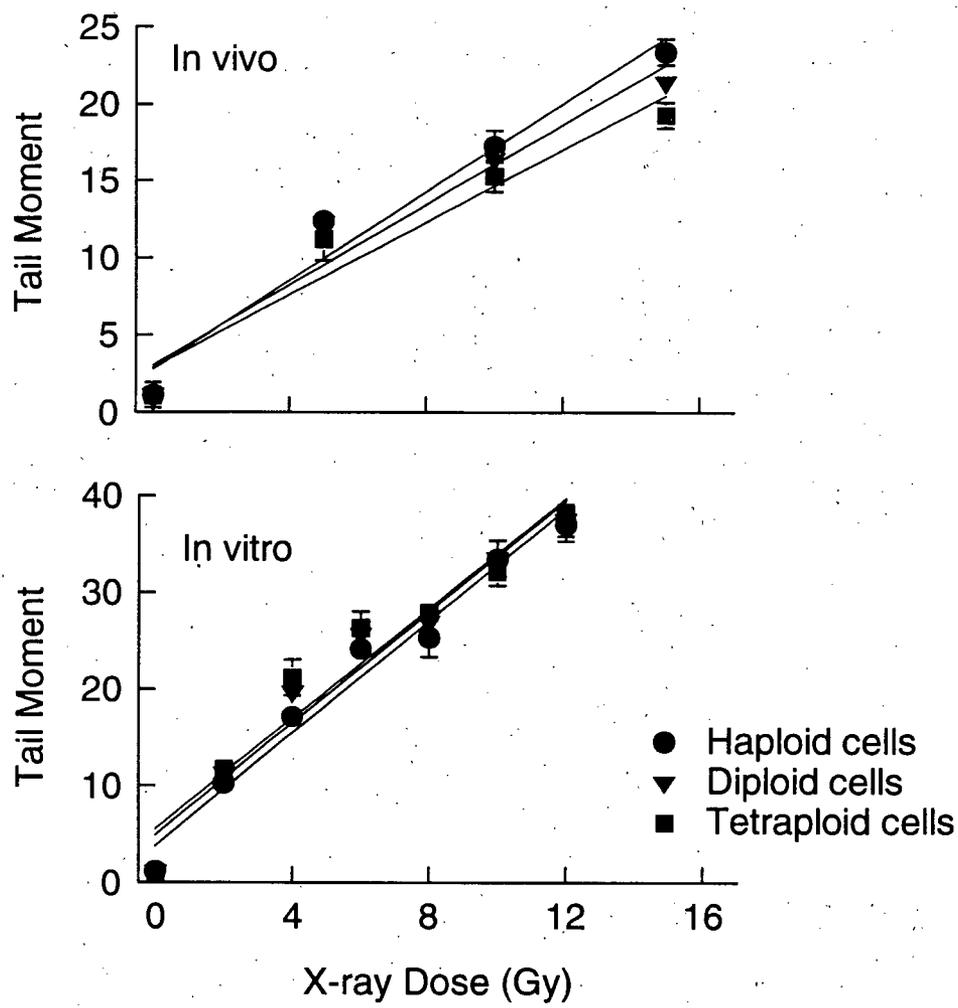


Fig. 6: Dose response relationships for testicular cells irradiated in vivo or in vitro. Cells were treated in the same way as seen in Fig. 4. The means and standard errors for three different experiments are shown.

For all tissues examined with the exception of the cerebellum, the majority of strand breaks were rejoined by the fast component (Fig. 7). Some differences were evident among tissues in rate of SSB rejoining which could be best described by measuring the half-time of repair. Table 4 gives the overall half time of repair calculated as the time required to rejoin half of the initial strand breaks after subtracting the background damage. It can be seen that SCCVII tumour cells and testis cells had the fastest rates of rejoining with  $T_{1/2}$ =9 minutes and 10 minutes respectively, followed by jejunum, liver, thymus, spleen and bone marrow with  $T_{1/2}$  ranging from 12 to 22 min. Cells from cerebellum (and cerebrum) were very slow to rejoin breaks (Fig. 7,8). The half time of rejoining was 48 minutes for cerebellum and was significantly longer than for other normal tissues. On the other hand, analysis of the slopes for the fast component of rejoining (defined as the first 3 points on the curves) indicated no significant difference in rejoining rate between these tissues (Table 4). Rejoining was almost complete after 4 hours of repair in all tissues. By 24 hours, there was no significant difference in tail moment between cells from irradiated mice or unirradiated mice.

**TABLE 4. KINETICS OF SSB REJOINING FOLLOWING IONIZING RADIATION**

<b>Tissue</b>	<b>Half-time<sup>1</sup> (min) (95% conf. limits)</b>	<b>Slope (min<sup>-1</sup>) (95% confi. limits)</b>
Tumor	11 (8.8-14)	-.027 (-.034-.021)
Testis	12 (9.6-14)	-.026 (-.031-.022)
Marrow	14 (11-23)	-.021 (-.028-.013)
Jejunum	18 (13-27)	-.017 (-.023-.011)
Liver	20 (16-30)	-.015 (-.019-.010)
Thymus	23 (20-27)	-.013 (-.015-.011)
Spleen	25 (21-23)	-.012 (-.014-.010)
Brain	45 (32-74)	-.0067 (-.0095-.0041)

<sup>1</sup> The half-time was calculated from the slope of the exponential part of the SSB rejoining curve (measured immediately after irradiation up to 15 to 30 min after irradiation), without taking into account background damage and assuming no repair prior to removal of the tissues. The true half-time is therefore expected to be shorter. Note that the confidence limits for brain do not overlap those for the other tissues.

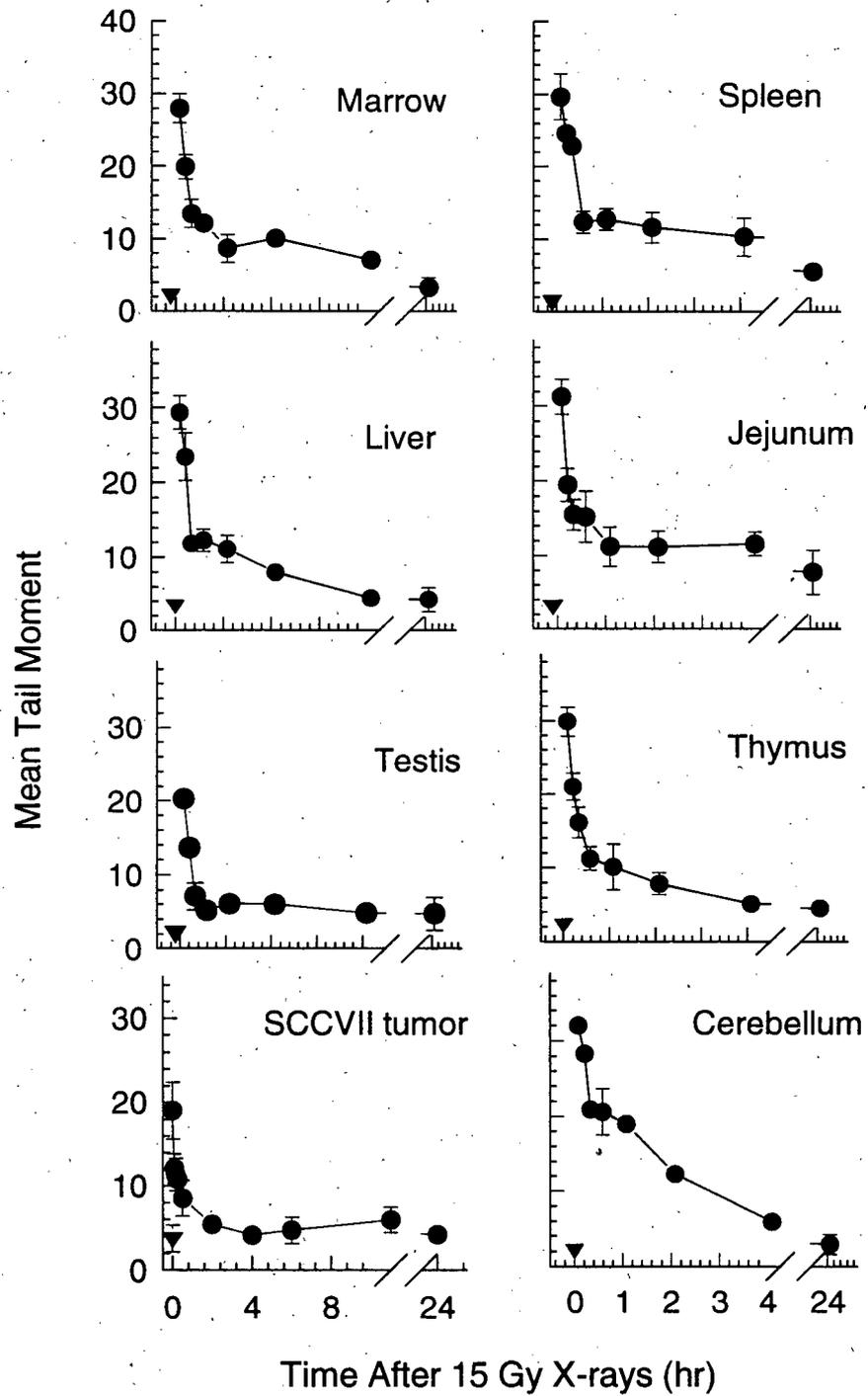


Fig. 7: SSB rejoining kinetics for cells from tissues in C3H mice. Mice were exposed to 15 Gy, killed and tissues were excised at the time indicated. The means and standard errors for three experiments are shown. " ▼ " in each panel represents the control.

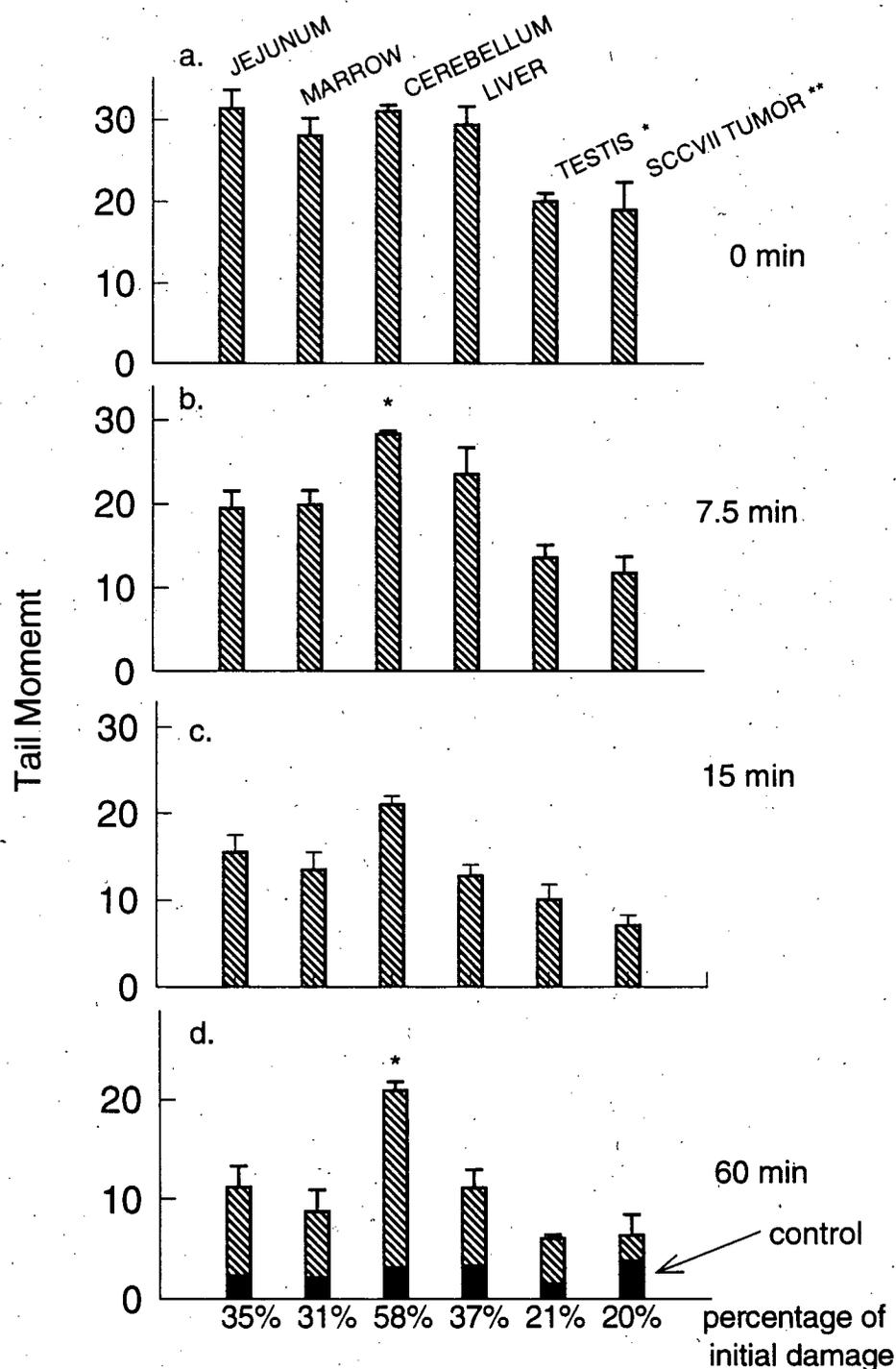


Fig. 8: Rejoining of DNA SSBs in different tissues of C3H mice after 15 Gy. Means and standard errors of 3 experiments are shown. \* & \*\* in (a) : values are significantly different from that of jejunum, marrow, brain and liver. \* in (b): the value is significantly different from that of jejunum, marrow, testis and tumor. \* in (d): the value is significantly different from that of the other tissues.

### 3.2.2 Heterogeneity in DNA repair

As was observed for initial DNA damage, heterogeneity in damage during the course of rejoining was minor (Fig. 9). Although at early times (15 minutes after irradiation) a small fraction of cells from some tissues, e.g. bone marrow and spleen, appeared to be slower in repairing than the general population, this displacement was small and undetectable at later times (Fig. 9). Twenty-four hours after irradiation, less than 5% of cells in testis and spleen demonstrated a high tail moment. However, a population of heavily damaged cells was seen in all tissues 48 hours after irradiation. The fraction of cells which retained a normal tail moment varied for each tissue. Cerebellar cells in general showed much less damage than other tissues at this time and seemed to be relatively homogenous in response compared to other tissues.

Cells with different ploidy from testis showed no significant difference in the overall process of strand break rejoining (Fig. 10a). The difference in tail moment between cells with different ploidy disappeared later when the rejoining process neared completion (data not shown). But DNA degradation 48 hours after 15 Gy was more severe in tetraploid and diploid cells, and haploid cells showed less damage (Fig. 10b).

### 3.2.3 DNA damage and repair in SCID mice

The dose response measured in SCID bone marrow and testis did not differ from that of C3H mice (Fig. 11). When 15 Gy was used for measurement of SSB rejoining, the rejoining rate for cells from tissues of SCID mice was found to be identical to the response of cells from tissues of C3H mice (Fig. 12). Damage at times later than 24 hours was not measured.

An attempt was made to measure DSB by exposing mice to 50 Gy X-rays and analyzing cells for damage using the neutral comet assay (Olive et al., 1991). However, due to the relatively low numbers of DSB produced by this dose when repair could not be inhibited (the average tail moment was only around 10), it was difficult to resolve a difference relative to background damage (data not shown).

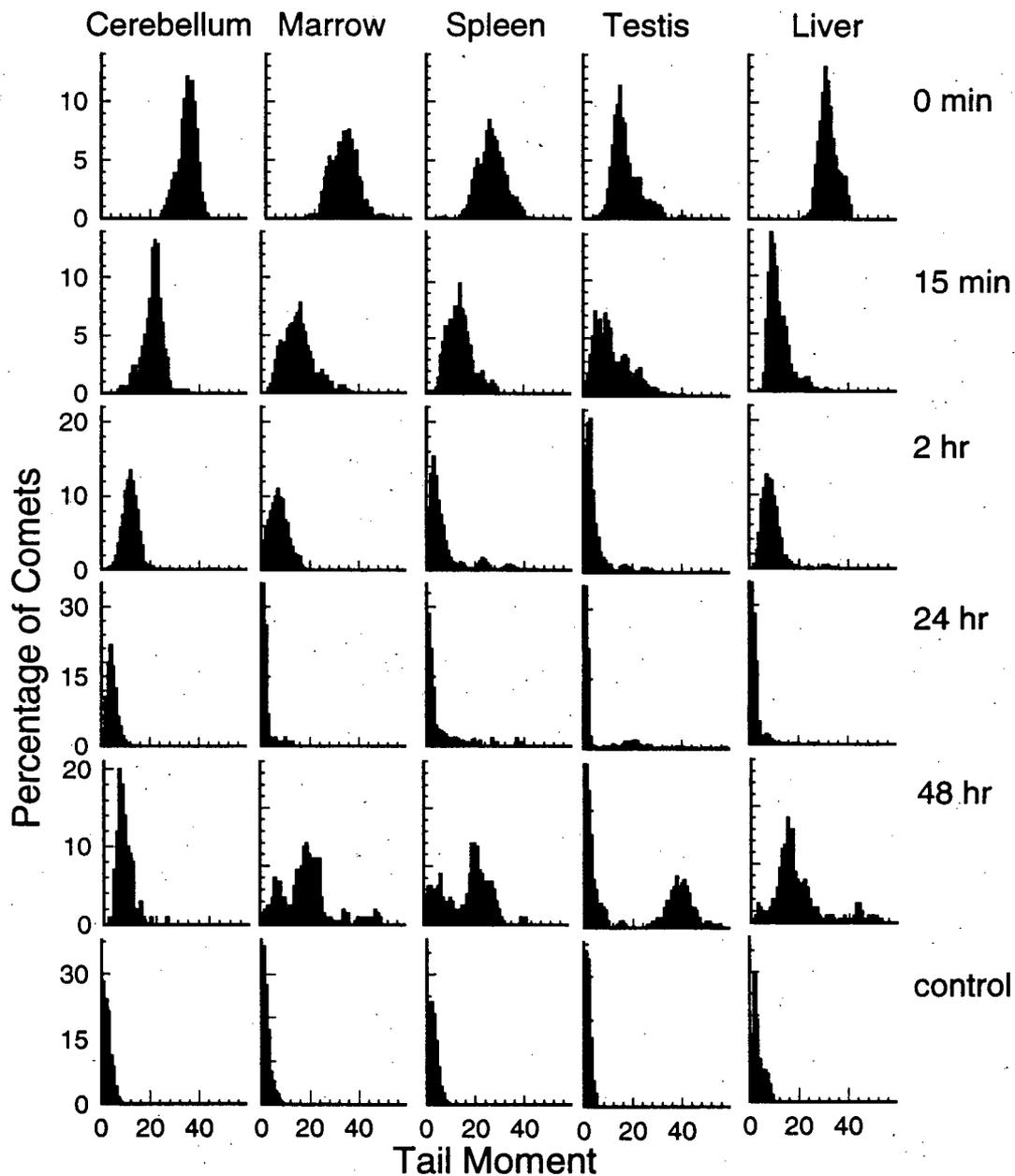


Fig. 9: Heterogeneity in DNA damage and repair in tissues of C3H mice following exposure to 15 Gy. After irradiation, tissues were excised and single cells were prepared for the comet analysis at the time indicated. Representative histograms are shown for approximately 200 individual cells/comets for each tissue obtained from one irradiated mouse.

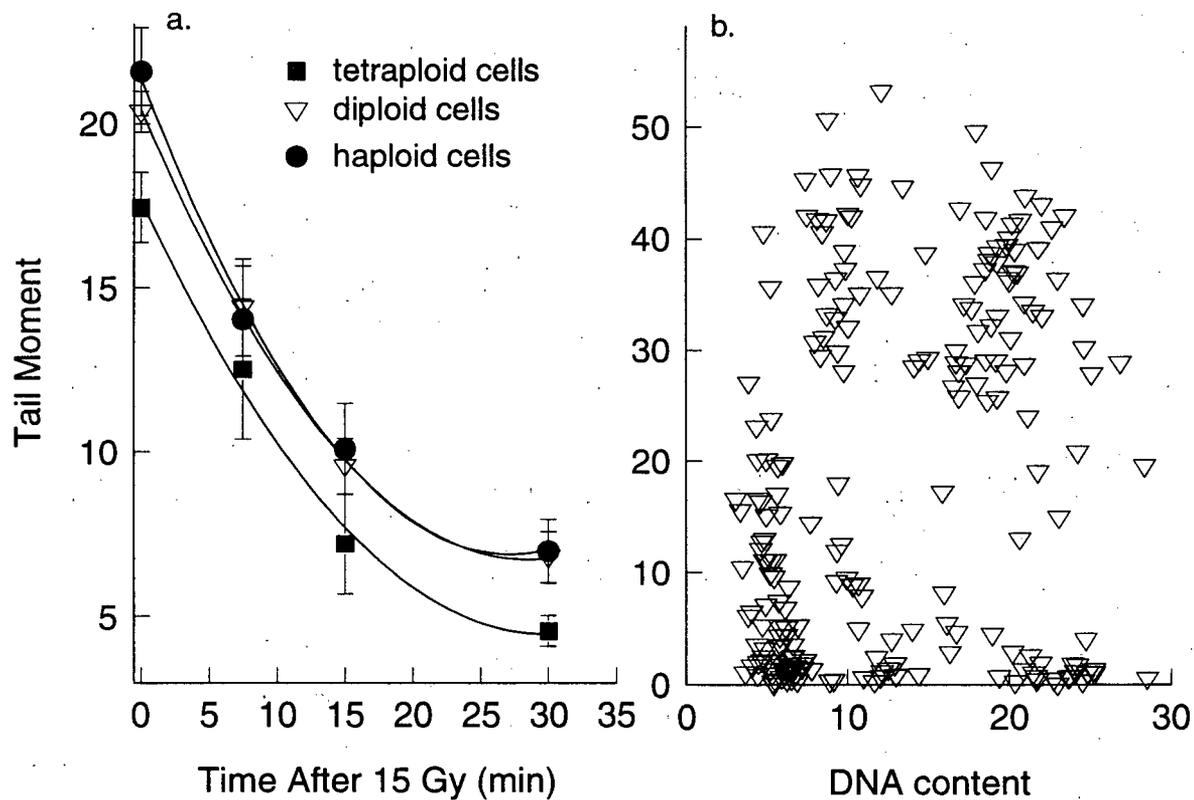


Fig. 10: a). DNA repair kinetics in testicular cells. Testis were excised at the time indicated after exposure to 15 Gy, and single cells were prepared for the comet analysis. The means and standard errors for 3 animals are shown. b). A representative bivariate plot for testicular cell damage 48 hrs after 15 Gy. Each symbol was from one comet/cell.

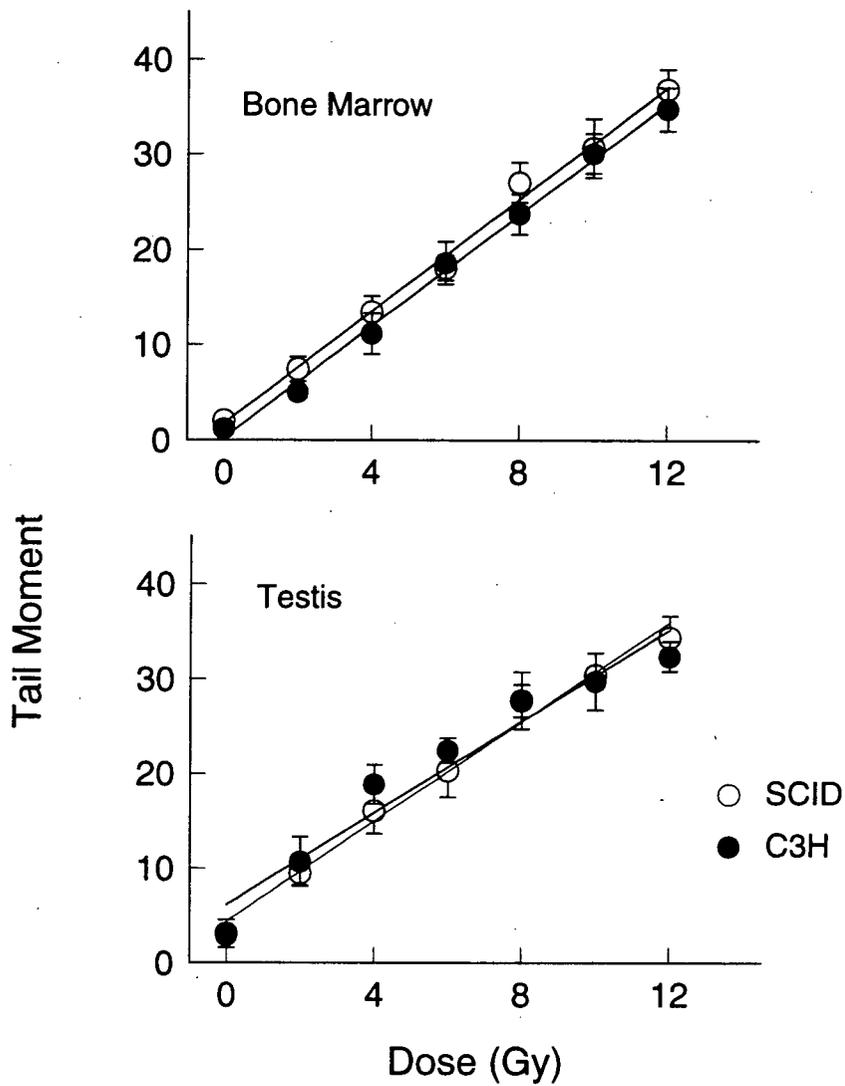


Fig. 11: Comparison between the responses of C3H and SCID mice to ionizing radiation administered to a single cell suspension on ice. The means and standard errors for 3 animals per dose are shown.

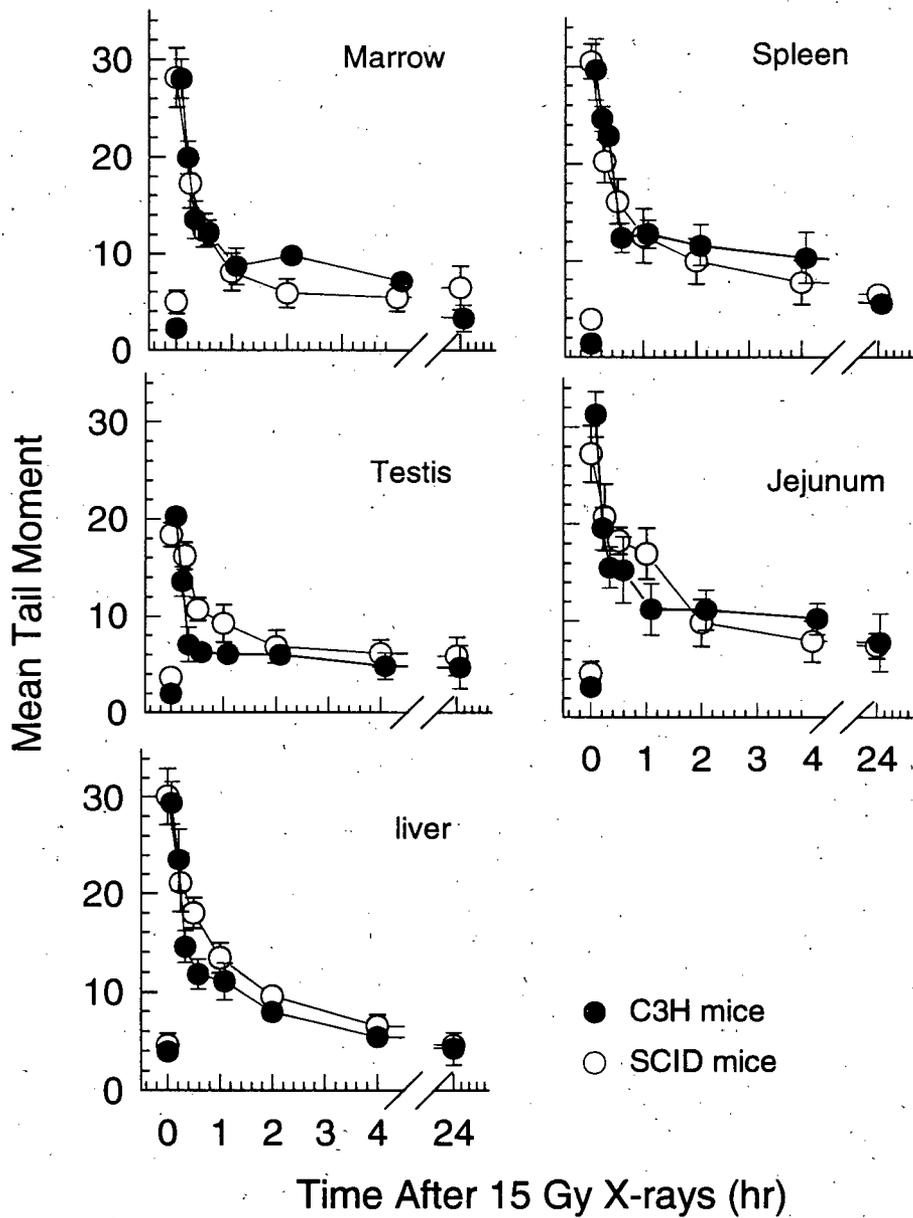


Fig. 12: Comparison of the DNA SSB rejoining kinetics between C3H and SCID mice exposed to 15 Gy. Mice were whole-body irradiated and killed at the times indicated. Tissues were excised to make single cells for comet analysis. The means and standard errors for 3 animals are shown. Single symbols at time 0 were from control animals.

### 3.3. The effect of radiation dose rate

Differences in the rate of repair of SSB between tissues will influence the amount of initial DNA damage measured after irradiation *in vivo*. A  $^{137}\text{Cs}$  irradiator was used in order to observe whether a higher dose rate could reduce the amount of DNA rejoining occurring during irradiation. The dose rate for the Cs source was 9.32 Gy/minute, three times higher than that of the X-ray unit. As expected, irradiation at the higher dose rate resulted in more DNA damage measured immediately after irradiation. For the tissues studied, tail moment in testis after 10 Gy from the Cs source was 26.5, compared with 17 for the same dose of X-rays (Fig. 13). A similar effect was also seen in tumour, bone marrow and other tissues, although the difference was smaller.

### 3.4. The effect of oxygen concentration on strand break induction by radiation

#### 3.4.1 Low oxygen tensions

Another possible explanation for differences in initial DNA damage in tissues of irradiated mice is that some tissues, like testis and tumours, contain hypoxic cells. If mice are asphyxiated prior to irradiation, differences between DNA damage in different tissues should be minimized. In fact, when tissues were anoxic at the time of irradiation (i.e., mice were killed 5-10 minutes before irradiation), differences between tissues were reduced, and, as expected, the amount of DNA damage produced in all tissues was significantly less than it was under air-breathing conditions. In spleen and bone marrow cells, damage was reduced to about one third when tissues were completely anoxic. However, anoxia reduced damage by only a factor of two in testis, consistent with the theory that this tissue is already partially hypoxic (Fig. 14).

When mice were allowed to breathe 10% oxygen before and during irradiation, bone marrow and testis showed more DNA damage than observed under anoxic conditions, but less damage than under air breathing conditions (Fig. 14). In well-oxygenated tissues like spleen, jejunum, and brain, breathing 10% oxygen produced a similar response as breathing air.

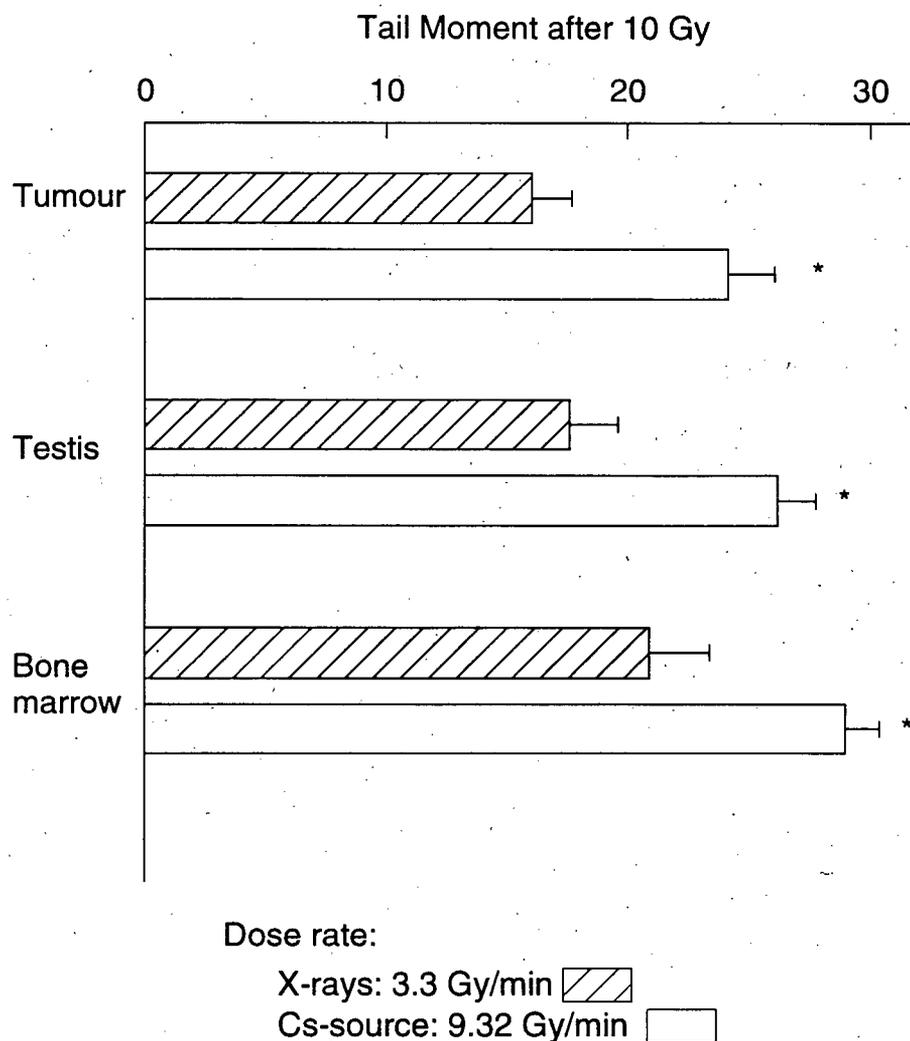


Fig. 13: Comparison of DNA damage produced by X and gamma rays in C3H mice. Mice were whole body irradiated with the same radiation dose either by X-rays or gamma rays. Tissues were taken immediately to measure SSBs using the comet assay. Mean and standard errors for 3 animals are shown. \* :  $p < 0.01$  compared with the value produced by X-rays in the same tissue.

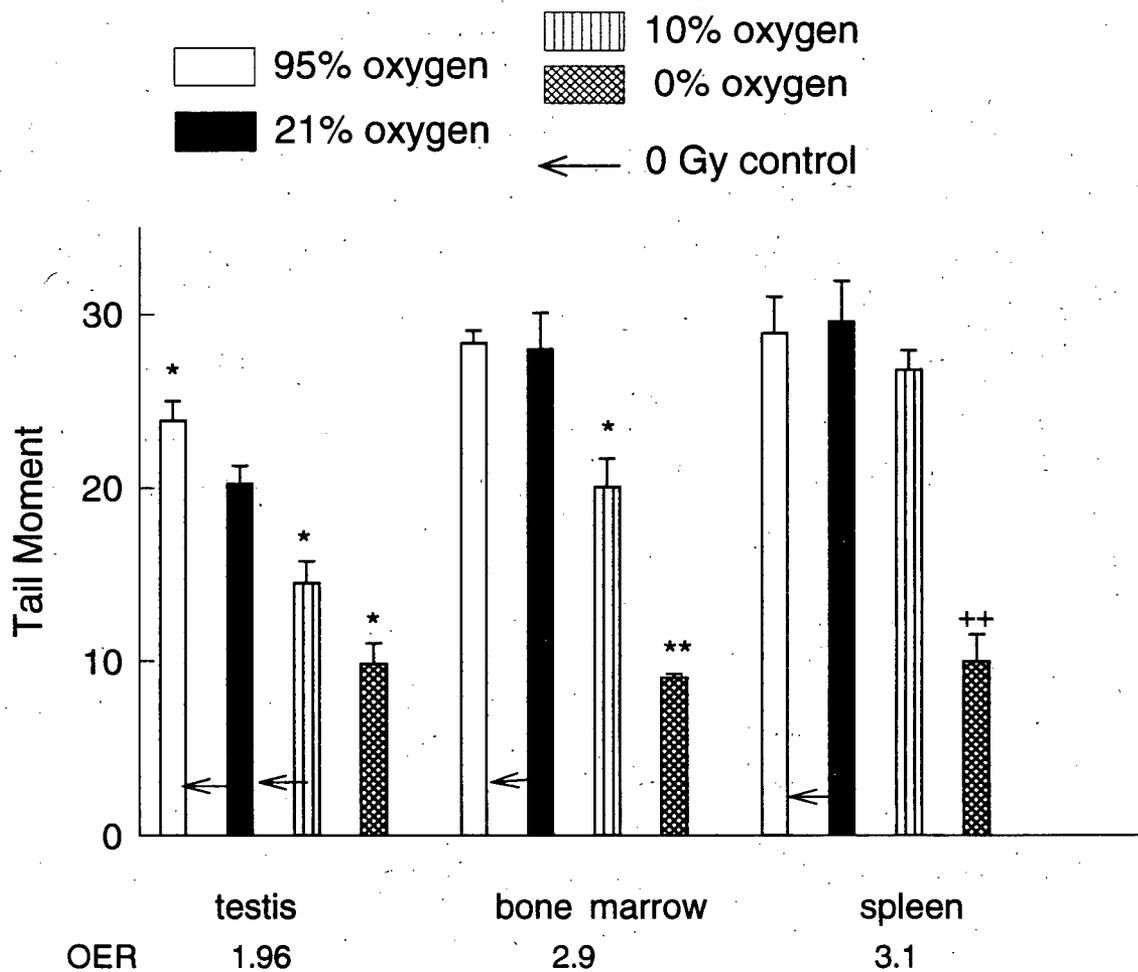


Fig. 14: Effect of different oxygen concentrations on SSB induction by 15 Gy X-rays. Mice were allowed to breathe different concentrations of oxygen 20 min before and during irradiation. Tissues were taken immediately after 15 Gy. DNA damage was measured using the comet assay. Mean and standard errors from 3 or more animals are shown. \*: the value is significantly different from the data of 21% oxygen for the same tissue. \*\* and ++: the values are significantly different from that of both 21% and 10% oxygen in the corresponding tissue.

### 3.4.2 High oxygen tension

When mice were allowed to breathe carbogen (95% oxygen + 5% carbon dioxide) for 15 minutes before and during exposure to 15 Gy, only testis showed an increase in tail moment compared to air-breathing conditions. No increase was seen in other tissues examined (Fig. 14).

Results using different gas mixtures indicate that some testis cells are likely to be radiobiologically hypoxic. The degree of DNA damage was therefore examined in various testicular cell types. As previously shown (Fig. 5), tail moment varies with ploidy in testicular cells of air-breathing mice. However, this difference became negligible when mice were either asphyxiated prior to irradiation, so that all cells were hypoxic, or when mice were allowed to breathe carbogen so that all cells were well-oxygenated (Fig. 15). In the latter case, there was a wider distribution in the range of tail moments for each cell population with regard to their ploidy, but there was no difference in the mean value of tail moment from haploid cells to diploid or tetraploid cells.

### 3.4.3 The interaction of bioreductive drug with hypoxic cells

Another approach to examine the degree of hypoxia in testis and bone marrow is to examine DNA damage by a bioreductive agent, RSU 1069, which produces extensive SSB in aerobic cells but interstrand DNA crosslinks only in hypoxic cells (Olive 1995c). Approximately 90 minutes after i.p. injection of 50 mg/kg of RSU 1069, mice were killed and testes as well as other tissues were removed to measure DNA damage using the comet assay. Damage was reduced in each of the populations in testis when compared to other tissues (Fig. 16b). The mean tail moment for the whole population was well below the average response of jejunum, spleen, and bone marrow. Compared with these normal tissues and SCCVII tumours which had a very low value of tail moment, testis demonstrated an intermediate degree of SSB production by RSU-1069 (Fig. 16a).

When 10 Gy was subsequently administered to cells pretreated in the same way (*in vitro* irradiation), testis did not show cells with significantly crosslinked DNA (i.e., no cells had very low tail moments, Fig. 16c).

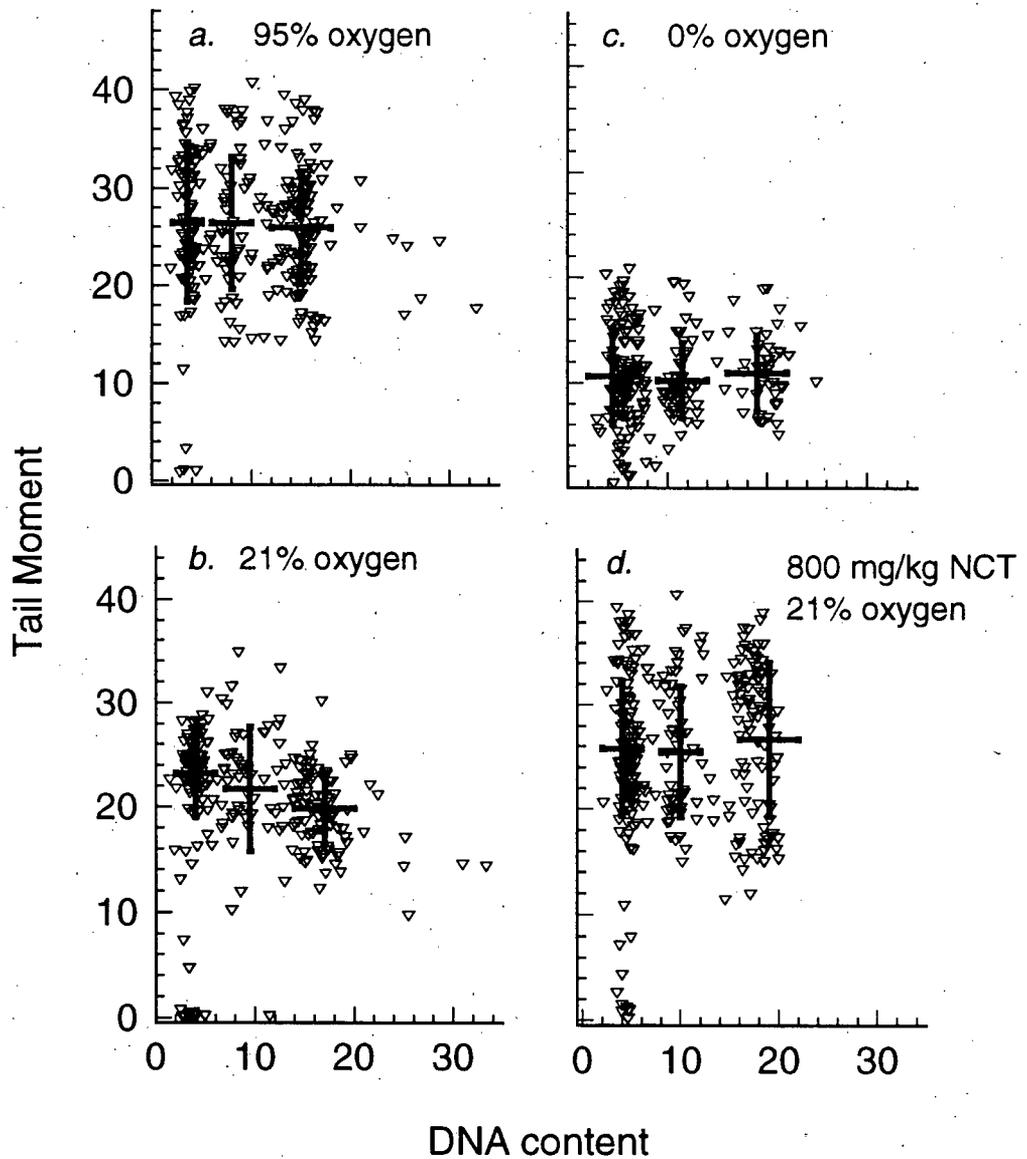


Fig. 15: Bivariate plots showing DNA SSB induced in testicular cells by 15 Gy. Mice were allowed to breathe 95%(a), 21%(b) or no oxygen (killed, c) 20 min before and during 15 Gy irradiation. Panel (d) shows the effect of nicotinamide given 60 min before irradiation. Testis were excised immediately after irradiation and single cells were prepared for comet analysis. Each panel was data collected from 200-250 comets from one sample. Data were divided into 3 ranges, and the means and standard deviations are shown for comets within each range.

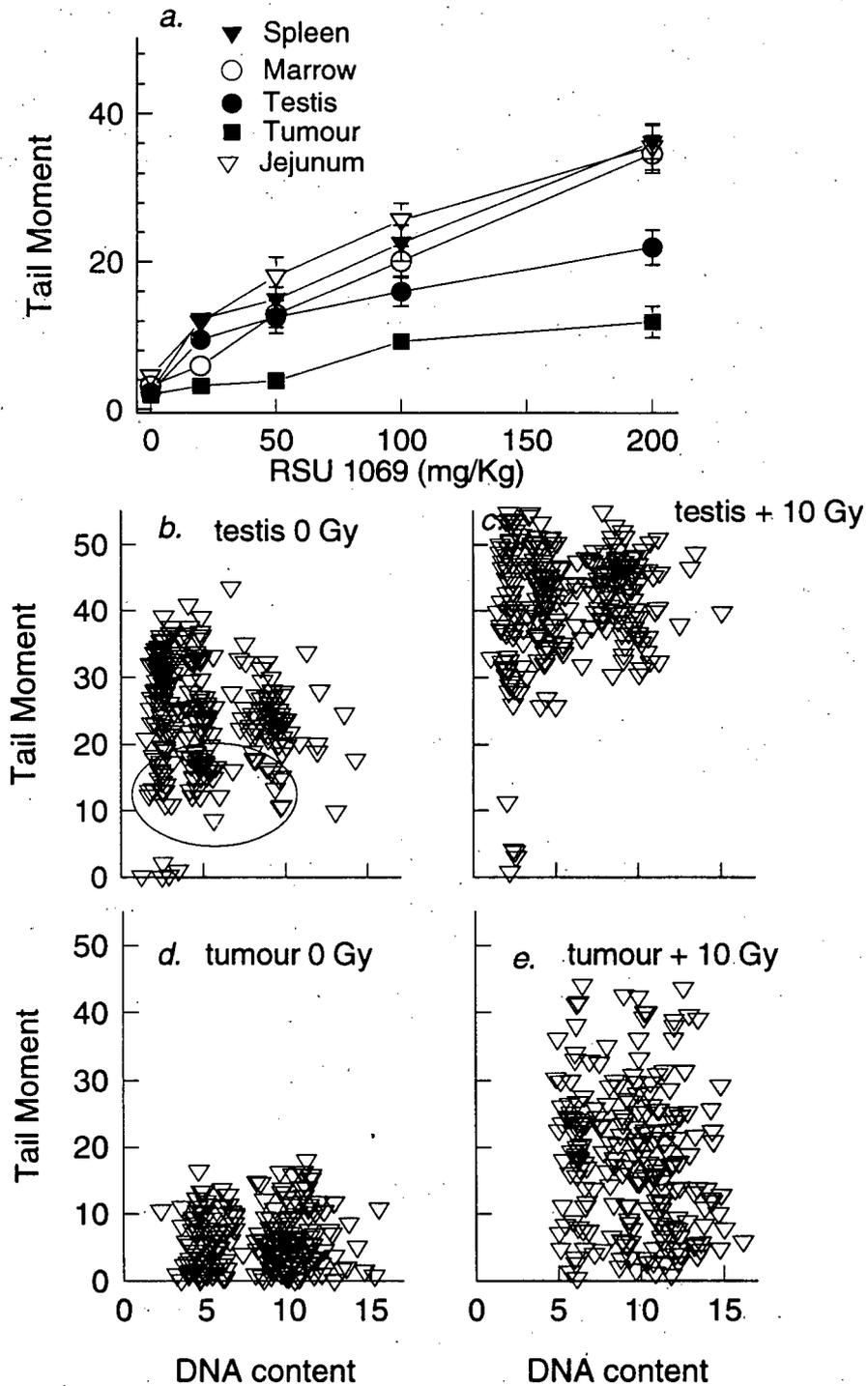


Fig. 16: Induction of SSBs by RSU-1069 in normal tissues and tumours. Mice were injected with RSU-1069 90 min before sacrifice, and tissues were removed to analyze DNA damage using the comet assay. Panel (a): the mean and standard deviation for 3 independent experiments are shown for each point. Panels (b-c): DNA damage in testicular cells 90 min after 50 mg/kg drug injection. Panels (d-e): bivariate plots for tumour cells treated by the same drug dose. Each panel of (b), (d) represents data from one of the tissue samples used in (a). (c) and (e) were obtained by irradiating cells from sample (b) and (c) respectively.

All three populations of cells had a homogenous pattern of damage production with the exception of elongated spermatids which were not lysed during preparation. Since RSU 1069 produces crosslinks only

in cells containing very low oxygen concentrations (Koch, 1993), these results support the idea that some testis cells are poorly oxygenated, but are not as hypoxic as some SCCVII tumour cells. This can be clearly seen in Fig. 16d, e. After 10 Gy irradiation, there was a wide range of distributions of tail moment in tumour cells: some had a high tail moment as seen in aerobic tissues but some had a very low tail moment, indicating that these cells were typically hypoxic and their DNA was crosslinked by RSU 1069.

### 3.5. Radiation-induced apoptosis in normal tissues

C3H normal tissues were examined for radiation-induced apoptosis. The comet assay and DNA gel electrophoresis were used to identify apoptotic cells (Olive et al. 1993a). Few heavily damaged cells were observed, perhaps due to apoptotic cell loss during preparation of a single cell suspension from the tissue and due to the low occurrence of apoptotic cells in normal tissues of C3H mice relative to other strains of mice (Nomura et al. 1992).

DNA gel electrophoresis showed typical nucleosomal ladders from spleen and thymus 4 hours after irradiation. The appearance of the ladders was not altered by radiation since 2 Gy, 5 Gy and 10 Gy all induced the same pattern and the bands were of the same intensity (Fig. 17). The amount of DNA remaining in the wells was not measured. This typical ladder pattern was not found in testis, jejunum or liver. At 2 hours after irradiation, no ladders were observed in any tissue, and after 10 hours, ladders were barely visible.

### 3.6. Effects of nicotinamide on radiation-induced damage production

#### 3.6.1 Toxicity of nicotinamide

Up to 1000 mg/kg nicotinamide was delivered to mice i.p. to examine DNA damage at early times (60-90 minutes) after irradiation (Fig. 18). Nicotinamide was relatively non-toxic but produced some DNA strand

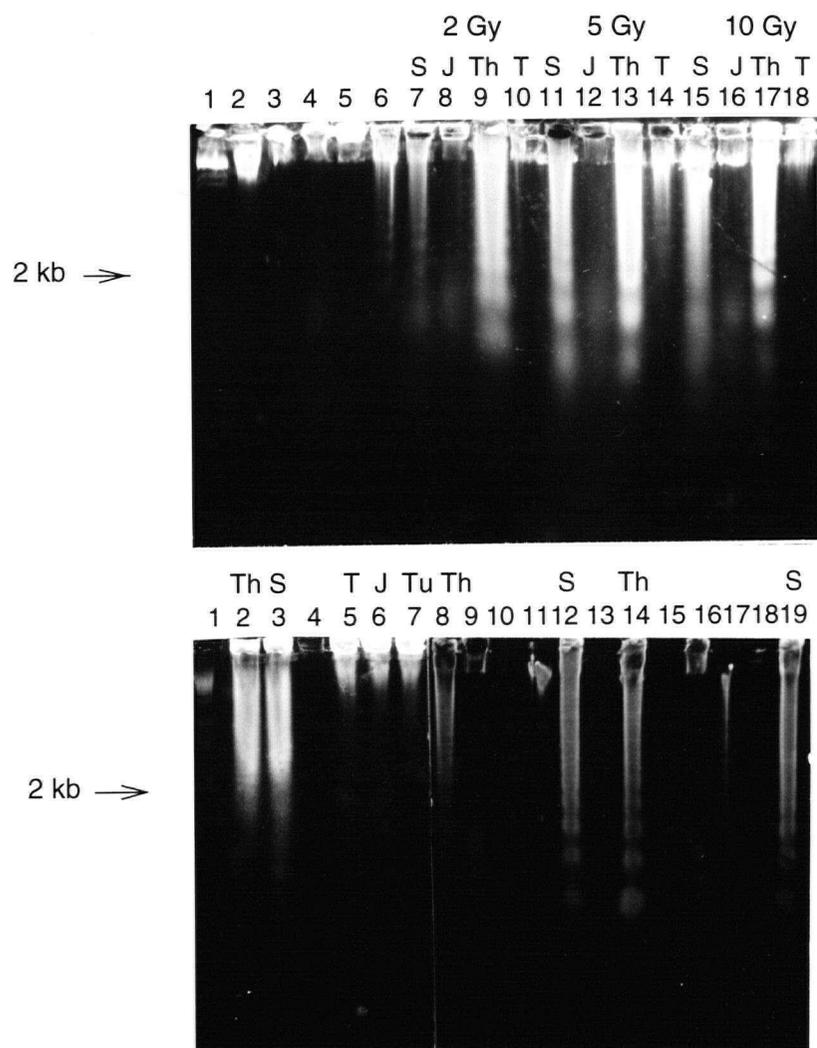


Fig. 17: DNA gel electrophoresis of C3H tissues irradiated in vivo. Upper panel: 4 hours after 2, 5, 10 Gy. Lane 1-marker; lane 2 to 5 - control; lane 6 - apoptotic TK6 cells. J-jejunum, S-spleen, T-testis, Th-thymus. Lower panel: tissues from mice treated with nicotinamide 800 mg/Kg prior to irradiation. Lane 1-marker, lane 2 to 7-24 hours after 10 Gy. Tu-tumour. Lane 8,12,14 and 19-4 hours after 5 or 10 Gy.

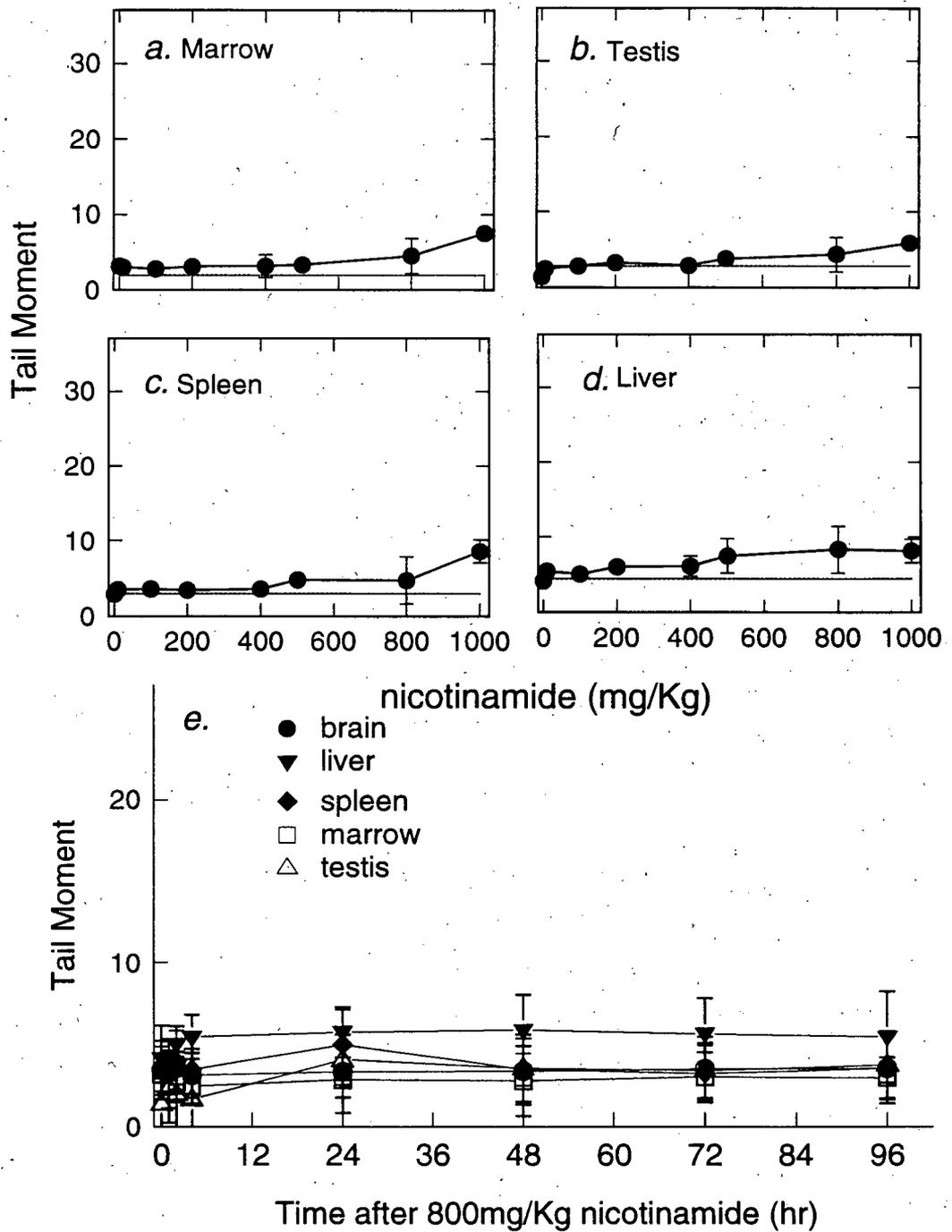


Fig. 18: Toxicity of nicotinamide one hour after 0 - 1000 mg/kg (a to d) or 0 to 4 days after 800 mg/kg measured as DNA SSB in different tissues. The means and standard errors for 3 experiments are shown. Dotted lines in panels (a-d) show the control value for each tissue.

breaks at doses greater than 800 mg/kg. Response as a function of time after i.p. injection was also examined following a single dose of 800 mg/kg nicotinamide. Within the first two hours, some mice which had received the higher doses were not as active probably due to reduced blood pressure, but mice had recovered considerably by the third hour post injection. DNA strand breaks showed no change with time and treated animals were not significantly different from controls.

### 3.6.2 Reduction of hypoxic cell fraction in tumours by nicotinamide

For mice injected with 200 mg/kg to 800 mg/kg of nicotinamide 30 - 90 minutes before 15 Gy of radiation, all tumours showed a decrease in the fraction of hypoxic cells as measured by the comet assay (Fig. 19). The hypoxic fraction decreased from 18.4% to 4.4% in mice given 200 mg/kg which was similar to the response to 500 mg/kg. Hypoxic fraction was reduced further to 2.9% for tumours treated with 800 mg/kg nicotinamide. Nicotinamide alone seemed as effective as nicotinamide plus carbogen, at least when the higher dose of the drug was given (Table 5). The time between nicotinamide injection and sacrifice was altered for different drug doses because of the fact that the peak nicotinamide drug concentration occurs earlier after exposure to lower drug doses (Chaplin et al.1993).

Initial DNA damage measured as mean tail moment also increased from 18.99 to 26.95 when 800 mg/kg nicotinamide was delivered to mice before 15 Gy of irradiation (Fig. 20). This result is also consistent with a decrease in hypoxic fraction.

**TABLE 5. HYPOXIC FRACTION OF SCCVII TUMOURS MEASURED USING THE COMET ASSAY**

Treatment	% Hypoxic cells (mean $\pm$ SD, # of tumours)
0 mg/kg nicotinamide (NCT)	18.4 $\pm$ 6.6 (15)
30 min before irradiation 200 mg/kg NCT	4.4 $\pm$ 7.6 (10)*
30 min before irradiation 500 mg/kg NCT	4.6 $\pm$ 7.8 (7)*
60 min before irradiation 800 mg/kg NCT	2.4 $\pm$ 2.8 (6)*
1 hr before irradiation 800 mg/kg NCT + carbogen before irradiation	2.7 $\pm$ 4.4 (4)*

\* Values significantly different from 0 mg/kg (p<0.005)

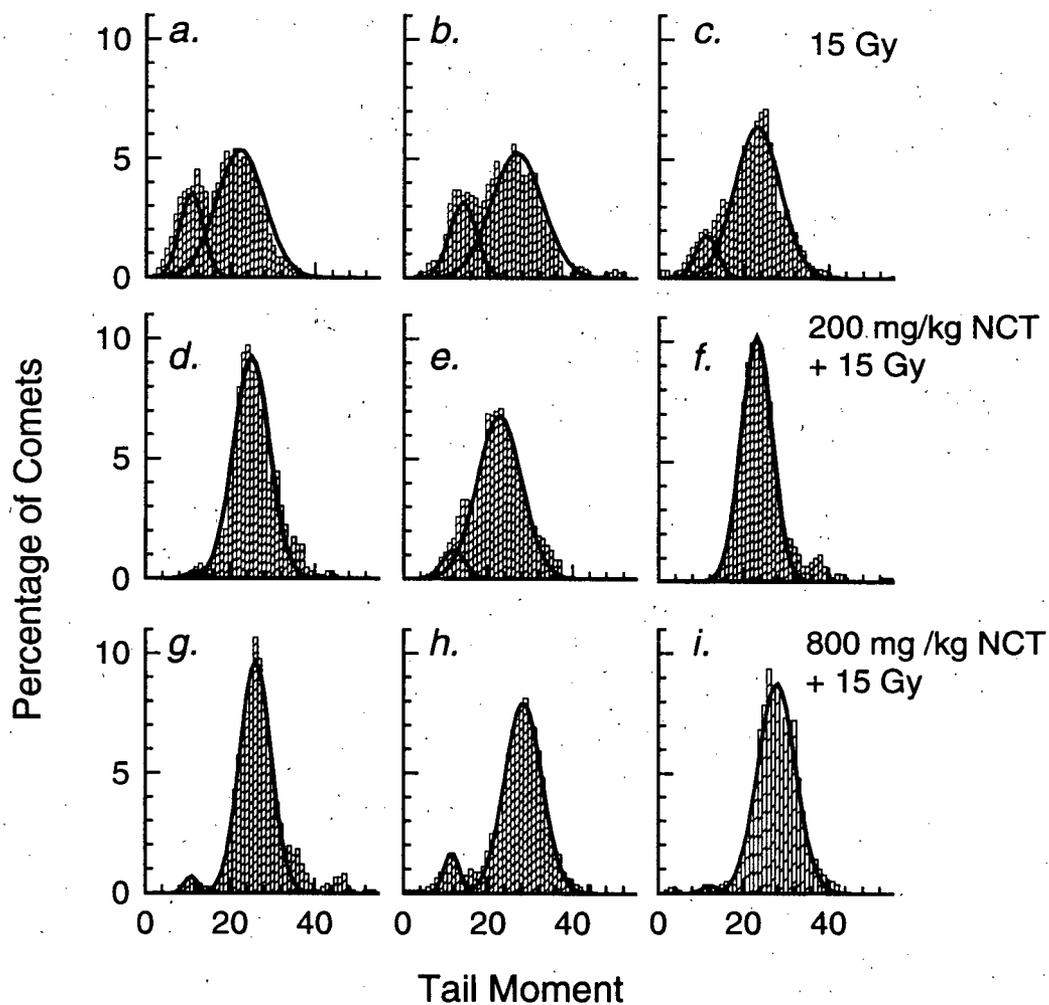


Fig. 19: Representative histograms of DNA SSB induction by 15 Gy x-rays irradiated in vivo. Tumour were excised immediately after irradiation and disaggregated into single cells for comet analysis. Panels (a-c): radiation only. Panels (d-f): 200 mg/kg nicotinamide i.p. 30 min before 15 Gy. Panels (g-i): 800 mg/kg drug injected 60-90 min before 15 Gy. The hypoxic fractions are listed in Table 4.

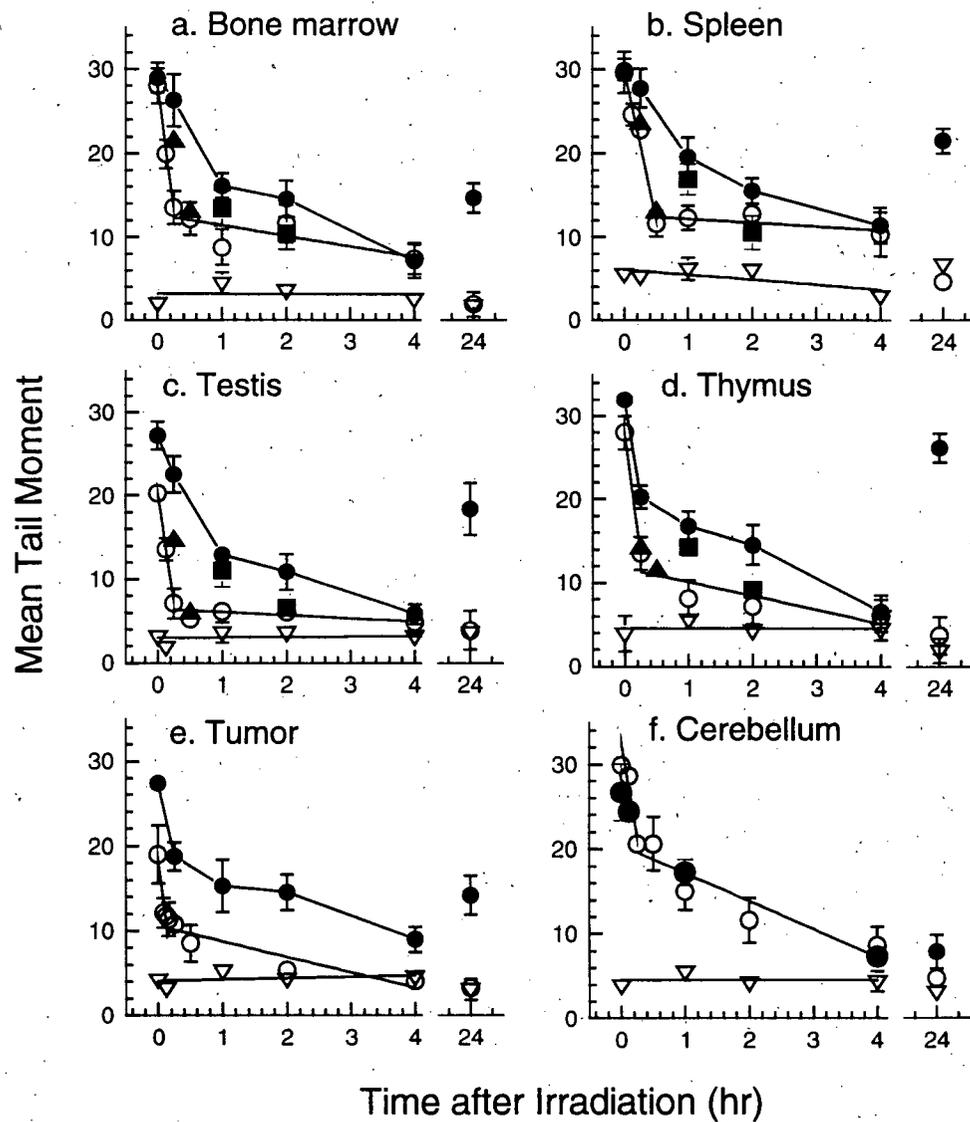


Fig. 20: DNA repair kinetics in C3H tissues after 15 Gy only ( ○ ) or 200 mg/kg ( ▲ ), 500 mg/kg ( ■ ), 800 mg/kg ( ● ) nicotinamide administered 60 min before 15 Gy. ▽ : 800 mg/kg nicotinamide alone. The means and standard errors for 3 experiments are shown.

### 3.6.3 Reduction of normal tissue hypoxia by nicotinamide

For studies performed in normal tissues, only testes showed an increase in initial strand break production when 15 Gy followed nicotinamide treatment. Mean tail moment increased from 20.29 to 26.56 immediately after 15 Gy in the testis of mice given 800mg/kg nicotinamide (Fig. 20). Unlike tumours which had an easily detectable hypoxic fraction, testis did not show such a population. Cells of all three DNA contents had the same degree of damage after drug treatment (Fig. 15d). Furthermore, all the cells had an increased tail moment following nicotinamide treatment. No effect was seen in bone marrow cells at this dose. However, at lower nicotinamide doses (below 500 mg/kg) the sensitization effect was not observable in testis. This result suggests that nicotinamide promotes reoxygenation of hypoxic cells which appear to be present in cells of all ploidies in testis.

### 3.6.4 Inhibition of DNA repair *in vivo* by nicotinamide

The effect of nicotinamide on DNA SSB rejoining kinetics was also studied in normal tissues. Fig. 20 indicates that nicotinamide prolonged the rejoining process in all the tissues except brain during the first three hours after irradiation. The half-time of SSB rejoining was significantly increased in all these tissues (Table 6). In tumour, rejoining half-time increased from 11 minutes to 84 minutes while in other normal tissues it increased from 12-25 minutes to 45-89 minutes. The half-time for tumour was increased about seven-fold by the presence of nicotinamide, and about 3-4 fold for most normal tissues. However in brain, the SSB rejoining process was not altered by nicotinamide. With depletion of nicotinamide in the plasma, rejoining improved. Four hours after irradiation, the tail moment was similar for nicotinamide treated mice and mice that had received 15 Gy only.

The ability of nicotinamide to inhibit SSB rejoining appeared to be dose dependent. Although only 2 time points were studied for 200 mg/kg and 500 mg/kg nicotinamide, these data indicate that 200 mg/kg nicotinamide was ineffective in inhibiting rejoining, and 500 mg/kg produced less inhibition than 800 mg/kg (Fig. 20).

**TABLE 6: INHIBITION OF REJOINING OF RADIATION-INDUCED SSB BY NICOTINAMIDE**

Tissues	Half-time (min) [95% conf. limits] <sup>1</sup>	Slope (min <sup>-1</sup> ) [95% cofi. limits]	Ratio <sup>2</sup>
Tumour	84 (75-92)	-.0036 (-.0050-.0031)	7.6
Testis	45 (38-50)	-.0067 (-.0079-.0057)	3.7
Bone marrow	63 (53-74)	-.0048 (-.0056-.0040)	4.5
Thymus	82 (71-94)	-.0037 (-.0042-.0032)	3.6
Spleen	89 (77-101)	-.0034 (-.0039-.0029)	3.5
Brain	48 (35-61)	-.0063 (-.0086-.0049)	1.0

<sup>1</sup>Mice were injected with 0.8 g/kg nicotinamide 60-90 min before irradiation and SSB induction was analyzed using the alkaline comet assay. The half-time of rejoining was calculated from the slope of the exponential part of the rejoining curve as described in Table 4 (see page 44) using times up to one hour.

<sup>2</sup>The ratio of the slopes of the repair curves in the presence and absence of nicotinamide is shown using the slopes for radiation only calculated in Table 4.

### 3.6.5 Promotion of DNA degradation in irradiated tissues by nicotinamide

Twenty-four hours after nicotinamide administration followed by 15 Gy, all normal tissues except brain showed a large amount of DNA degradation. Average tail moment increased to 15 - 27 in different tissues. The mean tail moment was highest in thymus and spleen followed by testis. For each tissue there were both heavily damaged cells and less damaged cells present (Fig. 21, 22), producing a wide range of tail moments which resembled the pattern seen in cells 48 hours after 15 Gy alone (Fig. 9). The peak appearance of these cells was around 24 hours after irradiation and this peak appeared to be independent of radiation dose or drug dose (Fig. 23). Nicotinamide alone produced no damage. Tail moment decreased to 10 after 30 hours (Fig. 23a). These results suggest that the process of cell degradation by radiation was accelerated by nicotinamide (Fig. 23b). The decrease in weight of spleen after irradiation with or without nicotinamide also supports this hypothesis (Fig. 24). However, while weight of testis also decreased following irradiation, this process did not appear to be promoted by nicotinamide. Cell death and clearance in testis was apparently not as rapid and extensive as seen in spleen. This conclusion was also supported by the histological results (see Section 3.6.6.3).

Interestingly, when nicotinamide was administered immediately after irradiation, the population of heavily

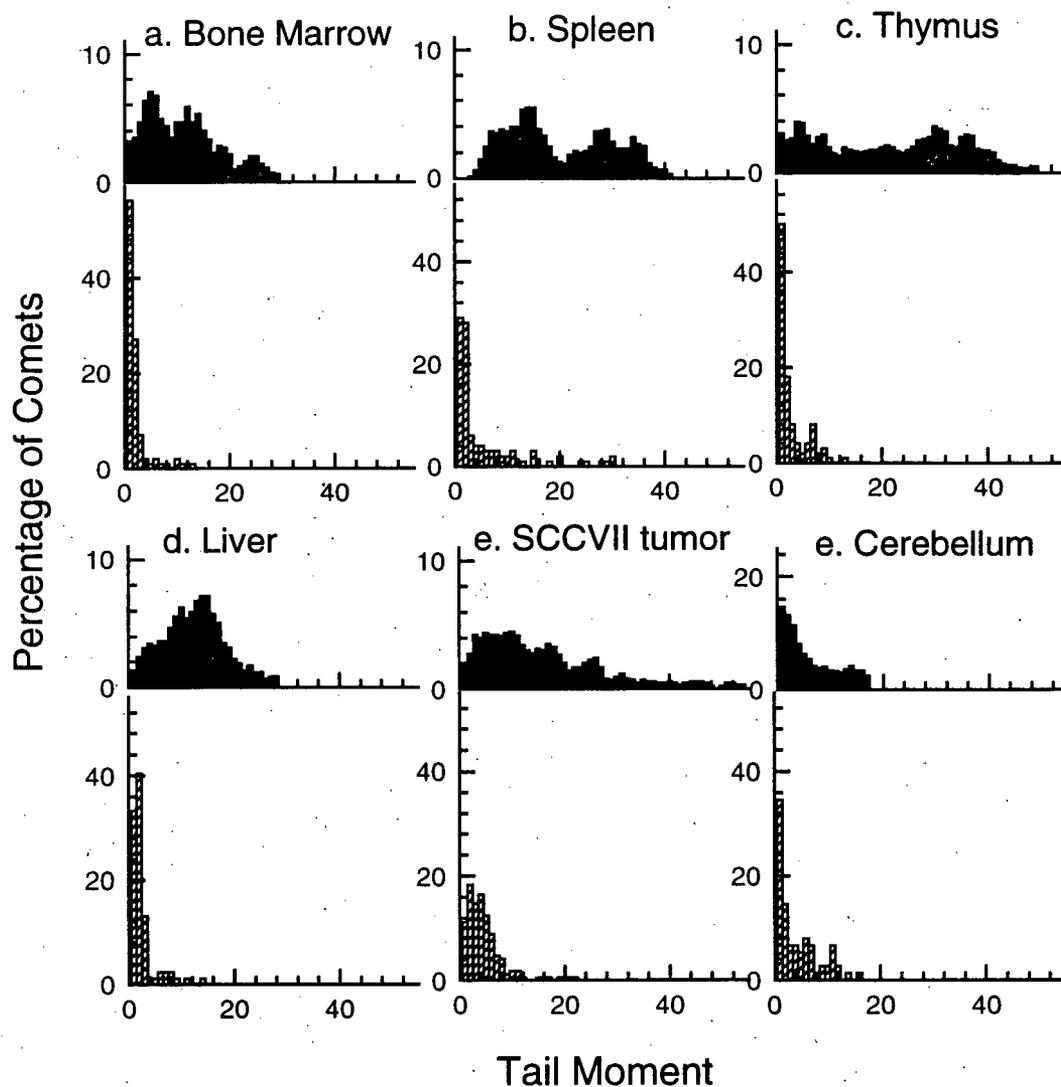


Fig. 21: Effect of nicotinamide on promotion of DNA degradation in tissues 24 hours after treatment. Shaded bars - irradiation only; filled bars - 800 mg/kg nicotinamide i.p. one hour before 15 Gy.

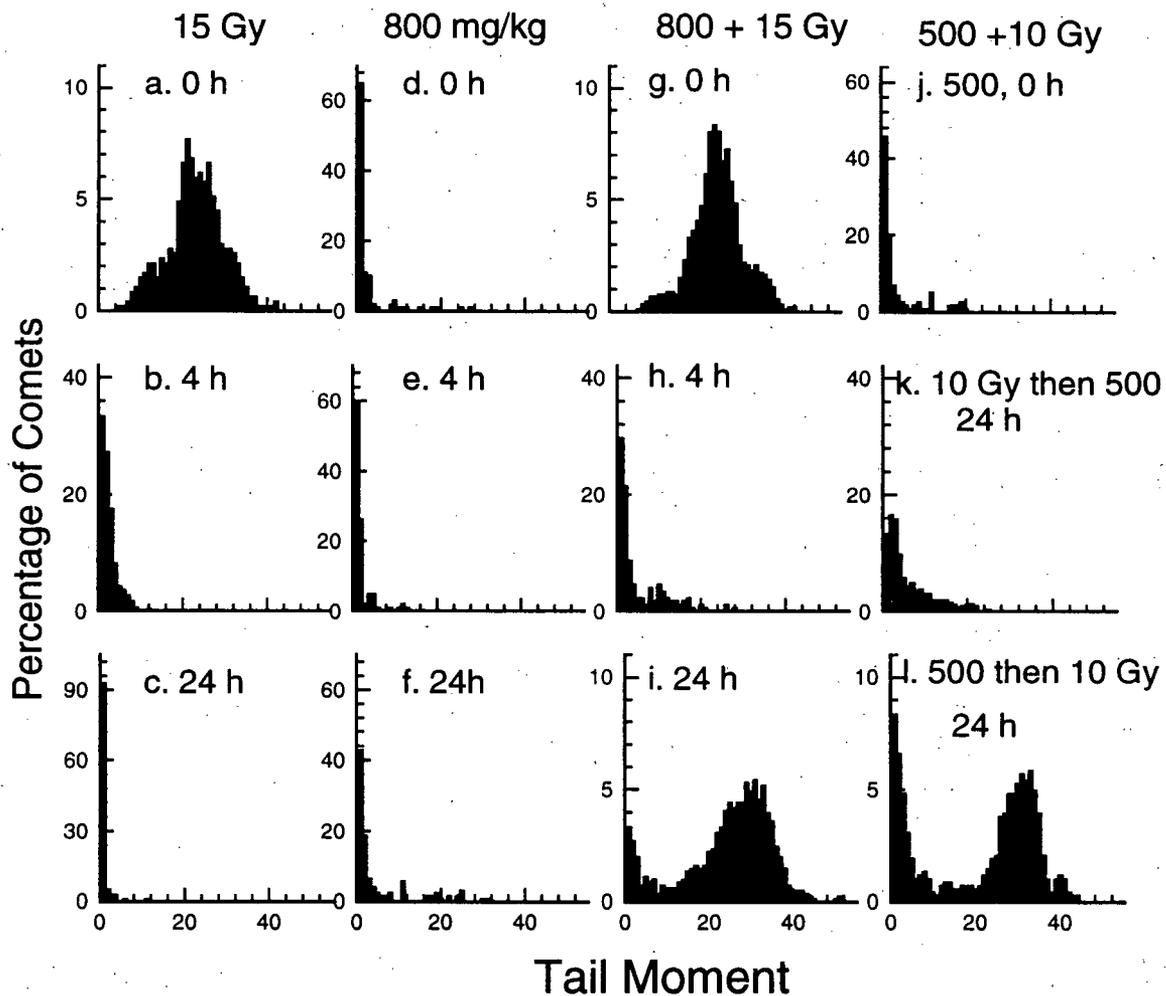


Fig. 22: Effects of 15 Gy only (a-c), nicotinamide only (d-f) or the combination of the two on DNA repair and damage in testis (g-i and j-l). Note that in (k) and (l) the order of treatment was reversed. The time of analysis or drug treatment is indicated in each panel which represents the response of a single animal.

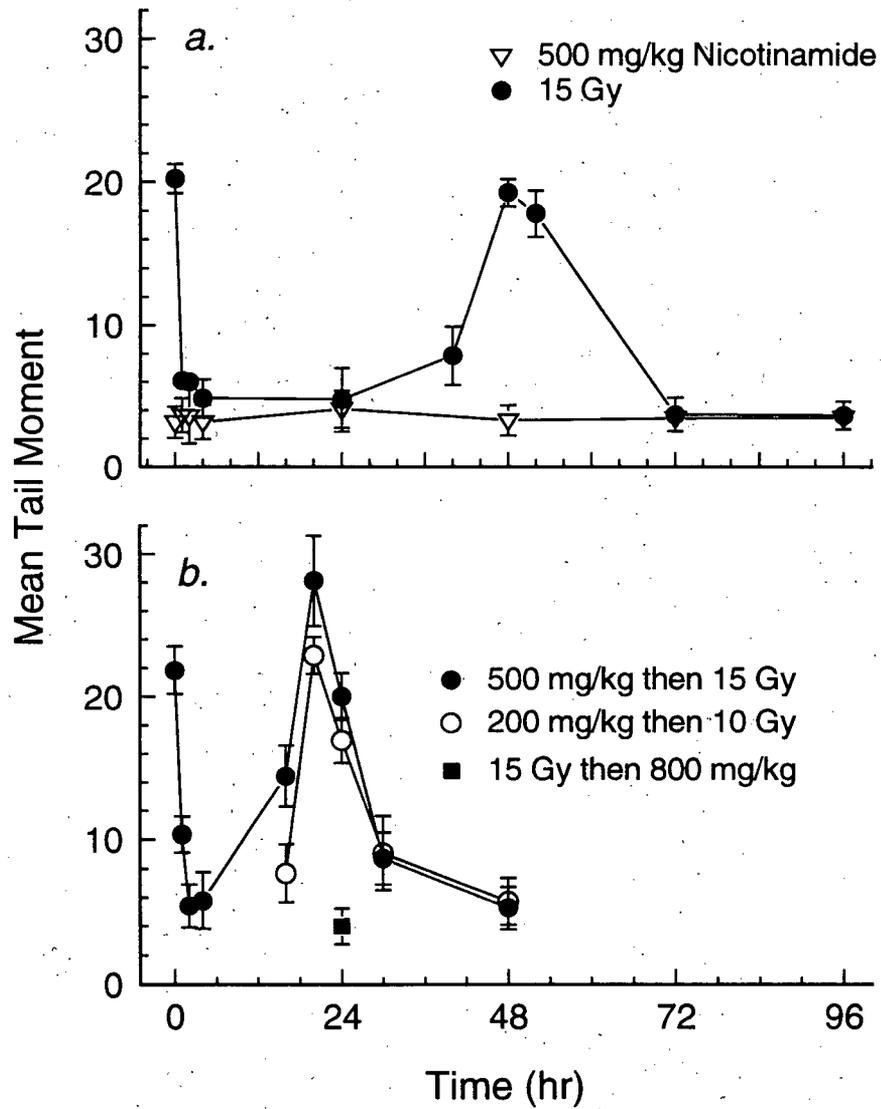


Fig. 23: Time course of the appearance of DNA degradation in testis by irradiation only or 500 mg/kg nicotinamide alone (a). Panel (b) shows the exposure to combined treatment of nicotinamide and radiation. The means and standard errors for 3 experiments are shown.

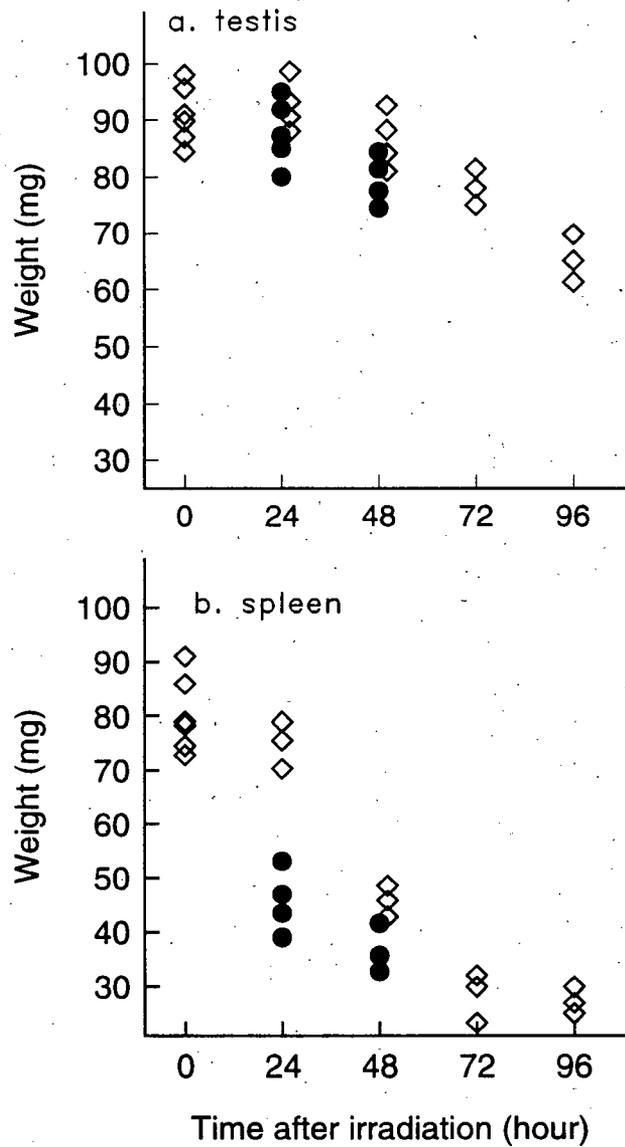


Fig. 24: The change of weight in testis (a) and spleen (b) after 15 Gy irradiation only ( ◇ ) or 800 mg/kg nicotinamide i.p. followed by 15 Gy 60 min later (filled circles). Mice were killed at the time indicated and the whole organs were excised, and weighed immediately. Symbols at 0 hour were from non-irradiated tissues.

damaged cells was not observed at 24 hours (Fig. 22k), although some less damaged cells were still present. This result suggests that nicotinamide must be present during irradiation and during the early DNA rejoining process in order to promote the earlier appearance of damaged cells.

The degree of extensive DNA damage in these tissues was dependent both on X-ray and nicotinamide dose. The higher the dose of either agent, the higher the percentage of heavily damaged cells and therefore the higher the tail moment (Fig. 25). This general relationship was seen in all tissues.

Heavily damaged cells were not observed for cultured mammalian cells treated in a similar fashion. Following the results of Ben-Hur and Elkind (1985), when 20 mM nicotinamide was given to the cultured Chinese hamster V79 and mouse EMT6 fibroblasts and incubated for one hour before and one hour after exposure to 10 Gy, no DNA damage was observed 24 hours later (data not shown).

### 3.6.6 The nature of cell death following radiation and nicotinamide

Several methods were used to attempt to determine whether the cell degradation seen using the comet assay was the result of apoptosis or necrosis. Mice were exposed to nicotinamide prior to irradiation with 15 Gy, then tissues were removed 24 hours later for analysis.

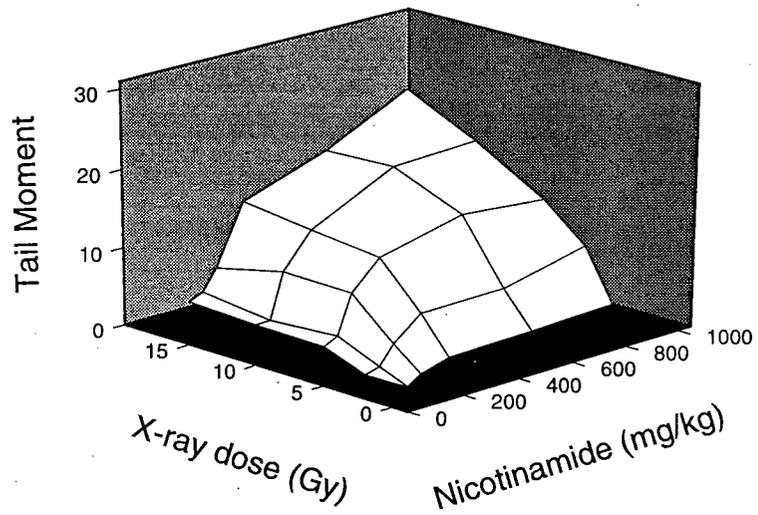
#### 3.6.6.1. DNA gel electrophoresis

DNA from spleen, thymus and other tissues was examined using standard agarose gel electrophoresis. Ladders indicating an apoptotic response were only seen in spleen and thymus 4 hours after irradiation whether or not nicotinamide was given (Fig. 17). However, no apparent DNA ladder but a smear more indicative of necrosis was seen in these tissues 24 hours after irradiation with or without nicotinamide.

#### 3.6.6.2. Terminal deoxynucleotidyl transferase labelling

When DNA fragments in cells from spleen and testis were 3-OH' end labelled with terminal deoxynucleotidyl transferase and examined using flow cytometry, it was found that less than 5% of the cell population

a. Testis



b. SCCVII Tumor

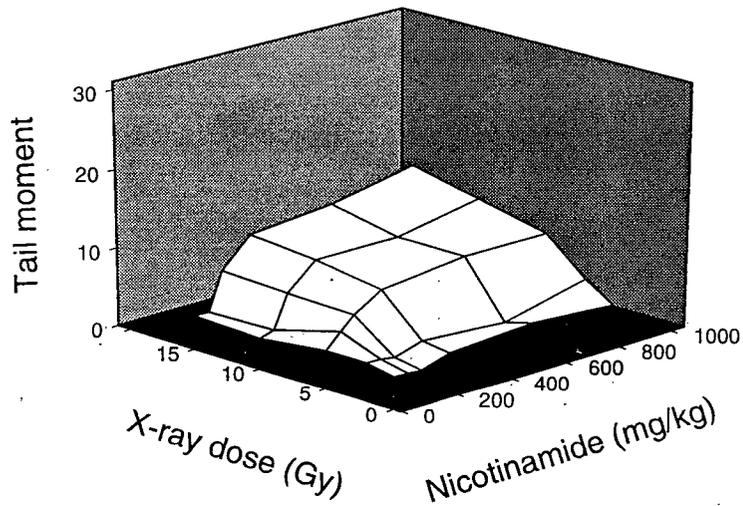


Fig. 25: DNA degradation in testis and SCCVII tumours 24 hours following exposure to nicotinamide and irradiation. Mice were injected with nicotinamide 60 min prior to irradiation. They were killed 24 hours later, and single cells were obtained from these tissues and prepared for comet analysis. Each point was the mean for 2-3 independent experiments.

exhibited extensive fragmentation in testicular cells (Fig. 26). In spleen, about half of the cells were labelled, but were not very well separated from the unlabelled (undamaged) cells (Fig. 27). The pattern was not different from the cells irradiated with 15 Gy only. This result suggests that the fragment size was much larger in spleen cells than in testis cells, but more cells were damaged in spleen than testis.

#### 3.6.6.3. Histological studies

Spleen, testis and tumour were prepared for histological examination. For the haematoxylin/eosin stained tissue sections (Fig. 28), spleen showed many degenerating cells with pyknotic nuclei in the white pulp one day after 15 Gy, and the number of such cells was increased after combined nicotinamide and radiation treatment. These degenerating cells formed small foci containing on the order of 10 cells. Occasionally single dying cells were also observed scattered in the white pulp. Some cells had the appearance of apoptotic cells: shrunken cells with condensed chromatin. However, in testis no significant changes were apparent in the cells 24 hours after 15 Gy only or after nicotinamide and 15 Gy (Fig. 29). Only minor changes in the cytoplasm of testis cells were seen suggesting a possible effect of irradiation. No significant morphological changes were observed in tumours after nicotinamide and radiation (data not shown), perhaps due to the large necrotic fraction associated with this tumour before any treatment.

#### 3.6.6.4. Size measurement of the DNA fragments in testis cells.

The size of DNA fragments in individual cells may help to distinguish apoptotic from necrotic cells (Olive and Banath, 1995b). Different concentrations of mechlorethamine ranging from 0 to 10.4  $\mu\text{M}$  were used to create DNA interstrand crosslinks in the degenerating cells of testis 24 hours after nicotinamide and radiation. After incubation with mechlorethamine for 30 minutes, cells were examined for DNA breaks using the comet assay. Fig. 30 shows the gradual decrease in tail moment in testicular cells treated with different concentrations of mechlorethamine, since crosslinks produced by mechlorethamine prevent DNA from migrating in this assay. About 3.15  $\mu\text{M}$  of mechlorethamine was necessary to decrease the tail moment to 10. Using a previous calibration curve prepared from irradiated cells (Olive and Banath 1995b), it could be calculated that the amount of SSB produced in these cells was equivalent to the effect of 44.5 Gy of radiation, or about  $2.23 \times 10^4$  DSBs/cell. Assuming there are  $3 \times 10^9$  base pairs in each mammalian cell

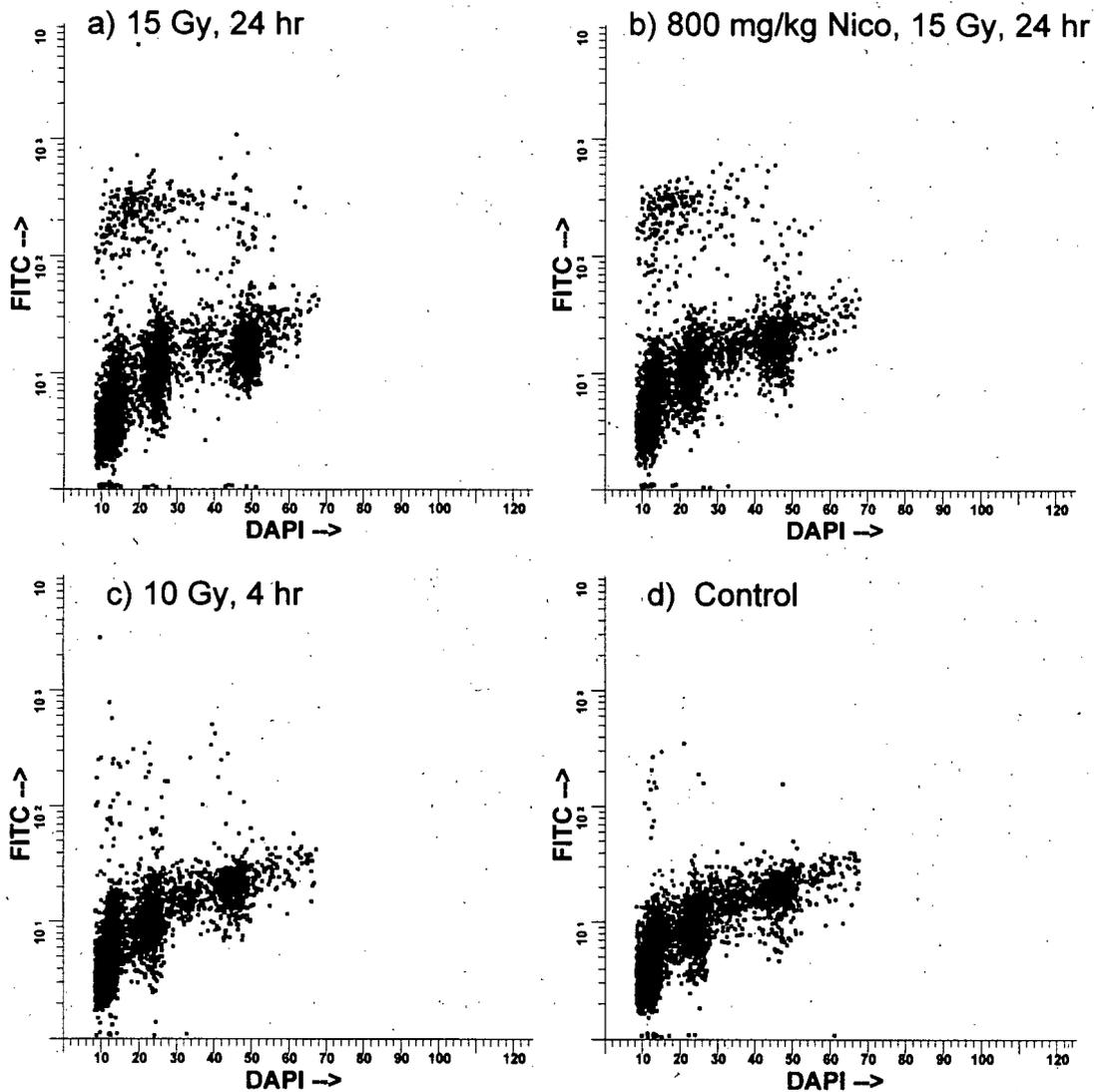


Fig. 26: Apoptosis in testis cells measured using the terminal deoxynucleotidyl transferase method. Testis cells were harvested after the treatments indicated, fixed and permeablized, and then assayed for the presence of "nicks" using the TdT assay. Analysis of the percentage of positive cells gave 6.1% in a, 5.3% in b, 0.9% in c, and 0.4% in d.

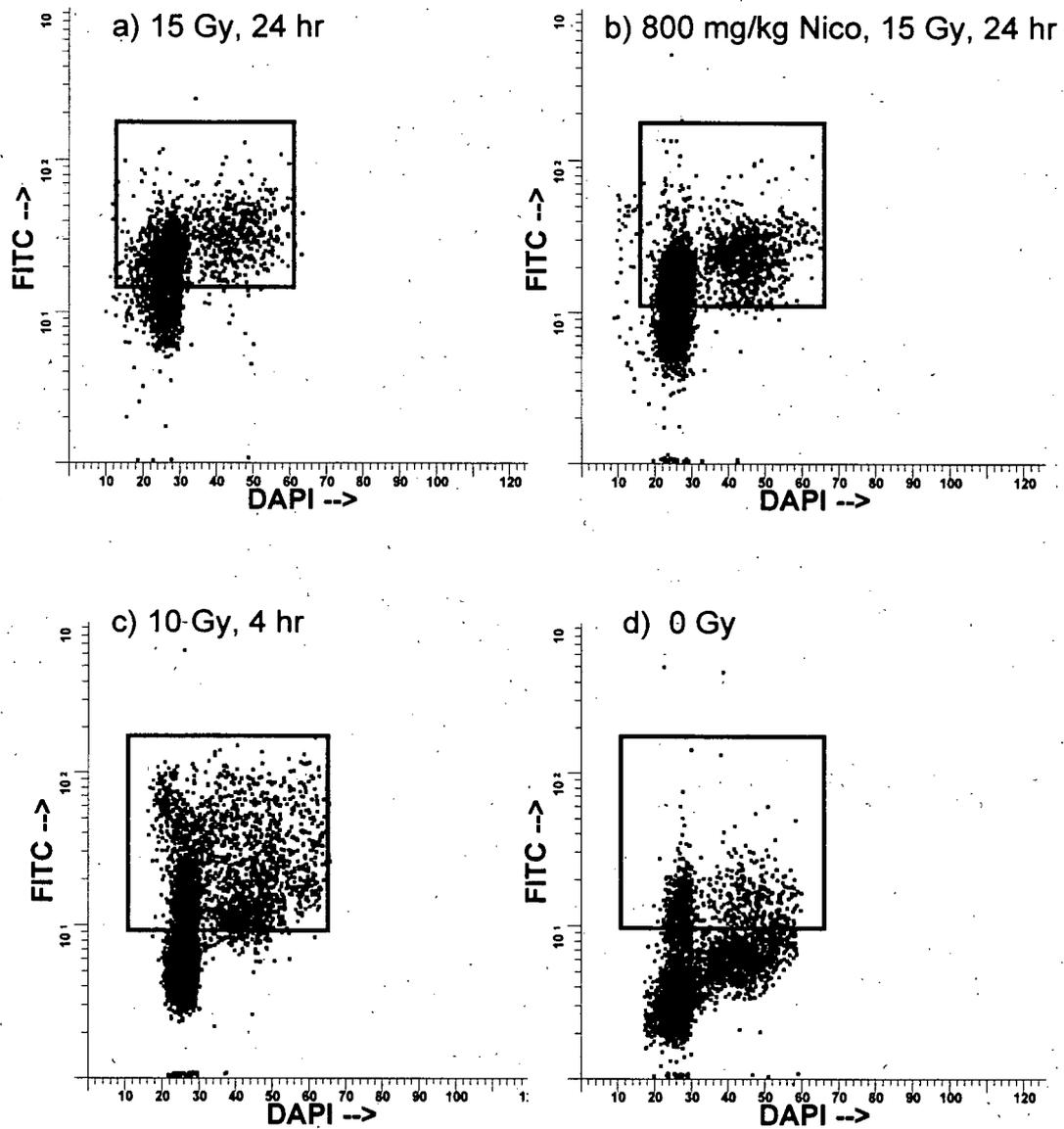
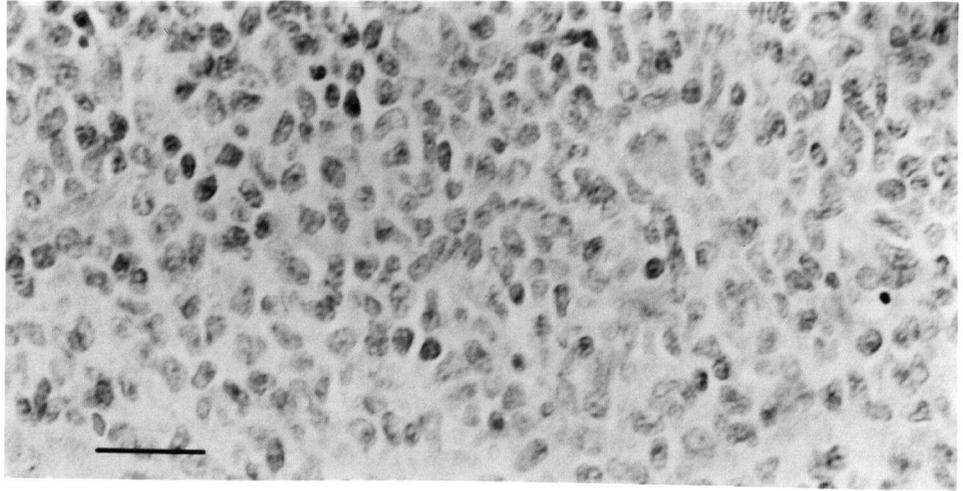
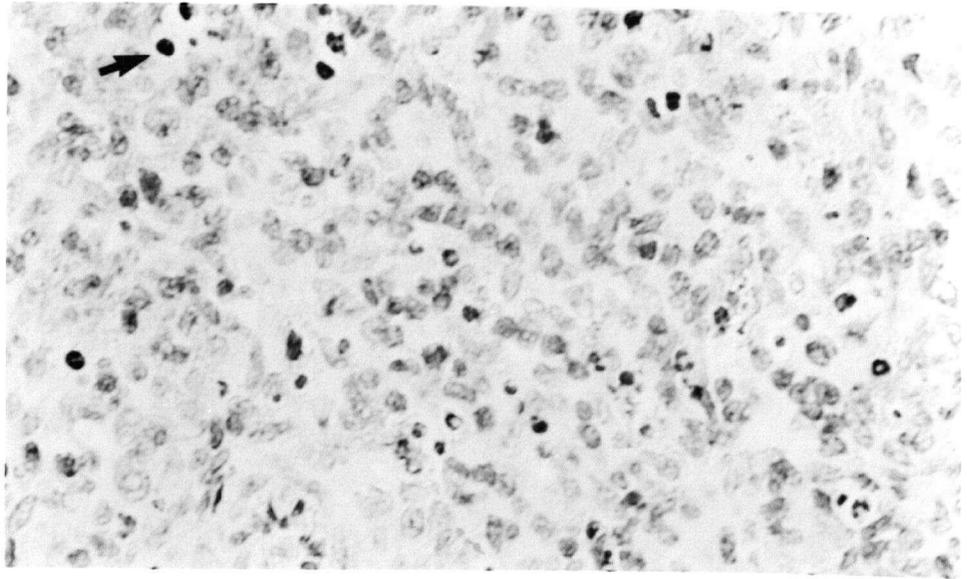


Fig. 27: Apoptosis in spleen cells measured using the terminal deoxynucleotidyl transferase method. Spleen cells were harvested after the treatments indicated, fixed and permeabilized, and then assayed for the presence of "nicks" using the TdT assay. Analysis of the percentage of positive cells approximated by the boxes gave: 70% in a, 78% in b, 44% in c and 21% in d.

a



b



c

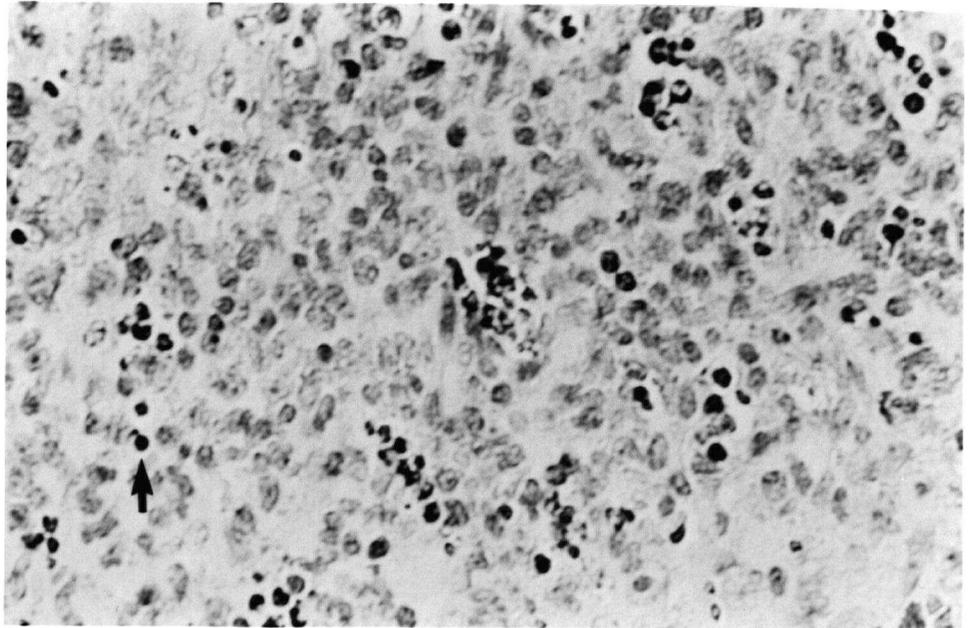
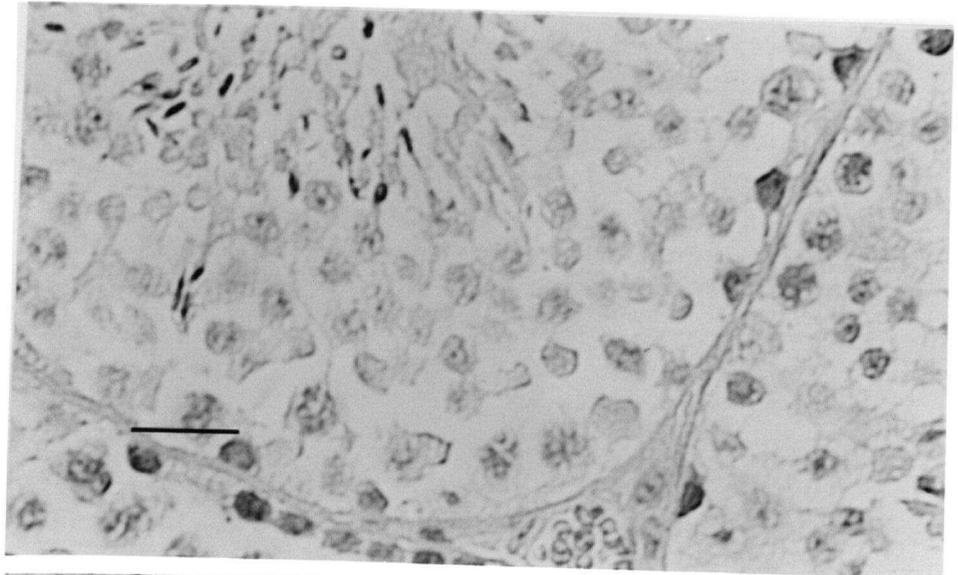


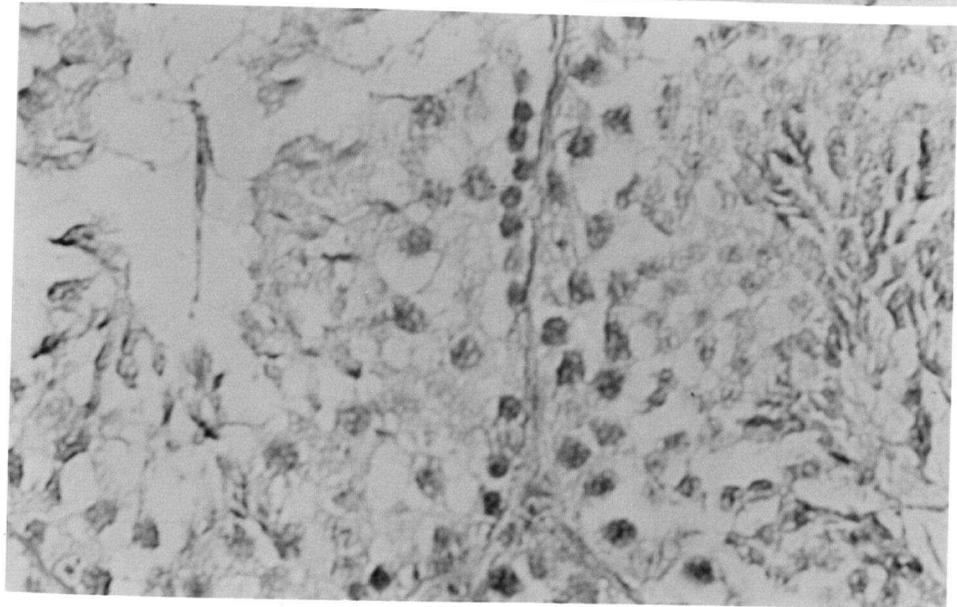
Fig. 28 (previous page): Histological appearances of spleen from untreated mouse (a), 24 hours after 15 Gy only (b) or 24 hours after 800 mg/kg nicotinamide followed by 15 Gy. Each of the picture was taken from the white pulp of the spleen. Arrows in panel (b, c) indicate probable apoptotic cells. Hematoxylin and eosin stain, x 40. Bar in panel (a) represents 20  $\mu$ m.

Fig. 29 (next page): Histological appearances of testis from mice after different treatments. (a) no radiation; (b) 24 hours after 15 Gy; (c) 24 hours after 800 mg/kg nicotinamide followed by 15 Gy. Different generations of germ cells can be seen in each seminiferous tubule. Panels (a-b) show the cross sections of the tubules, panel (c) shows a longitudinal section. Hematoxylin & eosin stain, x 40. Magnification bar in (a) represents 20  $\mu$ m.

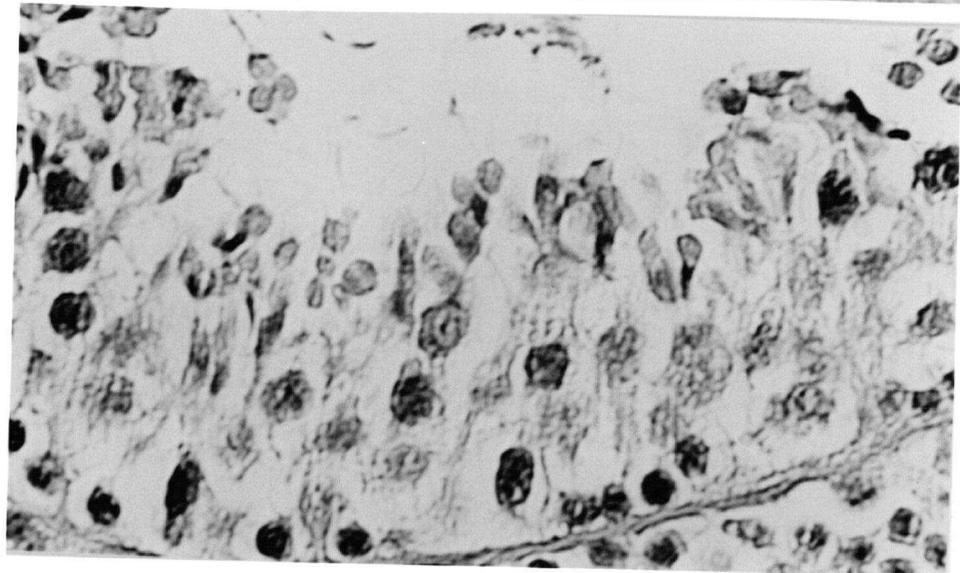
a



b



c



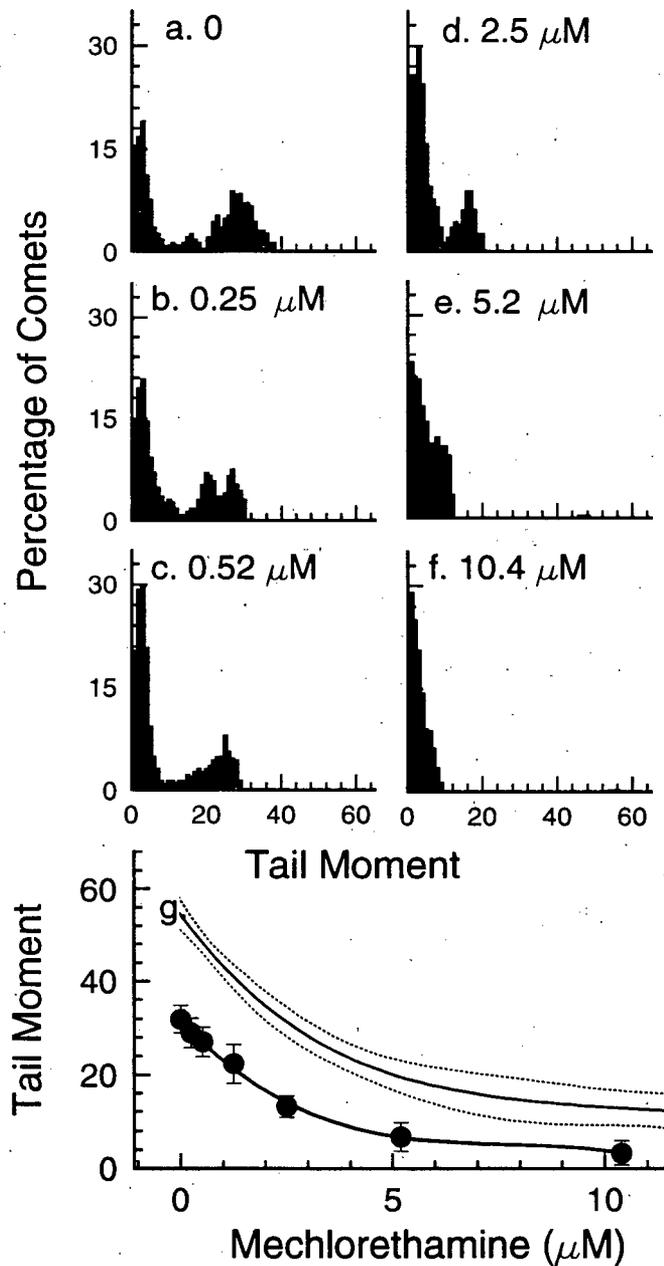


Fig. 30: Sizing the DNA fragments in testis induced by nicotinamide and X-rays. Cells were incubated with different concentrations of nitrogen mustard, a DNA crosslinking agent, to reduce fragment migration in the electric field (and therefore decrease the tail moment) as shown in (a - f). Panel (g) shows the standard curve generated from TK6 cells (Olive & Banath 1995) and the curve made from testicular cells ( ● ). See text for the calculation of fragment size.

(Kornberg 1980), the size of each fragment would be about 135 kb. This value is somewhat larger than the 50 kb size of apoptotic fragments estimated by others in different cell types (Olive and Banath 1995b; Oberhammer et al. 1993).

#### 3.6.7. Clonogenicity in tumour cells treated with radiation and nicotinamide

Five hundred mg/kg nicotinamide given i.p. 90 min prior to 10 or 15 Gy increased cell killing in tumour cells excised immediately after irradiation. Clonogenicity was reduced in these cells compared with those treated with radiation alone (Fig. 31a). However, the increased cell killing was mainly seen in the populations of tumour cells containing a higher concentration of the fluorescent dye, Hoechst 33342, that is, from tumour cells closer to "functional" blood vessels. This population should be primarily aerobic, but will also contain cells affected by transient changes in perfusion (Chaplin et al., 1987). Nicotinamide itself was not toxic to cells. In order to see the possible effect of nicotinamide on sensitization of cells without the interference of fluctuation of cell oxygenation, tumours were clamped 90 minutes after injection of same dose of nicotinamide and then exposed to 15 or 20 Gy. No further increase in cell killing was observed compared with tumours treated with radiation alone (Fig. 31b).

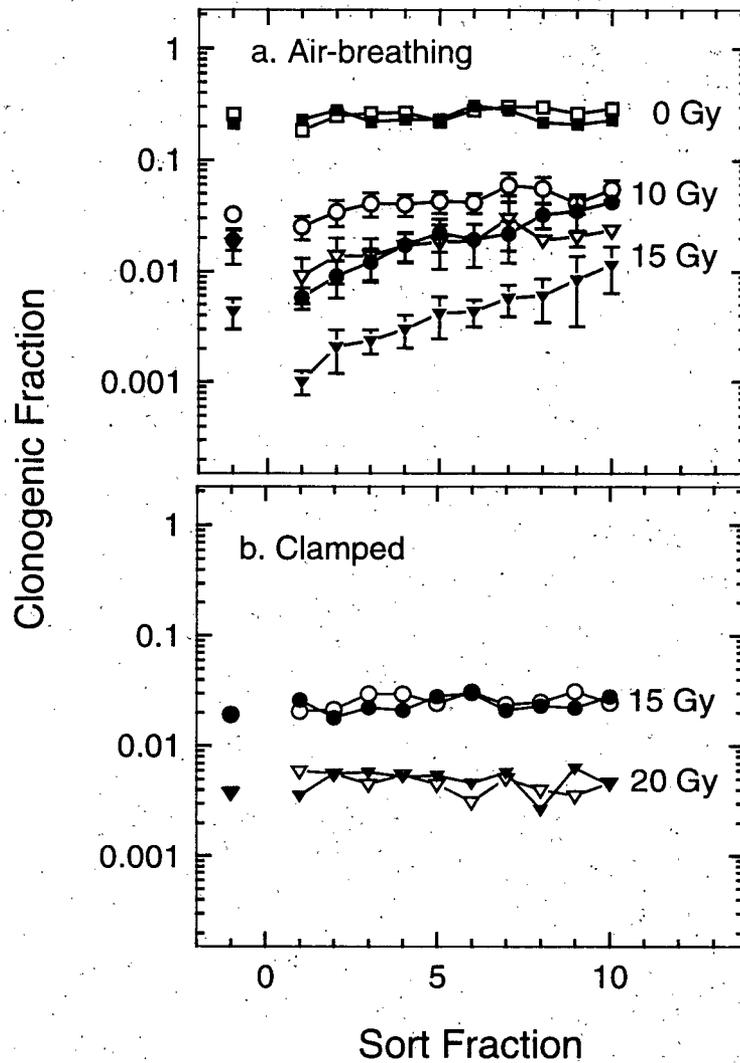


Fig. 31: Effect of nicotinamide and radiation on SCCVII tumour cells measured as clonogenic fraction. a) Mice were injected with 500 mg/kg nicotinamide followed by i.v. injection of Hoechst 33342 80 min later. Radiation was given 10 min later. Tumours were excised immediately and single cells were prepared for cell sorting according to the Hoechst 33342 fluorescence gradient. Fraction 1 represents the most fluorescent cells in the tumour and therefore closest to functional blood vessels. Fraction 10 was the cells most distant from blood supply. The means and standard deviations for 3-4 animals are shown. b) Mice were treated in the same way as in (a) but tumours were clamped before 15 or 20 Gy. Open symbols were from radiation alone, closed symbols were from the combination with nicotinamide. The means of 3-5 experiments are shown. Symbols near the ordinate were from unsorted samples.

## 4. DISCUSSION

### 4.1. Radiation-induced DNA damage

#### 4.1.1 SSB induction is dose-dependent but cell type independent

In agreement with many previous studies conducted using a variety of methods (e.g. Sakai and Okada 1984; Vexler et al. 1982; Vanankeren and Meyn 1988; McMillan et al. 1990), the number of radiation-induced DNA SSB increased linearly as a function of radiation dose *both in vitro* and *in vivo* (Fig. 4). Damage produced by X-ray doses higher than 20 Gy was beyond the range of sensitivity of the alkaline comet assay, but the assay is sufficiently sensitive to measure DNA damage produced by clinically relevant doses (i.e., 0.5-2 Gy) (Singh et al 1994).

For all the cells irradiated *in vitro*, the slopes of the dose-response relationships were not significantly different from each other. Although cells from these different tissues vary in proliferation and differentiation status, it is clear that these factors are not relevant in the production of *initial* DNA damage by ionizing radiation. The amount of radiation energy required to produce a SSB appears to be identical in all mammalian cells, with the exception of late stage spermatocytes. The values calculated by different investigators vary from 30 - 100 eV/SSB (Lett et al., 1970; Ormerod, 1975). Therefore, these results confirm that the induction of SSB by ionizing radiation *in vitro* is independent of cell type and state of differentiation. While some methods show variations in damage between cell lines possibly caused by differences in chromatin conformation (Olive, 1992), the alkaline comet assay is not sensitive to such differences.

Clearly what is unique about the comet assay is the ability to measure the response of individual cells. However, heterogeneity in initial response in all tissues was small. For irradiation performed *in vitro*, there was no indication that subpopulations of cells were present that were responding differently to ionizing radiation.

#### 4.1.2 The radioresponse *in vivo* is different from that of *in vitro*

##### 4.1.2.1 Effect of DNA repair

Fig. 4 clearly showed a significant difference in the slopes of the dose response curves between cells irradiated *in vitro* and *in vivo*. Under the latter circumstance the measure of damage was always lower. The difference was largest in tumour and testis cells which demonstrated a ratio greater than 2 for the two slopes. The values for the remaining tissues varied around 1.6-1.7 (Table 2). The experimental differences suggest that several factors can modify radiation induced DNA damage *in vivo*. First, the initial SSB rejoining process is very rapid, and will be occurring as the radiation dose is delivered. About 4.5 minutes was required to administer 15 Gy X-rays and another 30 seconds was needed to sacrifice the mouse, excise the tissue, and immerse the tissue in ice-cold PBS to inhibit repair. During irradiation, a significant number of SSB will be rejoined. When cells are irradiated on ice *in vitro*, however, little or no SSB rejoining can occur, so the tail moment for these cells was always higher. Therefore, it is not surprising to see such a difference between the *in vitro* and *in vivo* responses in this and previous studies (e.g. Ono and Okada 1974, Meyn et al. 1980 & 1986).

Repair-related differences between the *in vitro* and *in vivo* responses should be minimized if the radiation dose is delivered within a shorter time. To demonstrate this, a radiation source which provided a higher dose-rate ( $^{137}\text{Cs}$ -gamma rays) was employed. When 10 Gy was delivered to mice in only 1.08 minutes rather than 3.3 minutes using the X-ray unit, the initial damage was higher in each of the tissues including the SCCVII tumours (Fig. 13). This result confirms that a large part of the difference between the *in vitro* and *in vivo* response is due to SSB rejoining which occurs *in vivo*.

Although SCID mice are known to be sensitive to ionizing radiation, the same level of SSB was induced in SCID mice as in C3H mice (Fig. 11, 12). Presumably this indicates that while SCID cells are known to be defective in rejoining DSB (Biederman et al. 1991), they rejoin SSB at normal rates (see section 4.2.3).

#### 4.1.2.2 Effect of oxygen

Heterogeneity in DNA damage was not observed for *in vitro* exposures, however, for irradiation performed *in vivo*, there was an important exception. Hypoxic cells in tumours and testis demonstrate fewer SSB than aerobic cells. This property forms the basis for the use of the comet assay to detect hypoxic cells in human tumours (Olive et al., 1993b). The slope of the dose-response curve for testis was 1.14 and was the lowest among all the normal tissues examined. Tumour cells were even lower with a slope of 1.02. For the other tissues, slopes varied between 1.72-1.87. This *in vivo* response is in agreement with results by Meyn and colleagues (1986) who concluded that hypoxia was responsible for differences in damage induction. Like other solid murine tumours, SCCVII murine tumours have been found to contain a high proportion of hypoxic cells (Chaplin et al. 1987). Once the tumour size reaches 0.4-0.5g, the fraction of hypoxic cells reaches 11.2% - 28.6% measured using the comet assay, and this result is consistent with the conventional paired-survival curve method (Olive and Durand 1992). But unlike cells irradiated *in vitro* which show clearly a factor of 3.3 for the oxygen enhancement ratio using this assay, the situation for tumour cells irradiated *in vivo* is rather complicated. As described previously, hypoxia in tumours develops in two different ways. In addition to those chronically hypoxic cells which lack oxygen due to inadequate diffusion through the tumour cord, intermittent blood flow in tumours causes an acute, perfusion-limited hypoxia in some tumours (Brown 1979, Chaplin et al. 1987, 1989). Such hypoxic cells can even exist adjacent to blood vessels which may function at some times, but not others. Little is known about the exact time for the opening and closing of these vessels although some studies showed that changes in perfusion occur every twenty minutes on average and that such transient non-perfusion can last 4-5 minutes (Trotter et al. 1989). Therefore, during exposure to 15 Gy X-rays which lasted 4.5 minutes in the experiment, many such transient changes in perfusion may have occurred, resulting in the appearance of some cells with intermediate levels of oxygen when averaged over the duration of the exposure. The net effect is to reduce the apparent displacement between the aerobic and hypoxic peaks, so that the oxygen enhancement ratio appears to be reduced. As shown on the histogram of DNA damage (Fig. 19a-c), the hypoxic cells (the peaks with lower tail moments) overlapped the better oxygenated cells which had higher tail moments. The proportion of cells with an intermediate oxygen content will vary as a function of tumour size and dose rate.

Hypoxic cells are frequently seen in murine tumours and have been shown to be present in some human tumours (Denekamp 1983, Vaupel et al. 1992; Olive et al. 1993b). Since anoxic cells exhibit only one-third the amount of DNA damage following irradiation, they have a much better chance of survival than aerobic cells irradiated with same dose, and they may retain clonogenic capacity following radiotherapy (Denekamp 1983). As shown in Fig. 19a-c, the presence of hypoxic cells reduces the mean value of tail moment compared to that of normal tissues, which means tumour cells are protected from radiation-induced DNA damage by hypoxia.

The effect of hypoxia is not only significant for tumours, but also for some normal tissues. Meyn and Jenkins (1983) suggested that different degrees of DNA SSB induction in tissues from whole-body irradiated mice might be due to biological hypoxia in some tissues. In order to evaluate the effect of oxygen using the comet assay, mice were allowed to breathe different concentrations of oxygen before and during irradiation. As shown in Fig. 14, the effect was most obvious for cells from testis of mice allowed to breath different oxygen mixtures. Breathing 10% oxygen in nitrogen decreased tail moment in testis and also in bone marrow cells, but not in spleen and other tissues. When mice were killed before 15 Gy to deprive all the tissues of oxygen, all the tissues showed the same low tail moment. In spleen this value was one-third of that observed for air-breathing mice, indicating an approximate oxygen enhancement ratio of 3 for this tissue. The same result was seen for liver, bone marrow and jejunum (Olive et al. 1994), which means that these tissues are well oxygenated under normal air-breathing conditions.

In comparison to other tissues, the oxygen enhancement ratio for testis was only about 2. This suggested that the oxygenation of testis is bordering on radiobiological hypoxia, and the organ therefore may contain hypoxic cells. Support for this conclusion comes from results described in the following paragraph, and also from results in the literature by Setchel (1978). First, the testicular artery is so long in relation to its diameter that this in itself poses an upper limit to blood flow. Second, according to the measurement of oxygen tension, the venous blood leaving the testis contains less oxygen than the mixed venous blood. Since there are no capillaries in the seminiferous tubules and tubular cells have an appreciable consumption of oxygen, the oxygen tension inside the tubules is 7 mmHg lower than that in the interstitium,

usually 7-12 mmHg in many mammals and around 2 mmHg for the human according to Max (1994). Such a low oxygen tension easily poises the testicular cells on the brink of radiobiologically hypoxia. The other tissue possibly bordering on hypoxia is bone marrow, since it exhibited a decrease in comet tail moment when the mice breathed a gas mixture containing only 10% oxygen. Other tissues showed no change in tail moment relative to air-breathing conditions. Therefore, bone marrow is adequately oxygenated under air-breathing conditions, but verges on hypoxia. Increasing the oxygen supply to the tissues by breathing carbogen (95% O<sub>2</sub> + 5% CO<sub>2</sub>) affected only the response of testis, confirming that testis is poorly oxygenated and likely to be resistant to radiation damage. The slightly less oxygenation of bone marrow cells, however, may not affect their response to radiation. In this study, however, only cells from the bone cavity were analyzed. Cells from the compact bone, which can only be released by collagenase digestion, were not examined. In the study of Allalunis et al. (1983), CFU-C (granulocyte-macrophage precursor cells) were found to be more hypoxic only if they resided in the compact bone.

The degree of biological hypoxia in SCCVII tumours and normal tissues was further evaluated using RSU 1069, a dual-function bioreductive drug which has been used to detect subpopulations of hypoxic cells in spheroids and tumours (Olive 1995b,c). The extent of tissue hypoxia can be estimated by observing the proportion of cells containing RSU 1069 induced DNA crosslinks. As expected because of the presence of hypoxic cells, the average tail moment for irradiated tumours was significantly lower than for other tissues. Tail moment for testis was intermediate between that of tumour and other normal tissues, indicating it has an intermediate degree of hypoxia (Fig. 16a). Analysis of individual tail moment histograms was also informative. Testicular cells did not show as low a tail moment as seen in tumours (Fig. 16b,d), although some of the cells had relatively low tail moments (fraction in the oval, Fig. 16b), suggesting they are probably more hypoxic than the remaining cells. Exposing these cells *in vitro* to 10 Gy irradiation shifted all the cells to higher tail moments in testis and other normal tissues, indicating that there were too few crosslinks in these cells to prevent DNA from migrating in the electric field. Only some of the tumour cells retained a very low tail moment after 10 Gy, indicative of the presence of crosslinks in hypoxic cells (Fig. 16e).

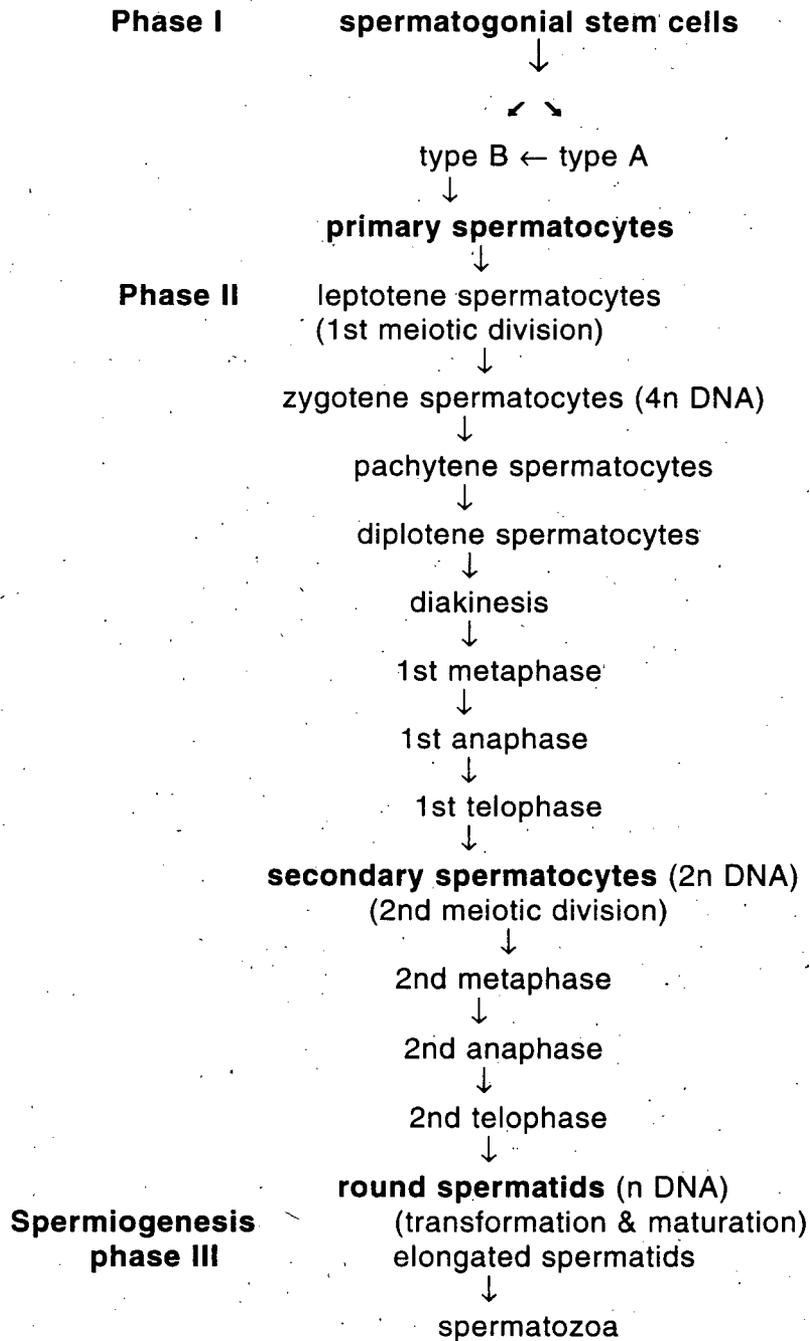
#### 4.1.2.3 Response of testis to radiation-induced DNA damage

Significant heterogeneity in radiation-induced SSB induction was observed among the different subpopulations of cells in testis. The pattern of cell development in this tissue is unique. As shown in the diagram in Fig. 32, spermatogonial cells undergo two mitoses and two meioses before the mature sperm are formed. All the cells develop in the seminiferous tubules and gradually move to the centre of the tubules as they mature. The developing sperm cells are nourished by surrounding Sertoli cells in the tubules. During cell development, DNA content increases from  $2n$  (primary spermatocytes) to  $4n$  after the first meiosis (tetraploid primary spermatocytes). At the end of the first meiotic division, secondary spermatocytes are formed containing  $2n$  DNA. They undergo a second meiosis to produce round spermatids with haploid ( $n$ ) DNA content. Then a massive condensation of the chromatin occurs when DNA is packaged into a small volume and transformed into an elongated spermatid. During the process of maturation and transformation, cytoplasm is partly shed and chromatin composition is extensively changed (Setchell 1978, Mezquita 1985). In the course of spermatogenesis, the amount of the testis-specific forms of histones TH2A, X2, TH2B, TH3 increases as the somatic forms decrease. Non-histone proteins decrease significantly but basic proteins change only slightly. At the end of spermatogenesis protamines are the only basic nuclear proteins in spermatids as well as in mature sperm (Mezquita 1985).

Testis cells can be grouped into three subpopulations according to their DNA content as determined using the comet assay. DNA content is defined as the total fluorescence of a comet image after staining with the DNA binding dye propidium iodide. The three populations should contain the following cells: 1) tetraploid cells comprised mainly of tetraploid spermatocytes and a small fraction of spermatogonia in  $G_2$  phase before mitosis; 2) diploid cells consisting of spermatogonia, primary and secondary spermatocytes, and a few supporting Sertoli cells as well as Leydig cells; and 3) haploid cells consisting of round to elongated spermatids. Germ cells later than this stage leave the testis and move into the epididymis, so spermatozoa will not be included in the samples analyzed using the comet assay. As seen in the bivariate plot for DNA content and tail moment, tetraploid cells showed the lowest amount of DNA damage after irradiation (Fig. 5). It is possible that this is an artifact of cell staining in the comet assay since a similar

Fig. 32 Diagram: stages of male germ cell differentiation

**Spermatogenesis**



pattern has been observed for cultured cells (Olive and Banath, 1993). Alternatively, the 4N cells may be more radioresistant than the haploid cells. The second explanation seems more likely since the difference in response of testis sub-populations was only observed among cells irradiated *in vivo*. When *in vitro* dose response was examined this difference disappeared (Fig. 6).

A possible explanation for the apparently greater resistance of the tetraploid cells to radiation damage is that they are more hypoxic. Support for this hypothesis was given when the difference between the sub-populations disappeared in mice killed before irradiation or when mice breathed carbogen before irradiation (Fig. 15a,c). The second indication that hypoxia was involved came from mice treated with nicotinamide before irradiation. As a radiosensitizer, this drug is believed to reduce tissue hypoxia and therefore enhance the effect of radiation (discussed in Section 4.4). Fig. 15d indicated that the difference in tail moment among the three cell populations disappeared after nicotinamide treatment. Tetraploid cells may be more hypoxic than diploid and haploid cells because they are rapidly proliferating, actively-metabolizing cells and need more energy to cope with physiological changes. The lower oxygenation of testis may barely provide adequate energy for such metabolically active cells. In diploid cells, some of the spermatogonial stem cells were also suggested to be hypoxic (Suzuki et al. 1977, Schlappack et al. 1987) although they were very low in number. As for the haploid cells, lower energy levels, and presumably lower respiratory rates may lead to better oxygenation. But for elongated spermatids, the chromatin is tightly packaged by histones and protamine which are crosslinked to each other by disulfide bridges formed by cysteine residuals and by phosphodiester chains (Balhorn 1982). Such a conformation makes DNA inaccessible to many intercalating agents such as actinomycin D (Gall and Ohsumi 1976). It was impossible to remove the nuclear protein in these cells using the normal alkaline comet assay lysis solution. Decondensation of these nuclei could be achieved by treating cells with the sulfhydryl reducing agents dithiothreitol or 2-mercaptoethanol (Gall and Ohsumi 1976). In my study, significant swelling of treated nuclei was observed under the microscope, but such treatment was not acceptable since it introduced a high level of SSB in untreated cells examined using the comet assay. Therefore, elongated spermatids remained unlysed in the regular comet assay and the results appear just as though no strand breaks were induced by radiation. Some previous studies suggested that these elongated spermatids demonstrate less

DNA damage after irradiation, and it was postulated that they are protected from attack by hydroxyl radicals by the highly compact nuclear structure (Ono and Okada 1976, 1977; Van Loon et al 1991, 1993). It was also suggested that these cells are protected by multiple cysteine residuals from protamines (Joshi et al. 1990). Cysteines are supposed to be hydrogen donors and able to protect DNA from radiation. But according to Balhorn's study (1982), most of the cysteines are used to form inter-molecular disulfide bonds and therefore not many hydrogen groups are available to act as hydrogen donors. If this is true for spermatids and haploid cells, no protection will be obtained from cysteine residues of protamines after histones are replaced. This conclusion is supported by the measurement of cellular thiol levels from haploid, diploid and tetraploid cells. No difference was observed using a flow cytometry technique and a fluorescent thiol probe (data not shown). Furthermore, according to the study of Deh Boer et al. (1989, 1990), thiol protection in spermatids is still primarily due to non-protein thiol GSH, the major thiol protector seen in other tissues.

Most of the previous published studies measured only the average damage for a population of cells instead of damage to individual cells, and the relatively high background damage from mechanical dissociation of tissues is likely to decrease the accuracy of these data. One paper previously analyzed damage to the testis using the single-cell gel electrophoresis assay, which differs from the comet assay primarily in terms of the lysis solution (Singh et al. 1989). In that study, the lysis solution contained a 10 fold higher concentration of NaOH and a higher concentration of NaCl (2.5 M, compared to 1 M in this study). Also, 2-mercaptoethanol was used in the lysis buffer. Results from that study showed that mouse epididymal and human sperm cells had a large amount of SSB after lysis which were not seen in lymphocytes after the same treatment. The suggestion that all testis cells have a high level of background damage was not confirmed in this study and could be due to the higher salt and alkali concentration which makes DNA molecules more vulnerable to breakage. Furthermore, the use of mercaptoethanol may change the DNA-protamine structure and expose alkali-sensitive sites to produce additional DNA breaks.

Results from this study are consistent with those of Joshi et al. (1990) which indicated that the slope of the dose response for SSB induction was similar from spermatogonia to round spermatids. A reduction in

damage was only seen in the late spermatid stage at the start of elongation. An advantage of their study was to radiolabel the cells in a way so that each stage could be differentiated at the time of irradiation. The same method has been used in many other studies which were designed to study cell survival instead of initial DNA damage, since surviving fraction and  $D_0$  value (the radiation dose required to kill 63% of the cells) are important in evaluating cell radiation sensitivity (Weichselbaum 1991). Some differences among these cells have been found: 1) the most radiosensitive cells are fetal spermatogonia and the sensitivity decreases in prepubertal and adult mouse (Vergouwen et al. 1994, 1995); 2) in adult mouse, spermatogonial stem cells remain the most sensitive among all the germ cells, but variations are found in different stages of the cell cycle: type B and proliferating type A spermatogonia are most sensitive.  $D_0$  values are 0.37-0.81 Gy for them but 2.29 Gy for quiescent spermatogonia A. The  $D_0$  value for cells at later stages is increased further to 5.3 Gy for preleptotene spermatocytes and even higher for the next generations (van der Meer et al. 1992). 3) elongating and elongated spermatids have reduced damage and therefore they are relatively resistant to radiation. But they also have a reduced capacity to repair DNA damage (Van Loon et al. 1993) probably due to inaccessibility of the condensed chromatin damage to repair enzymes and/or due to a deficiency in the DNA repair system. 4) the non-parenchymal cells, Sertoli and Leydig cells are minimally affected by radiation (Bustos-Obregon and Rodriguez 1991) probably because they are terminally differentiated. This pattern fully agrees with the previous description about the relationship between cell differentiation and radiosensitivity.

#### 4.2. The efficiency of DNA repair is tissue dependent

In comparing the response of different tissues, the rate of rejoining of SSB is more important in determining the overall effect of radiation than is a possible difference in the initial yield of lesions. Previous studies have suggested that there is a correlation between lethality and the level of residual unrepaired DSB (Peacock et al. 1989, Weichselbaum 1991). The level of such unrepaired breaks may be determined by the competition between repair and the fixation of these lesions. Consequently, the rate of DNA repair may represent an important factor in modifying lethality which is especially critical in radiosensitive cell types (George and Cramp 1988). It is very interesting that the degree of degradation observed 48 hours after 15 Gy in irradiated testis cells was apparently influenced by cell ploidy. The radioresistant haploid cells

showed a significantly smaller proportion of damaged cells than the diploid and tetraploid cells (Fig. 10) (see section 4.2.4).

#### 4.2.1 Some characteristics of rejoining kinetics

SSB rejoining kinetics is approximated reasonably well by a two component repair curve (Fig. 7). Due to the repair which unavoidably occurred during irradiation, the initial tail moment measured was only about 2/3 of that measured for cells irradiated *in vitro*. Without knowing the actual initial damage, rejoining half-times can only be approximated for comparison purposes.

The tissues studied can be classified into three groups: 1) rapidly proliferating and differentiating cells (bone marrow, spleen, testis, jejunum), and SCCVII tumour cells are also included in this group although the degree of differentiation is questionable; 2) reverting postmitotic and well-differentiated cells, capable of limited proliferation under the appropriate stimulus (liver); 3) terminally differentiated cells which are incapable of proliferation in any circumstance (most cerebellar and cerebral cells). The general pattern of rejoining for all these tissues is similar with two components, but important differences in rate of rejoining were observed. Tumour cells completed the rapid phase of repair in less than 30 minutes which was shorter than any normal tissue. With the exception of cerebellum and cerebrum, the normal tissues had a similar repair half-time (Table 3). However, cerebrum required 45 minutes to rejoin half of the SSB. All of the repair curves seemed to be complex and composed of at least two components. The majority of the SSB were rejoined by the fast component, but one hour after irradiation the tail moment was still high. The residual damage (calculated as the percentage of initial tail moment minus the value for control) at the end of 1 hour was 31%-37% in liver, marrow and spleen, 20%-21% for tumour and testis; but 58% for cerebellum (Fig. 8). The breaks remaining at this time would be rejoined by the "slow component". Therefore a larger fraction of the DNA damage in cerebellar cells would be repaired by the slow component. Wheeler and Wierowski (1983) also showed a biphasic pattern of DNA SSB rejoining in cerebellar cells and 9L tumour cells. They hypothesized that the different speeds of SSB rejoining represent the removal of lesions from regions of the genome that vary in their accessibility to repair enzymes. Alternatively, slower rejoining may represent repair of a different lesion, such as the DSB

(George and Cramp 1987). Furthermore, the slow component was found to be saturable both in neurons and tumour cells (Wheeler and Wierowski 1983); the half time for repair of this phase is dose dependent.

Rejoining kinetics of cultured cells generally indicates a two component-pattern (e.g. Epstein et al. 1974, Baumstark-Khan et al. 1992). However, this has not been observed for cultured cells using the comet assay where a pure exponential rejoining curve was observed for repair-proficient cells (Olive and Banath, 1993). Because cells are obtained from tissues using a harsh mincing and filtering method, it may not be surprising that there is a higher level of background and residual damage level *in vivo*. The fast component of repair for cultured cells appears to be much faster (3-5 minutes) than was observed in these studies. However, a strict comparison is not appropriate. Because of the uncertainties regarding the level of initial damage *in vivo*, repair half-time was calculated in an unusual manner for this study which considered both components of the repair curve as well as the background damage. The conclusion from these studies is that the overall process of DNA repair measured by the comet assay is slower *in vivo* than *in vitro*, a result consistent with previous research using different methods such as zonal rotor centrifugation, alkali unwinding or alkali elution.

The state of differentiation of a cell has been shown to influence DNA SSB rejoining rates. Murray and Meyn (1987) radiolabelled the epithelial cells of small intestine and found that crypt cells rejoined SSB the fastest, and that the repair rate decreased as cells matured. However, one cannot yet exclude the possibility that there is a reduced level of repair enzymes or a reduced activity of these enzymes. Liver, as a post-proliferative tissue, did not show a slow rate of SSB rejoining perhaps related to its special property of regeneration or high metabolic rate. The involvement of liver in drug metabolism may mean that it is more likely to have more active DNA repair mechanisms to handle toxic chemicals. Until now there has been no evidence of elevated activity or increased amount of repair enzymes in liver, although liver has very high levels of the radioprotective molecule, reduced glutathione (GSH) (Murray et al. 1984). In spite of high GSH however, liver did not show a reduction in initial SSB after irradiation. The two types of fibrosarcoma studied by Murray et al. (1984) repaired DNA damage faster than all of the normal tissues, in agreement with results from this study using SCCVII tumours. Therefore, the major determinants in rate

of DNA repair seem to be metabolic activity and degree of cell differentiation. Wheeler (1983) calculated that the neuronal genome has less accessible sites that are three times more prevalent than in the tumour genome. Assuming that residual damage at the end of one hour after irradiation is indicative of the accessibility of lesions for repair, then cerebellar cells had three times more residual damage than tumour cells (Fig. 8d). This explanation disregards any possible differences in DNA repair enzyme amount or activity which cannot be excluded at this point.

#### 4.2.2 The inhibitory effect of nicotinamide on DNA repair

DNA repair inhibitors are of considerable interest as possible modifiers of chemotherapy and radiotherapy of malignant tumours. In this study, the effect of nicotinamide on DNA SSB rejoining was examined. Initially, nicotinamide received attention as a radiomodifier because of its structural similarity to 3-aminobenzamide, a potent but toxic inhibitor of poly(ADP-ribose) polymerase (PARP). As a chromatin-bound enzyme, PARP can be activated by DNA strand breaks induced by various DNA damaging agents or physiological events such as DNA recombination or sister chromatin exchange (Berger 1985, Oleinick and Evans 1985). When activated by ionizing radiation-induced DNA strand breaks, the enzyme can recognize the damage by binding to these sites and at the same time catalyze the formation of poly(ADP-ribose), a negatively charged polymer that modifies DNA binding proteins and nuclear enzymes including PARP itself (Shall 1994). ADP-ribosylation of histones helps to unwind the DNA strands and exposes the damaged sites to repair enzymes. This modification is further believed to recruit repair enzyme(s) to the damaged sites although it may also inhibit these enzymes within the first few minutes (<0.5-1 minutes) after irradiation for reasons that are still unknown. It was found that DNA polymerase is inactivated at a lower degree of poly(ADP-ribosyl)ation while activated after a higher degree of modification (Shall 1994). PARP activity increases linearly with radiation dose (Ben-Hur and Elkind 1985). However, modification inactivates PARP itself and these polymers are immediately degraded, releasing PARP from the damaged sites before the repair process is initiated (Sato et al. 1994). Interestingly, in cultured Chinese hamster cells irradiated with 7.5 Gy, in addition to the short activation which lasts <10 minutes postirradiation, the enzyme activity was found to increase again 1-3 hours after irradiation (Oleinick and Evans 1985). The second peak of

activation is probably due to the DNA repair process involving secondary strand breaks introduced by repair of base damage and DNA recombination although no direct proof has been obtained.

Inhibition of the ADP-ribosylation reactions by nicotinamide and other agents has been observed in cultured cells (Yamamoto et al. 1982, Berger 1985). The degree of inhibition of DNA repair by nicotinamide that was observed *in vivo* is similar to the effect of other PARP inhibitors, although all those studies were performed using cultured cells. Addition of nicotinamide to irradiated cells is known to inhibit DNA strand break rejoining and increase cell killing (Ben-Hur and Elkind 1985) although it is less effective than 3-AB. The low toxicity of nicotinamide makes it more appealing as a radiosensitizer in the clinic, although in current Phase I and II studies, nicotinamide and carbogen have shown a dose-limiting ototoxicity (T. Pickles, personal communication). In this study doses of 200 mg/kg to 800 mg/kg of nicotinamide were given to mice in order to determine the effect of nicotinamide on rejoining of radiation-induced SSB *in vivo*. A dose of 200 mg/kg did not inhibit DNA repair, while a significant inhibition was observed when 800 mg/kg was given 60-90 minutes before irradiation. An intermediate degree of inhibition was shown by 500 mg/kg nicotinamide (Fig. 20). SSB rejoining time was increased in all tissues except brain. The half-time of rejoining appeared to increase most significantly in the SCCVII tumour, since the rejoining half-time increased by a factor of 7 if nicotinamide were given before irradiation, while it increased 3-4 fold in other tissues (Table 3). While this suggests a significant therapeutic gain, the influence of nicotinamide on tumour reoxygenation must also be considered (see Section 4.4). The only tissue unaffected by nicotinamide was cerebellum, perhaps due to the slow SSB rejoining already occurring in this tissue or to the fact that less of the drug reaches the brain because of the blood-brain barrier. <sup>14</sup>C-labelled nicotinamide and its metabolites were found to be at a much lower level in brain tissue than in liver, kidney and lymph nodes (Anderson et al. 1994).

The amount of damage remaining 4 hours postirradiation was similar with or without nicotinamide pretreatment suggesting that the rate of SSB rejoining, but not the extent of rejoining is reduced by nicotinamide. Results also indicate that nicotinamide only inhibits rejoining when given at high doses which are not clinically relevant. It is possible that rapid elimination of the drug *in vivo* prevents a sufficiently high

intracellular accumulation. Alternatively, PARP may not be a vital enzyme in DNA SSB rejoining, a likely explanation especially since transgenic mice lacking the PARP gene do survive to adulthood (Wang et al. 1995). These mice can even repair DNA damage induced by alkylating agents (base excision repair) and UV-light (nucleotide excision repair). It was postulated that the function of PARP has been replaced by other enzymes in these mice, although for the time being it is hard to examine the exact mechanism. However, PARP gene knock-out mice are prone to develop skin disease as they become older (>6 months). Skin is an organ exposed to environmental stress, and mice lacking PARP/poly(ADP-ribosylation) are not able to eliminate these damaged cells and are therefore susceptible to skin abnormalities as they age. However, DNA SSB rejoining, which involves a different repair pathway, has not been examined in these mice; the effect of nicotinamide on PARP- mice might be quite informative.

It was expected that nicotinamide would inhibit DNA repair during irradiation so that the tail moment measured immediately after irradiation would be increased. Surprisingly, tail moment in most of the tissues pretreated with nicotinamide was unchanged relative to radiation only. Another repair inhibitor, metoclopramide, which inhibits DNA polymerase (Olsson et al. 1995) gave a similar result (data not shown). It is likely that the tail moment is already sufficiently high after 15 Gy that it is difficult to see any additional damage. Another more interesting explanation is that only the slow component of rejoining may be affected by nicotinamide pretreatment. Unfortunately, without knowledge of the actual level of initial damage, it is not possible to distinguish between these two explanations.

#### 4.2.3 SSB repair is proficient in SCID mice

It was interesting that no significant difference was observed in SSB rejoining in tissues of irradiated SCID and C3H mice. There was no delay of repair, nor any indication of increased residual damage in SCID compared to the same tissue from C3H mice. It is known that SCID cells are deficient in DSB rejoining (Fulop and Phillips 1990, Beidermann et al. 1991), but apparently SCID cells are able to repair SSB and crosslinks (Hendrickson et al., 1991). SCID cells do rejoin some of the DSB induced by X-rays, and the percentage of unrejoined breaks increases with the radiation dose. Furthermore, the initial process of DSB rejoining was found to be similar to normal cells (Chang and Brown 1993). The mechanisms governing

repair of SSB and DSB obviously differ, and the fact that SCID cells are deficient in the p350 (p450) subunit of DNA-dependent protein kinase (Kirchgessner et al. 1995) indicates that this kinase is not required for SSB rejoining. Therefore, while DSB do constitute about 2-4% of the total SSB measured, the sensitivity of the method is unlikely to reveal this small number of unrepaired breaks against a background level of damage caused by the tissue disaggregation methods.

#### 4.2.4 Heterogeneity in DNA repair

Heterogeneity in DNA repair during the first four hours of repair was not readily apparent for any of the tissues studied. Previous experiments with cultured cells revealed a G<sub>1</sub> population which rejoined breaks more rapidly than cells in other phases of the cell cycle (Olive and Banath, 1993), but such a population was not observed in these studies. However, only a small population of G<sub>2</sub> cells was observed in bone marrow and jejunum, therefore it was not possible to detect differences in rejoining rate in relation to cell cycle position.

Although radiation-induced apoptosis occurred during the repair period after irradiation, these dying cells were not detected using the comet assay, probably due to their loss during tissue preparation (see Section 4.3). However, by 48 hours after 15 Gy, some heavily damaged cells could be detected in spleen and some other tissues (Fig. 9), indicating significant DNA degradation in these tissues. Histological studies revealed that most of these cells were necrotic. Some cells in spleen, however, did appear to have the properties of apoptotic cells. The peak time of appearance of these heavily damaged cells was 48 hours after 15 Gy irradiation, and damaged cells could be observed in almost all tissues except brain. All of the tissues, regardless of proliferation status or differentiation status, showed a pattern of degradation at the same time, as though the process resulting in this damage was a generalized tissue breakdown. Mice irradiated with 15 Gy would be expected to die within 4-5 days from damage to the gastrointestinal system (Hall 1978).

Again the response of the cerebellum differed from the other tissues in terms of the extent of degradation. Neurons are well-known to be radioresistant in spite of their slow repair of DNA damage. Hopewell (1983)

showed that 30-40 Gy was needed to produce selective white matter necrosis after a latent period of 8-9 months. The degree and the time of appearance of damage is dose dependent. Twenty Gy introduced only vascular changes in both grey and white matter after a much longer latent period. Doses lower than 20 Gy can cause permeability in the blood-brain barrier, but changes are not observable for at least 18 months (van der Kogel 1991). Two main pathways have been suggested for this pathogenesis, one involving predominantly the progressive loss of glial cells and the other involving vascular injury. The organ tolerance dose, which is determined by the most radiosensitive vital cells in that organ, was found to be 20-30 Gy for brain, 10-15 Gy for GI tract, 15-20 for liver, 2-10 Gy for bone marrow and >20 Gy for testis (Constine and Rubin 1988). These data provide a profile of the whole organ tolerance for the acute and late effects of whole body radiation. Since in this study heterogeneity in tissue damage was examined two days after irradiation, the damage observed should be classified as an acute effect. At this stage no visible degeneration of neurons should be expected. The only observable change at this time is probably the electrophysiological characteristics of neurons, examined by Pellmar and Lepinski on guinea pig brain 30 minutes to 3 days after 5-15 Gy (1993).

Each tissue is composed of many different types of cells and each cell type will exhibit a different radiosensitivity. As seen in Fig. 10, testis clearly demonstrated two populations of cells 48 hours after irradiation; one was heavily damaged and the other was apparently undamaged as measured using the comet assay. The LD<sub>50</sub> for proliferating spermatogonia is 20-23 cGy, and it increases to 200-500 cGy for primary spermatocytes, 15 Gy for spermatids and 600 Gy for spermatozoa (Searle 1974). Although initial DNA damage is relatively independent of cell type, the population of cells with a very low tail moment after 48 hours was, in fact, the late stage (haploid) germ cells (Fig. 10b). Some degree of heterogeneity in other tissues also existed at this time, probably representing different cell types varying in rate of cell death.

#### 4.3. Radiation-induced apoptosis is tissue specific

As discussed above, extensive DNA damage was observed 48 hours after irradiation in most tissues. Histology, DNA gel electrophoresis and size measurement using nitrogen mustard suggested that this DNA degeneration was not a result of apoptosis. A generalized systems failure could be responsible.

When heterogeneity of DNA repair was examined at earlier times after irradiation, no significant number of heavily damaged cells was seen. However, according to published studies, doses of 2.5 Gy-20 Gy should induce apoptosis in some normal tissues (Tsubouchi et al. 1974, Rydberg and Johanson 1975, Nomura et al. 1992). These authors were not able to detect DNA degeneration at the time of occurrence of apoptosis using a conventional method for detecting DNA strand breaks. A similar result occurred in this study. While the comet assay could easily detect apoptotic TK6 cells which are cultured *in vitro* (Olive et al. 1993a), *in vivo* apoptotic cells from tissues were undetectable. DNA gel electrophoresis, however, showed typical ladders of apoptosis measured at the same time (Fig. 17). The only explanation is that these apoptotic cells are fragile and they are easily damaged by the mechanical disaggregation method used to prepare cells for the comet assay. However, even fragile cells would be preserved as part of the entire tissue sample during preparation of DNA for gel electrophoresis.

DNA ladders were detectable 4 hours postirradiation. The spleen and thymus were the two major tissues displaying this phenomenon. Ladders were not observed for samples prepared from liver, testis and brain. Although previous studies also demonstrated apoptosis in the epithelial cells of small intestine (Rydberg and Johanson 1975), they represented a small fraction of total cells from the crypts. Since the epithelial layer of the jejunum was used for this study, the percentage of apoptotic cells was likely to be too low to demonstrate ladders. A similar reasoning applied to cells from bone marrow. Apparently only cells from bone marrow, lymphoblastic and rapidly proliferating tissues retain a strong capacity for radiation-induced apoptosis. Although apoptosis can be induced by many different triggers described already, for some cell types radiation-induced DNA strand breaks are the major trigger in p53-dependent apoptosis. An increase of p53 results when DNA strand breaks are induced (Nelson and Kastan 1994), and p53 has been found to play an important role in both cell cycle control and apoptosis (Wyllie 1993). Interference with cell cycle progression leading to apoptosis will obviously occur only in proliferating cells. In post-mitotic cells the level of p53 is probably low or undetectable such as seen in villous cells from small intestine (Merritt et al. 1994). Interestingly, germ cells from testis show less sign of apoptosis after whole body irradiation despite their rapid proliferation. The cells in this tissue reported to die after irradiation are spermatogonia especially type B cells, and spermatocytes at early stages. These cell types were found to be eliminated 3 days after 3-5

Gy irradiation (Setchell 1978, Van der Meer 1992). While the pattern of cell elimination is consistent with the importance of cell differentiation, the time of occurrence of cell death seems to be later than those of other tissues. As seen in Fig. 24, testis weight decreased more slowly than that of spleen. Only after 3 days did the decrease become significant in testes, but a decrease in weight was already observed one day after irradiation of spleen. The lack of a significant drop in testis weight one day after irradiation may not be surprising, since the sensitive cells constitute only a small fraction of entire germ cell population. Furthermore, cells further in differentiation than pachytene spermatocytes are known to be quite resistant to cell death. A further reduction in weight of spleen on the second day after irradiation indicated that cell death was still proceeding, whether by apoptosis or necrosis. However, the ladder pattern was not detectable 8 hours after irradiation; dead cells may be rapidly removed by macrophages. The fact that the mice received 15 Gy is also relevant since the occurrence of apoptosis or necrosis can be dependent on the radiation dose (Payne et al., 1992; Lennon et al., 1991).

Neurons in adult mice have lost the capacity to proliferate, consistent with the observation that radiation had a smaller effect on these cells. In fact, it is possible to induce apoptosis by radiation in the granular layer of cells in cerebellum, and in oligodendrocytes when they are still proliferating in young mice several days after birth (Vrdoljak et al. 1992, Fritsch et al. 1994). Once cells mature within two weeks after birth, radiation does not induce apoptosis. The molecular basis for this is believed to be *bcl-X<sub>L</sub>*, one member of the *bcl* gene family, which is highly expressed in these adult neurons as well as hepatocytes and can inhibit the pathway to apoptosis initiated by radiation and *p-53* (Alison and Sarraf 1995). However, in mature or post mitotic cells, apoptosis can still be induced by other agents presumably via different signal transduction pathways. Apoptosis can be induced in liver by several chemotherapeutic drugs, and villous cells from the small intestine can also be induced to undergo apoptosis (Potten et al. 1994). The mechanisms of cell damage induced by these chemicals are probably more complicated than just DNA strand breaks.

The lifetime of radiation-induced apoptotic cells *in vivo* is shorter than in culture. Although in this study no effort was made to observe the rate of disappearance of apoptotic cells, the time for detection of DNA ladders was considerably shorter than that of most cultured lymphoblast cells (Olive et al. 1993a).

According to the study of Nomura et al. (1992), the average duration of apoptotic bodies in spleen and thymus is 2 hours induced by 2.5-10 Gy, which is consistent with findings in this study. Furthermore, the induction seemed to be dose independent from 2 - 10 Gy of radiation. Although no signs of apoptosis were observed below 2 Gy, there was also no obvious increase in apoptosis as the dose increased from 2-10 Gy. This observation may simply indicate the insensitivity of the gel electrophoresis method which is not a quantitative assay for apoptosis.

Experiments with terminal deoxynucleotidyl transferase indicated that little end-labelling occurred in cells of testis either 4 hours after 10 Gy, or 24 hours after 15 Gy (Fig. 26). This result supports the gel electrophoresis and histological studies indicating that apoptotic cells are not produced in this tissue. However, in spleen, end-labelling was produced in a significant proportion of the irradiated cells. The discrimination between labelled and unlabelled cells was however more difficult in this tissue, indicating that the number of fragments per cell was smaller, and therefore presumably the size of DNA fragments was larger (Fig. 27). However, at 4 hours after 10 Gy, when ladders could be visualized using gel electrophoresis, some cells with smaller fragments were clearly present (Fig. 27c). The high background labelling seen in spleen (Fig. 27d) reduces the reliability of these data.

#### 4.4. Nicotinamide has multifunctions as a radiosensitizer *in vivo*

Nicotinamide was shown to inhibit SSB rejoining in murine tissues and SCCVII tumours. Previous results showed that the enhancement ratio (ER) for radiosensitization is higher in tumours than in normal tissues, suggesting that tumours are more responsive to nicotinamide than normal tissues. Using 1000 mg/kg of nicotinamide one hour before irradiation, Horsman (1989b) found the ER was 1.4-1.7 for EMT6 tumours, 1.5-1.6 for Lewis lung tumours and 1.2-1.6 for SCCVII tumours. But the ER for small intestine, skin and testis was only around 1.1-1.2. Similar results were also obtained by Kjellen et al. (1991) and Ono et al. (1993). Such a difference in ER is more likely caused by tissue oxygenation status rather than by inhibition of DNA repair, since these tumours are known to contain a high hypoxic fraction (Denekamp 1983, Olive and Durand 1992). Nicotinamide at 250 mg/kg had the same effect as 1000 mg/kg in increasing SCCVII tumour killing by radiation (Chaplin et al. 1993). However, lower doses of nicotinamide do not seem to

inhibit DNA repair (Fig. 20). It is important to note that inhibition of repair of SSB rejoining by nicotinamide is not the result of a subset of cells which do not rejoin damage. Using the comet assay, it was possible to conclude that all cells of the damaged tissues rejoin breaks more slowly after nicotinamide treatment.

Nicotinamide has been found to reduce the frequency of opening and closing of tumour blood vessels when given one hour prior to radiation (Chaplin et al. 1990, Hirst et al. 1995). This is believed to be caused by a reduced interstitial fluid pressure in tumours (Lee et al. 1992) secondary to the effect of reduced flow resistance and vessel constrictions after drug administration (Hirst et al. 1995). Increased blood flow and tumour perfusion have also been observed using laser Doppler flowmetry after 250-500 mg/kg of nicotinamide, and the increase was dose-dependent (Hill and Chaplin 1995). The increase in blood flow and reduction in arterial blood pressure begins 20 minutes after an injection of 500 mg/kg (Kelleher and Vaupel 1994b). The physiological basis for these changes is considered to include the effect of  $\text{NAD}^+$  in addition to the effects of nicotinamide itself.  $\text{NAD}^+$  is the major metabolite of nicotinamide and acts as the coenzyme for many important dehydrogenases. The resultant increase in lactate may also contribute to the increase in tumour blood flow.

In tumours, nicotinamide appears to act as a radiosensitizer primarily by improving tumour oxygenation. Although nicotinamide has consistently been shown to increase tumour blood flow, it is curious that conflicting results have been observed concerning the improvement of oxygenation status measured using oxygen electrodes (Kelleher and Vaupel 1994b, Lee and Song 1992). This difference may be caused by the insensitivity of the oxygen electrode to small changes in oxygen tension, or by changes in the oxygen consumption by the tumour cells. In the latter case, the increase in oxygen tension will not be detected when the supply is below a saturation level. But in Lee and Song's study, both of the murine tumours and three xenografted human tumours showed a significant increase in oxygen tension after nicotinamide treatment. One suggestion for the inability of some investigators to observe increases in tumour oxygen tension is that nicotinamide induces changes at the microregional level which cannot be detected using the oxygen electrode. Because the comet assay detects the effect of hypoxia in individual cells, microregional changes can be readily detected. DNA damage histograms on Fig. 19 clearly show that the fraction of

hypoxic cells was reduced in the tumours pretreated with nicotinamide. The hypoxic fraction fell from 18.4% for control to 4.4% in tumours injected with 200 mg/kg nicotinamide prior to radiation, and was further decreased to 2.4% in mice treated with 800 mg/kg (Table 4). In fact, reoxygenation by 200 mg/kg was almost as large as that produced by 800 mg/kg.

As for the case of testes, it seems that the whole cell population had an increased level of oxygenation after nicotinamide administration. Results in Fig. 15 and 16 showed no hypoxic fraction comparable to that seen in SCCVII tumours. This effect was probably due to the increased blood flow and therefore the increased oxygen supply to testis indicated by nicotinamide.

In the study by Price et al. (1995), an increase in oxygen tension was seen in cells intermediate in hypoxia but no alleviation was seen in the severely hypoxic cells ( $pO_2 < 2$  mmHg). Cell survival studies using SCCVII tumours also support this result since those cells closest to the blood vessels showed the greatest increase in radiation sensitivity following nicotinamide (Fig. 31). This may actually reflect the mechanism of drug action since severely hypoxic cells are more likely to be chronically hypoxic cells distant from blood vessels. These cells may still remain hypoxic even if blood flow improves and interstitial fluid pressure declines if they remain beyond the limit for oxygen diffusion. It has been suggested that chronic hypoxia can be alleviated by supplying oxygen gas at high tension to increase oxygen diffusion to regions distant from the blood supply. Nicotinamide appears to reduce perfusion-limited (acute) hypoxia so that the combination of the two has been proposed to overcome the two types of hypoxia in treatment of tumours - ARCON (accelerated radiotherapy with carbogen and nicotinamide) (Rojas, 1992). But in the SCCVII tumour studied here, this combination did not reduce the hypoxic fraction of below the value obtained when 800 mg/kg nicotinamide was used alone (Table 4). The significant benefit from nicotinamide alone may have prevented detection of any additional effect of carbogen. The SCCVII tumour is known to contain a significant proportion of acutely hypoxic tumour cells (Chaplin et al., 1987). Some fluctuations may still remain in some of the blood vessels following nicotinamide, e.g. 2% as detected by Chaplin et al. in a fibrosarcoma (1990). While carbogen may not enhance the oxygenation status when higher doses of nicotinamide (such as 800 mg/kg) are given as seen in this study, it was found to be effective with the

combination of lower doses (100-200 mg/kg) of nicotinamide: a further decrease in surviving fraction and increase in micronucleus frequency were obtained (Ono et al.1993). The combination with 500 mg/kg also showed an increase in tumour control when oxygen tension was increased from 21% to 95% (Kjellen et al. 1991). A plateau for radiosensitization was found when nicotinamide dose was increased further, which evidently supports the above explanation.

When the initial level of DNA damage is examined immediately after irradiation, nicotinamide appears to be most effective in enhancing damage in tumour and in testis, the two hypoxic tissues. If oxygenation in these tissues is improved by nicotinamide, DNA damage by radiation must increase. But when DNA repair inhibition is considered, tumours also show the most inhibition, suggesting that the therapeutic gain, related to DNA repair, is also enhanced in tumour. Radiation damage to tumours is therefore enhanced in two ways: reoxygenation and inhibition of repair. For the SCCVII tumour, the former appears to be most important at clinically relevant doses. Results shown in Fig. 31 indicate that nicotinamide does not enhance radiation-induced cell killing in SCCVII tumours asphyxiated prior to irradiation. This result suggests that the primary effect of nicotinamide on the SCCVII tumour is reoxygenation, not inhibition of repair. However, it should be remembered that the *in vitro* clonogenicity assay used to measure tumour cell survival requires excision of the tumour immediately after irradiation, and this procedure may not have allowed sufficient time for the full benefit of nicotinamide in inhibiting DNA repair.

In tissues treated with nicotinamide prior to irradiation, the dramatic degree of DNA degradation which occurred in cells of all tissues 24 hours after treatment was an unexpected observation. A similar pattern of degradation was found in tissues exposed to X-rays only (Fig. 9, 21), although in this case, damaged cells appeared between 48 and 72 hours after irradiation (Fig. 23). Apparently nicotinamide was able to accelerate radiation-induced cell death in all tissues, with the possible exception of brain. The lifetime of these damaged cells was about 24 hours. Their appearance in the comet assay was similar to that induced by radiation alone. Furthermore, the number of degenerating cells in a tissue and therefore the degree of elevation in tail moment for the entire cell population increased with both radiation dose and nicotinamide dose. Even at clinically relevant doses - 2 Gy X-rays and 200 mg/Kg nicotinamide - there was

still an observable effect (Fig. 25). Histology showed foci of degenerating cells in the white pulp of spleen, and some cells displayed signs of apoptosis with condensed chromatin and shrunken cell bodies (Fig. 28). Interestingly, gel electrophoresis did not indicate nucleosomal ladders at this time, although ladders were visible in gels 4 hours after irradiation when apoptosis could usually be detected in spleens treated with radiation alone. However, apoptotic cells at 24 hours postirradiation may have represented only a small proportion of dying cells. The TdT assay showed that a higher degree of label was seen in cells irradiated 4 hours earlier than that for 24 hours in spleen (Fig. 27). No obvious morphological changes typical of apoptosis or necrosis were observed in tissue sections from testis (Fig. 29), in spite of the presence of extensive DNA damage in a significant proportion of the cells. Apparently DNA degradation in testis is an early event, while gross cell degradation occurs later. Certainly cell death in testis was slower than that in spleen, as measured by decreases in organ weight (Fig. 24). Organ weight loss after radiation alone compared to radiation plus nicotinamide was not significantly different in testis, but was in spleen. It is worth noting that the use of enzymes to disaggregate tumours will probably ensure that these degrading cells are lost from analysis and would therefore not contribute to cell survival results from a tumour excision assay.

The alkaline comet assay has a working range from about 50 breaks per cell to about 15,000 breaks per cell. Fragmentation of DNA to smaller sizes will not be appreciated, so that the heavily damaged cells observed in tissues 24 hours after radiation and nicotinamide treatment may be fragmented far beyond 15,000 SSB per cell (equivalent to about 400 kb). Knowing the size of DNA fragments in dying cells can help to distinguish apoptotic cells from necrotic cells (Olive and Banath, 1995b). DNA from most apoptotic cells is fragmented to an initial size of 50-300 kb and eventually reaches sizes of 200 bp in many cell lines. The size of DNA fragments in necrotic cells is often much larger. A method has been developed to size fragments in individual apoptotic or necrotic cells by determining the amount of a crosslinking agent, such as nitrogen mustard, which is needed to reduce the tail moment to some specified (but arbitrary) level (Olive and Banath, 1995b). By generating a calibration curve for ionizing radiation, where the number of breaks induced per Gy is known, it is possible to convert the dose of the crosslinking agent needed to inhibit migration in the damaged population to an average DNA fragment size (Fig. 30). This experiment, when performed with testis cells obtained 24 hours after nicotinamide and radiation, gave a fairly large

fragment size, 135 kb, that could indicate that the damage to these cells was more likely to be the result of early necrosis rather than apoptosis.

The 3'-OH end-labelling assay when applied to these cells confirmed the absence of small DNA fragments in heavily damaged cells. Although many cells from spleen were labelled, they were not clearly separated from the rest of the cells, an indication that the DNA fragment size was large. Furthermore, few positive testicular cells were observed in this assay, indicating the absence of small fragments with 3'-OH ends which are characteristic of apoptotic cells. DNA fragments from cells dying by necrosis often do not label efficiently using this nick-translation method (Gorczyca et al. 1993b).

DNA degradation observed in murine tissues and SCCVII tumours 24 hours after treatment with nicotinamide and radiation seems to be related to inhibition of DNA repair by nicotinamide. This conclusion comes from analysis of the data in Fig. 20 and Fig. 22k. When nicotinamide was given after irradiation, heavily damaged cells were not observed indicating that nicotinamide must be present in the cells before irradiation to have this effect. Furthermore, no enhanced cell death in brain was observed, just as no DNA repair inhibition was observed in this tissue. Perhaps the SSB rejoining which occurs in the presence of nicotinamide is more error-prone and is therefore responsible for the earlier appearance of cells with heavily fragmented DNA.

The underlying mechanisms for the observed damage by nicotinamide plus radiation are still unclear, but are likely to be related to the effect of nicotinamide on the metabolism of poly(ADP-ribose). Synthesis of the poly(ADP-ribose) polymer by PARP will deplete cellular NAD<sup>+</sup> pools and consequently lower ATP levels due to the synthesis of new NAD<sup>+</sup> molecules. If the energy deprivation is severe, cells may not be able to cope with the stress and die (Berger 1985). But conflicting results have been reported by different authors. Depending on the situation and cell type, nicotinamide may inhibit (Manome et al. 1993), promote (Denisenko et al. 1989), or have no effect on apoptosis (Watson et al. 1995). PARP is also known to be inactivated as an early step in apoptosis in some cell types (Nicholson et al. 1995). *In vivo*, tissue NAD<sup>+</sup> level and ATP/Pi or ATP/ADP+AMP ratio were found to increase after nicotinamide injection, but this does

not seem to protect cells from damage since inhibition of PARP abrogates normal repair process. Therefore, all one can conclude is that inhibition of repair of DNA damage is possibly lethal to some cells although these cells do not necessarily die by apoptosis.

Results also indicate that the degradation seen 24 hours following nicotinamide and radiation *in vivo* does not occur *in vitro*. The same treatment of cultured V79 and EMT6 cells did not induce DNA degradation 24 hours after irradiation, although longer times were not examined. This suggests that during the first 24 hours after nicotinamide and irradiation, cells *in vivo* faced different challenges than those *in vitro*. Pharmacokinetic studies showed that the NAD<sup>+</sup> elevation in animal tissues and tumours can last as long as 16 hours after a dose as low as 100 mg/kg nicotinamide. This situation may not occur *in vitro* since nicotinamide was removed 1 hour after radiation to mimic the *in vivo* drug half-life. NAD<sup>+</sup> molecules may take part in cell metabolism via acting as a coenzyme for more than one hundred dehydrogenases. Therefore, abnormal metabolism may promote the toxic effects of radiation producing the earlier appearance of degenerating cells. This would not explain, however, why no heavily damaged cells were observed 24 hours later in tissues given nicotinamide *after* irradiation. Somehow, the accelerated cell death must be related to inhibition of SSB rejoining. In tumour cells, the degree of DNA degradation did not appear to be as high as in other normal tissues (Fig. 25), possibly due to the rapid "normal" turnover of cells bordering necrosis in solid tumours (cell loss rates of up to 90% are not uncommon in many solid tumours). Consistent with this hypothesis, cell survival results indicate that it is the tumour cells in better vascularized areas which are primarily sensitized by nicotinamide (Fig. 31a).

## 5. CONCLUSIONS

The comet assay allows measurement of DNA damage and repair at the level of individual cells. This property can be very valuable in understanding the radiation response of complex normal tissues which consist of several different cell types. In this study, six normal tissues of C3H mice and SCCVII tumours were studied for their response to ionizing radiation as measured using the comet assay. To return to the initial questions and hypotheses in Section. 1.1, some answers can now be provided. In agreement with previous studies, the number of SSB induced in cells from various tissues irradiated *in vitro* was found to be independent of both cell type and state of cell differentiation. The only exception was elongated spermatids which could not be adequately evaluated with the comet assay. However, for tissues from mice irradiated *in vivo*, important differences in the dose-response relationships were observed. Because of the repair which unavoidably occurs during irradiation, the level of DNA SSB per Gy produced *in vivo* was always lower than SSB produced *in vitro*. Differences between the *in vitro* and *in vivo* dose-response curves were primarily the result of differences in tissue oxygenation and repair capacity. While a separate hypoxic fraction was not present in testis as it was in tumours, several results indicate that testis cells are intermediate in oxygenation with the tetraploid cells of the testis appearing more hypoxic than other cell types. The first two working hypothesis have therefore been confirmed by these results, since heterogeneity in initial SSB induction was mainly found in tumour and testis and was the result of tissue hypoxia.

As proposed in initial hypothesis #3, SSB rejoining capacity appeared to be influenced by the state of cell differentiation, a conclusion which is consistent with previous studies. Terminally differentiated neurons were slowest to rejoin SSB, although reduced repair enzyme activity in these cells cannot be excluded as a factor contributing to slow repair. Conversely, rapidly proliferating tumour cells rejoined breaks faster than most normal tissues. Radiation-induced apoptosis may also be influenced to some extent by the state of cell differentiation since the occurrence of apoptosis also appeared to be consistent with differentiation status. Furthermore, apoptosis appeared to occur to a maximum extent at a time when most of the SSB had been rejoined (e.g., 4-8 hours after irradiation). Cells are destined to die if they can not repair radiation damage, but they must also retain the signalling pathways leading to apoptosis.

Nicotinamide administration led to reoxygenation of hypoxic cells of SCCVII murine tumours, even when administered at relatively low drug doses. Nicotinamide also promoted reoxygenation of testis cells confirming studies suggesting that blood flow may be inadequate in this tissue. An observation made previously using cultured cells was confirmed to occur *in vivo*: nicotinamide at doses of 500 mg/kg or greater significantly inhibited SSB rejoining in tumours and all normal tissues with the exception of brain. In addition, administration of nicotinamide led to an apparent acceleration of radiation-induced DNA degradation, an effect which was dependent on dose of both X-rays and nicotinamide. Interestingly, nicotinamide given after irradiation did not promote cell death suggesting a link between inhibition of SSB rejoining and appearance of extensively damaged cells. Histology, nick-translation and gel electrophoresis experiments support the conclusion that necrosis, not apoptosis, is occurring after 24 hours as a result of radiation damage to these tissues. In spite of these dramatic effects on DNA repair and degradation, clonogenic studies in SCCVII tumours indicate that the ability of nicotinamide to promote reoxygenation of tumours is still the dominant mechanism of radiosensitization by this drug.

Further studies are needed to clarify the importance of PARP in the effects of nicotinamide *in vivo*. To determine whether inhibition of SSB rejoining can actually promote cell killing, other inhibitors of different repair enzymes should also be studied. It is clear from the clonogenicity results for SCCVII tumors that applying a functional assay of cell response is critical since inhibition of SSB rejoining does not necessarily equate with an increase in cell killing. Experiments similar to those performed here, but using the PARP knockout mice, could be most informative. Moreover, while ionizing radiation has been shown in this study to produce a relatively homogeneous response in all cells of complex tissues, both in terms of initial SSB and rejoining of SSB, a similar pattern of damage is not likely to occur with other genotoxic agents where questions of drug delivery, activation and detoxification become relevant.

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