EFFECTS OF IONIZING RADIATION

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NOTE

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ANNEX C

Non-targeted and delayed effects of exposure to ionizing radiation

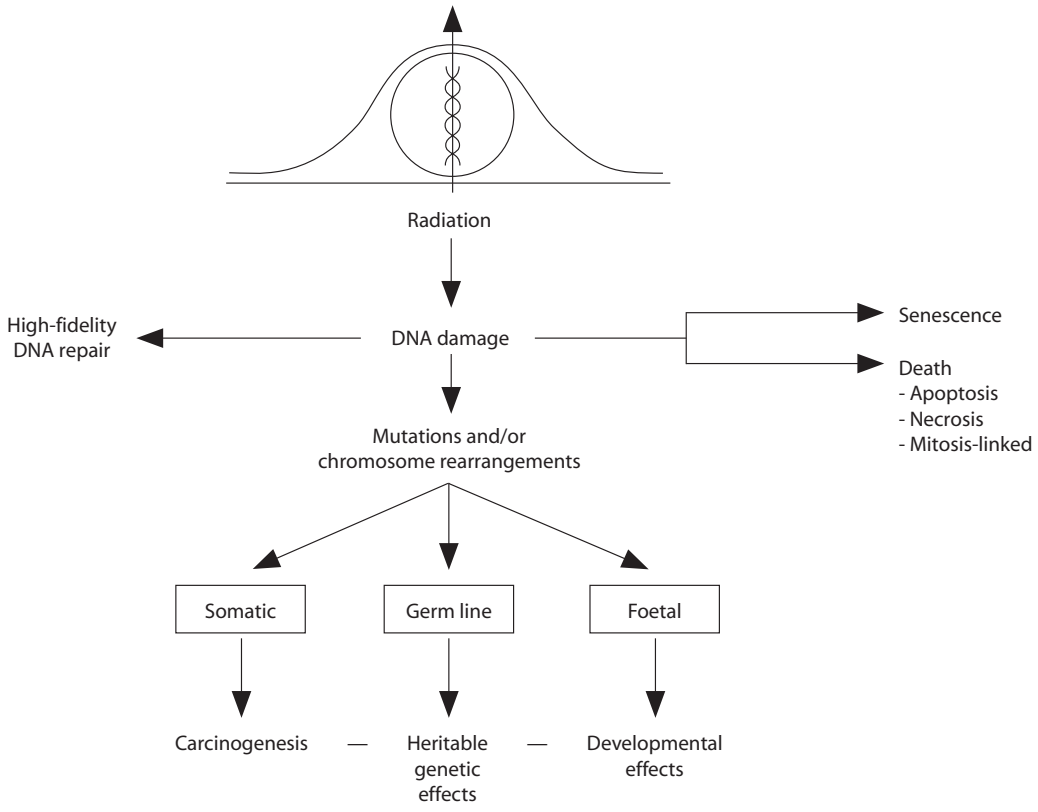
Contents

INTRODUCTION

1. The goal of this annex is to summarize the evidence for non-targeted and delayed effects of exposure to ionizing radiation in vitro and in vivo. Currently, human health risk estimates for effects associated with radiation exposures are based primarily on the view that the detrimental effects of irradiation occur only in irradiated cells. Over the years, a number of non-targeted effects of radiation exposure have been described that challenge this concept. These non-targeted effects include genomic instability occurring in the progeny of an irradiated cell, bystander effects, clastogenic factors produced in plasma from irradiated individuals that can cause chromosome damage when cultured with non-irradiated cells, and heritable effects of parental irradiation that can manifest across generations. This annex considers whether these effects pose new challenges to evaluating risks associated with radiation exposure, understanding radiation-induced carcinogenesis and interpreting epidemiological data on radiation exposure.

2. A central tenet in the radiation sciences has been that the energy from radiation must be deposited in the cell nucleus to elicit a mutagenic and/or clastogenic effect and thus be relevant for its potential to cause damage (figure I). It is implicit in this tenet that the biological consequences of cellular irradiation affect only the irradiated cell and that non-irradiated cells do not share the legacy of the radiation exposure.

Figure I. Prevailing paradigm for the biological effects of cellular exposure to ionizing radiation.
Ionizing radiation deposits energy in the nucleus of the cell. DNA damage is induced, and cellular responses to that damage can affect the fate of the irradiated cell. The damage can be removed and the genetic material restored by high-fidelity DNA repair. DNA repair systems may also eliminate the damage, but error-prone processing can result in gene mutations and clastogenic effects leading to chromosomal rearrangements. Depending upon the cell type, various cellular processes may be initiated that result in carcinogenesis in somatic cells, heritable genetic effects in germ line cells and developmental defects in foetal cells which may or may not be derived from mutational or clastogenic effects. DNA damage might activate cell cycle checkpoint control and cause the damaged cell to go into a protracted senescent state. Alternatively, if the damage is substantial, cell death may occur via a number of cellular pathways.



3. When ionizing radiation is absorbed in biological material, excitations and ionizations occur that are non-randomly distributed along localized tracks. The spatial distribution of these ionization/excitation events produced by different particles varies considerably depending on the quality of radiation. The term "linear energy transfer" (LET) is used to classify radiation quality according to the average energy transferred per unit length of the track. For the purposes of this annex, X- and gamma rays are considered to be low-LET radiation, protons and neutrons are considered to be intermediate LET radiation, and alpha particles and heavy ions are considered to be high-LET radiation.

4. In contrast to the risks associated with exposures to low doses of ionizing radiation (less than about 200 mSv, UNSCEAR 2000 Report [U2]), the risks of cancer after high and moderate doses of radiation are relatively well understood. This understanding is based on data from detailed epidemiological studies of the survivors of the atomic bombings in Japan and other exposed groups, e.g. clinically irradiated populations and those exposed as a result of the Chernobyl accident (UNSCEAR 2000 Report, annex I). However, risks at low doses are generally extrapolated from the high-dose data, applying dose and dose-rate effectiveness factors. Estimating risk is further complicated because environmental exposures are predominantly protracted, low-dose, low-dose-rate exposures, or high-dose-rate exposures delivered in small fractions (see annex A, "Epidemiological studies of radiation and cancer"). This contrasts with the majority of laboratory studies and clinical exposure situations, where exposures are usually acute, high-dose, high-dose-rate exposures. In addition, inherent in many models of radiation risk is that only those cells or tissues actually irradiated are burdened by the legacy of the radiation exposure. A number of non-targeted delayed effects of radiation exposure have been described; the purpose of this annex is to summarize the evidence for these effects and indicate present hypotheses on how they may affect the assessment of health hazards associated with radiation exposure and radiation-induced carcinogenesis.

5. For the purposes of this annex, "non-targeted effects" refers to radiation-induced effects manifesting in cells whose nucleus was not subject to a direct hit by the radiation, i.e. no ionization events due to cellular irradiation were deposited within the volume of that nucleus. In such instances the radiation may have hit the cytoplasm, or neighbouring cells, tissues or organs, or even cells in another culture vessel, and a response is communicated from these irradiated cells to non-irradiated cells to elicit an effect. It must be stressed at this stage that the non-targeted effects of ionizing radiation described in this annex do not imply that the well-documented targeted effects of radiation are irrelevant or unimportant, or that the concept of "dose" needs to be revised. That is not the case. Rather, the goal of this annex is to summarize the literature on non-targeted effects associated with exposure to ionizing radiation and, where possible, to evaluate how such effects may affect risks associated with radiation exposure, the understanding of radiation-induced carcinogenesis, and the mechanistic basis for interpreting epidemiological data on radiation effects.

I. RADIATION-INDUCED GENOMIC INSTABILITY

A. Radiation-induced genomic instability in vitro

6. Genomic instability is an all-embracing term to describe the increased rate of acquisition of alterations in the genome. As compared with the direct effects of radiation, i.e. those effects directly induced as a consequence of energy deposition, radiation-induced instability is observed in cells at delayed times after irradiation and manifests in the progeny of exposed cells multiple generations after the initial insult (figure II). Instability is measured as chromosomal alterations, changes in ploidy, micronucleus formation, gene mutations and amplifications, mini- and microsatellite (short tandem repeat) instabilities and/or decreased plating efficiency (summarized in table 1), and has been the subject of a number of reviews [K17, L14, M11, M12, M14, M48, W12]. These observed delayed effects can persist in unstable clones over time, and in some instances mimic those effects seen in tumour cells. There are likely to be multiple pathways for initiating and perpetuating induced instability [K8, L11], and the relative contributions of the different pathways involved probably depend on the genetic background of the target cell or organism [P3, W1] and on environmental factors (reviewed in references [K4, M32, M33]).

Figure II. Radiation-induced genomic instability.

A single cell survives irradiation and is clonally expanded. During clonal expansion, a number of the progeny of that irradiated cell die (through lethal mutations or delayed reproductive cell death), which results in a persistently reduced plating efficiency in this clone. Alternatively, or as a consequence of the presence of these dead and dying cells, instability events can occur in the progeny of the irradiated cell. These may result in chromosomal rearrangements, aberrations or gaps, micronuclei, mutations, gene amplifications and/or a failure of the cells to correctly separate their chromosomes at mitosis, resulting in aneuploid cells.

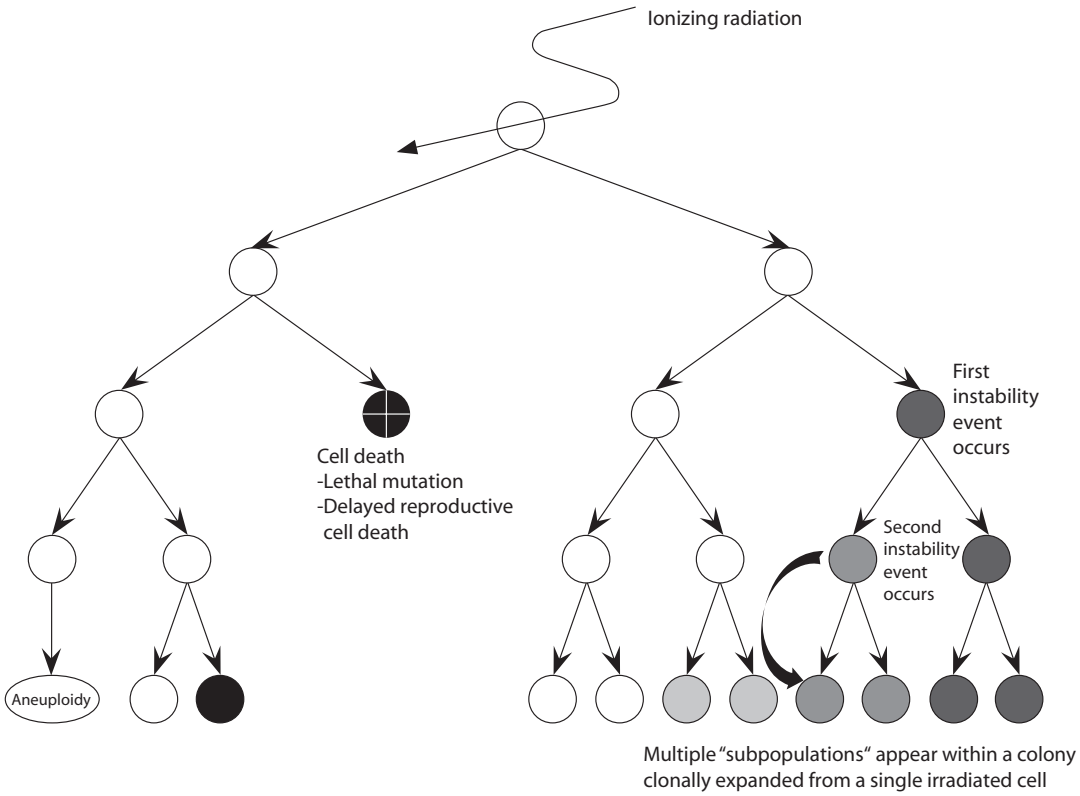


Table 1 In vitro studies of radiation-induced genomic instability

Year	*End point*	*Cell type*	*Radiation type*[a]	*Comments*	*Reference*
1991	Plating efficiency	Chinese hamster ovary cells	X-rays		[C13]
1992	Chromosomal aberrations	Murine haemopoietic stem cells	Alpha particles		[K3]
1992	Mutation frequency	Chinese hamster ovary cells	X-rays		[C12]
1992	Plating efficiency	Chinese hamster ovary cells	X-rays		[C12]
1993	Chromosomal instability	Human skin fibroblasts	Heavy ions: neon, argon	Dose response	[M30]
1993	Chromosomal aberrations	Human lymphocytes	X-rays		[H7]
1993	Chromosomal aberrations	GM10115 human–hamster hybrid cells	X-rays		[M2]
1993	Neoplastic transformation	HeLa × skin fibroblast human hybrid cells	Gamma radiation		[M31]
1993	Plating efficiency	HeLa cells	X-rays	Dose response	[F8]
1993	Plating efficiency	GM10115 human–hamster hybrid cells	X-rays		[M2]
1994	Chromosomal aberrations	HeLa cells	X-rays		[B20]
1994	Chromosomal aberrations	Human haemopoietic stem cells	Alpha particles		[K1]
1994	Giant cell formation	HeLa cells	X-rays		[B20]
1994	Plating efficiency	HeLa cells	X-rays		[B20]
1994	Plating efficiency	Human keratinocyte cells	Gamma radiation; alpha particles		[O5]
1995	Chromosomal aberrations	Human lymphocytes	X-rays		[H8]
1995	Chromosomal aberrations	Murine haemopoietic stem cells	Alpha particles; X-rays		[K2]
1995	Delayed TP53 mutation	Murine epithelial cells	Gamma rays		[S11]
1995	Plating efficiency	Murine haemopoietic stem cells	Alpha particles; X-rays		[K2]
1996	Apoptosis	V79 Chinese hamster ovary cells	X-rays (1–12 Gy)	Dose response up to 3–4 Gy	[J5]
1996	Apoptosis	Human keratinocyte cells; CHOK hamster cells	Gamma rays	Dose response	[L34]
1996	Chromosomal aberrations	Human epithelial cells	X-rays; alpha particles		[D18]
1996	Chromosomal aberrations	TK6 human lymphoblasts	X-rays		[G8]
1996	Chromosomal aberrations	V79 Chinese hamster ovary cells	X-rays (1–12 Gy)	Dose response up to 3–4 Gy	[J5]
1996	Chromosomal aberrations	Human lymphocytes	Alpha particles		[K5]
1996	Micronucleus frequency	V79 Chinese hamster ovary cells	X-rays (1–12 Gy)	Dose response up to 5 Gy	[J5]
1996	Morphological abnormalities	Human keratinocyte cells; CHOK hamster cells	Gamma rays	Dose response	[L34]
1996	Mutation frequency	TK6 human lymphoblastoid cells	X-rays		[G8]
1996	Plating efficiency	Human epithelial cells	X-rays; alpha particles		[D18]
1997	Chromosomal aberrations	GM10115 human–hamster hybrid cells	X-rays; gamma rays; ^{56}Fe ions; neutrons; gold ions	Dose response	[L30, L31]
1997	Chromosomal aberrations	V79 Chinese hamster ovary cells	Alpha particles; X-rays	Dose response	[M28]
1997	Chromosomal aberrations	Murine haemopoietic stem cells	Gamma rays		[P9, P15]

Year	*End point*	*Cell type*	*Radiation type*[a]	*Comments*	*Reference*
1997	Micronucleus frequency	V79 Chinese hamster cells	Alpha particles; X-rays	Dose response	[M28]
1997	Mutation frequency	Murine haemopoietic stem cells	X-rays; alpha particles; neutrons		[H12]
1997	Mutation frequency	Chinese hamster ovary cells	X-rays; alpha particles		[L29]
1997	Plating efficiency	V79 Chinese hamster cells	Alpha particles; X-rays	Dose response	[M28]
1998	Chromosomal aberrations	Human fibroblasts	X-rays; neutrons; alpha particles		[K18]
1998	Chromosomal aberrations	Human epithelial cells	Gamma rays; neutrons		[U20]
1998	Chromosomal aberrations	GM10115 human–hamster hybrid cells	X-rays		[D1]
1998	Chromosomal aberrations	Human lymphocytes	Gamma rays		[H9]
1998	Chromosomal aberrations	Murine haemopoietic stem cells	Alpha particles; X-rays		[K18]
1998	Chromosomal aberrations	GM10115 human–hamster hybrid cells	^{125}I		[K9]
1998	Chromosomal aberrations	Human lymphocytes	Gamma rays		[L32]
1998	Chromosomal aberrations	GM10115 human–hamster hybrid cells	X-rays; gamma rays; ^{56}Fe ions; neutrons; gold ions	Dose response	[L9]
1998	Chromosomal aberrations	Murine haemopoietic stem cells	Alpha particles		[L20]
1998	Chromosomal aberrations	GM10115 human–hamster hybrid cells	X-rays; gamma rays; ^{56}Fe ions; neutrons; gold ions	Dose response	[P14]
1998	Chromosomal aberrations	V79 Chinese hamster ovary cells	X-rays		[T3]
1998	Plating efficiency	GM10115 human–hamster hybrid cells	X-rays		[D1]
1998	Plating efficiency	Murine haemopoietic stem cells	Alpha particles; X-rays		[K18]
1998	Plating efficiency	Murine haemopoietic stem cells	Alpha particles		[L24]
1999	Apoptosis; micronucleus frequency	AGO I522B primary human fibroblasts	X-rays; alpha particles	Dose response	[B22]
1999	Chromosomal aberrations	GM10115 human–hamster hybrid cells	X-rays; gamma rays; ^{56}Fe ions; neutrons; gold ions	Dose response	[L6]
1999	Micronucleus frequency	Human SCL-II squamous carcinoma cells	X-rays	Dose response	[K20]
1999	Plating efficiency	AGO 1522B primary human fibroblasts	X-rays; alpha particles	Dose response	[B22]
1999	Plating efficiency	Human keratinocyte cells	Gamma rays; alpha particles		[C14]
1999	Plating efficiency	Human SCL-II squamous carcinoma cells	X-rays	Dose response	[K20]
2000	Chromosomal aberrations	HPV-G and HaCaT human keratinocyte cells	Gamma rays; alpha particles		[M29]
2000	Chromosomal rearrangements	HF19 human fibroblasts		No instability observed	[G25]
2000	Chromosomal aberrations	Human lymphocytes	Alpha particles; X-rays		[A6]
2000	Chromosomal aberrations	GM10115 human–hamster hybrid cells	X-rays; gamma rays; ^{56}Fe ions; neutrons; gold ions	Dose response	[L12]
2000	Plating efficiency	HPV-G and HaCaT human keratinocyte cells	Gamma radiation; alpha particles		[M29]
2001	Apoptosis	WTKI human lymphoblastoid cells	^{56}Fe ions; ^{137}Cs gamma rays		[S8]
2001	Chromosomal aberrations	TK6 human lymphoblasts	^{56}Fe ions; gamma rays		[E14]
2001	Chromosomal aberrations	Human lymphocytes	Gamma rays		[B11]

Year	*End point*	*Cell type*	*Radiation type*[a]	*Comments*	*Reference*
2001	Chromosomal aberrations	Human lymphocytes	^{56}Fe ions		[G9]
2001	Chromosomal aberrations	Human lymphocytes	Alpha particles		[K19]
2001	Chromosomal aberrations	GM10115 human–hamster hybrid cells	X-rays; gamma rays; ^{56}Fe ions; neutrons; gold ions	Dose response	[L10]
2001	Chromosomal aberrations	WTK1 human lymphocytes	^{56}Fe ions; ^{137}Cs gamma rays		[S8]
2001	Microsatellite instability; gene amplifications	BEP2D human bronchial epithelial cells	Alpha particles; ^{56}Fe ions		[P16]
2001	Minisatellite instability	4T1 murine mammary adenocarcinoma cells	Gamma rays		[L33]
2001	Mutation frequency	*Saccharomyces cerevisiae*	Gamma rays	Dose response	[B19]
2001	Mutation frequency	TK6 human lymphoblasts	^{56}Fe ions; gamma rays		[E14]
2001	Mutation frequency	Human lymphoid cells	Gamma rays (12 Gy)		[G9]
2001	Mutation frequency	4T1 murine mammary adenocarcinoma cells	Gamma rays (12 Gy)		[L33]
2001	Plating efficiency	*Saccharomyces cerevisiae*	Gamma rays	Dose response	[B19]
2001	Plating efficiency	Human lymphoid cells	^{56}Fe ions		[G9]
2001	Recombination frequency	Saccharomyces cerevisiae	Gamma rays	Dose response	[B19]
2001	Telomere shortening	WTKI human lymphoblastoid cells	^{56}Fe ions; ^{137}Cs gamma rays		[S8]
2002	Apoptosis	Mouse fibroblast clones; V79 Chinese hamster ovary cells	X-rays		[C11]
2002	Chromosomal aberrations	Human fibroblasts	Gamma rays		[B21]
2002	Chromosomal aberrations	Murine haemopoietic stem cells	Gamma rays		[B23]
2002	Chromosomal aberrations	Mouse fibroblast clones; V79 Chinese hamster ovary cells	X-rays		[C11]
2002	Chromosomal aberrations and response to second irradiation	TK6 human lymphoblasts	^{56}Fe ions; gamma rays		[E13]
2002	Mutation frequency	TK6 human lymphoblasts	^{56}Fe ions; gamma rays		[E13]
2003	Chromosomal aberrations	Normal diploid human fibroblasts	Low- and high-LET radiation	No instability observed	[D17]
2003	Chromosomal aberrations	TK6 human lymphoblasts	Gamma rays		[E15]
2003	Global gene expression	Primary human lymphocytes	Gamma rays		[F16]
2003	Chromosomal instability and radiation-induced delayed reproductive death	Haemopoietic stem cells (R-M26/2-1)	Gamma rays	Independent of *TP53* status	[M27]
2003	Delayed lethality and micro-nucleus formation	Human osteoblast cells	Depleted uranium		[M57]
2003	Chromosomal aberrations	TK6 and NH32 human lymphoblasts	Gamma rays	Dose response up to 5 Gy	[S9]
2003	Micronucleus frequency	Human peripheral blood lymphocytes	Gamma rays	Dose response	[J8]
2003	Chromosomal aberrations	Murine haemopoietic stem cells	Gamma rays		[M27]
2003	Delayed *TP53* activation	HTI 080 human fibrosarcoma cells	X-rays; gamma rays		[S24]
2003	Mutation frequency	TK6 human lymphoblasts	^{56}Fe ions; gamma rays		[E15]
2003	Plating efficiency	Murine haemopoietic stem cells	Gamma rays		[M27]

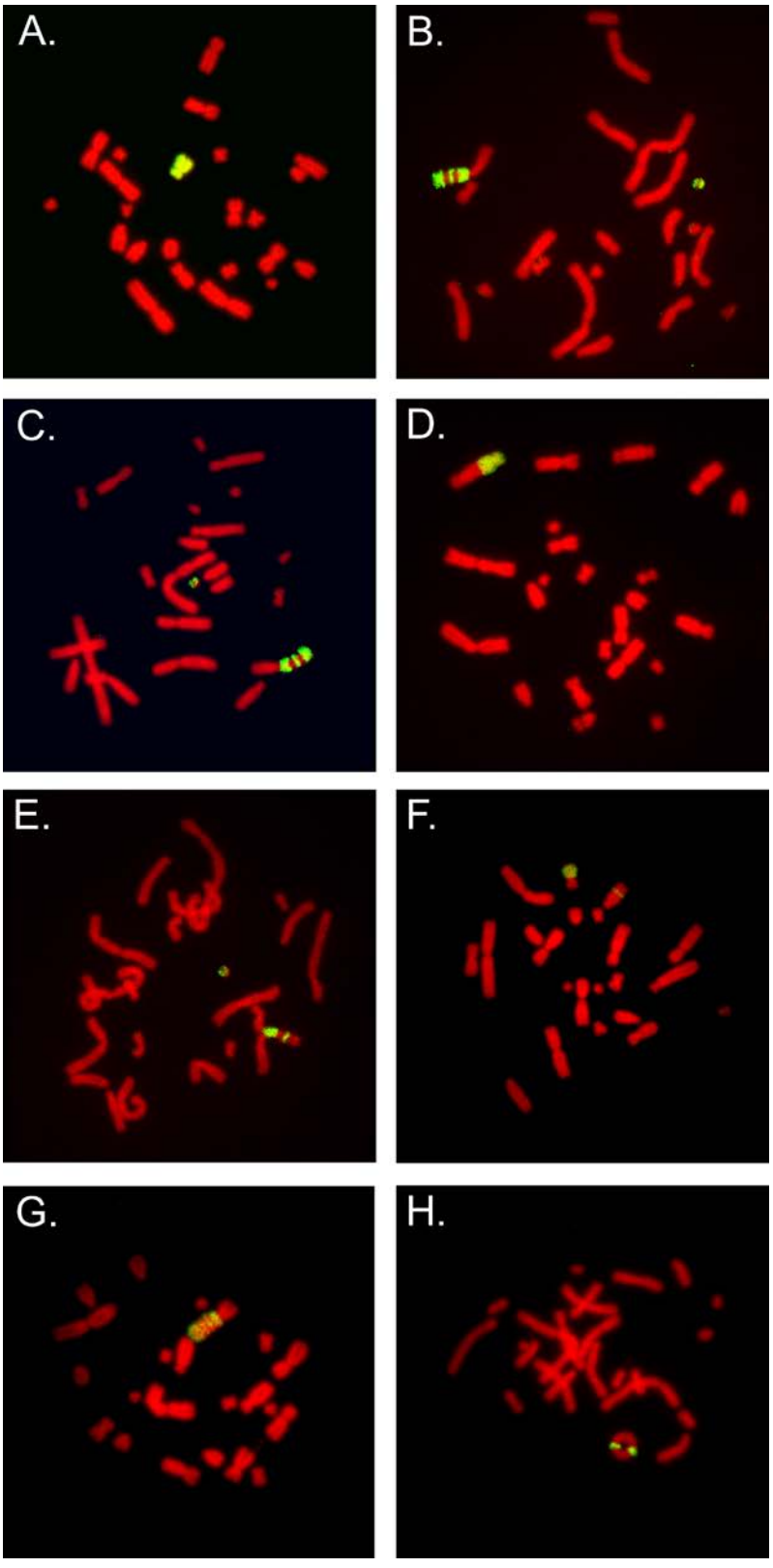
Year	*End point*	*Cell type*	*Radiation type*[a]	*Comments*	*Reference*
2004	DNA damage; comet assay	Chinese hamster ovary cells	X-rays	Low dose	[G23]
2004	GFP-based protein assay for homologous recombination	Human RKO cells	X-rays		[H24]
2004	Cell viability; apoptosis; changes in MAP and ERK signalling	Human lymphoblast cells	X-rays		[R13]
2004	Gene expression analysis	GM10115 human–hamster hybrid cells	X-rays		[S59]
2004	ROS production	Mouse m5S derived cl. 2011-14 cells	X-rays	Cell killing related to time after irradiation	[T6]
2004	Lethal sectoring; division delay	HeLa S3-9IV cells	X-rays; alpha particles		[S47]
2004	Chromosomal aberrations	*Scid* mouse cells	X-rays		[U26]
2004	Mutagenic radicals	Human–hamster hybrid A (L) cells	X-rays	High-LET	[W20]
2005	Chromosomal aberrations	Peripheral blood lymphocytes	Gamma irradiation		[B33]
2005	HPRT frequency; apoptosis; cell survival	Lymphoblastoid TK6 cells			[C20]
2005	H2AX phosphorylation	Human fibroblasts	Si and Fe ions	High-LET	[D26]
2005	Global gene expression profile	Human skin fibroblasts	X-rays	2 cGy and 4 Gy	[D27]
2005	Chromatid and chromosomal aberrations	Haemopoietic stem cells	Gamma rays		[G22]
2005	Micronuclei; chromosomal aberrations	Human peripheral blood lymphocytes	^{60}Co gamma rays	0–4 Gy	[J9]
2005	Genomic patterns of aberrations; radiation-induced mouse lymphomas	Mouse genomic BACs			[M55]
2005	Delayed apoptosis	CGL1 (HeLa × fibroblast) hybrid cells	X-rays	7 Gy	[M56]
2005	Chromosomal rearrangements	TK6 cells and clones with differing TP53 status	Gamma rays	2 Gy	[M54]
2005	Delayed apoptosis	GM10115 human–hamster hybrid cells	X-rays		[N29]
2005	Cell killing	V79 cells	X-rays	Low dose	[S65]
2005	Microarray analysis of isogenic clones to assay for gene expression	Human–hamster hybrid cells	X-rays		[S61]
2005	Gene expression influenced by *TP53* status	TK6; NH32; WTK1	Gamma rays	10 Gy at different times	[T7]

[a] It is not possible to include the range of doses used, as many studies used different doses, dose rates and radiation types within a single study. The reader is referred to the original study for information on the range of doses used.

7. Of the multitude of end points associated with radiation-induced instability, chromosomal changes are the best described. While some investigators describe chromosome gaps [S11, U20] or breaks [K3, L24] as the primary aberrations observed, it is unlikely that these contribute to long-term instability, as gaps have no known phenotype and breaks are generally lethal [C2, C3]. Of more significance are gross chromosomal rearrangements, particularly chromosomal translocations, duplications and partial trisomies [G8, M2, S8], which appear to involve amplification and recombination of large chromosomal regions by a currently unknown mechanism [D1, M16]. An example of chromosomal instability in human–hamster hybrid GM10115 cells clonally expanded from a single cell surviving X-irradiation is presented in figure III.

Figure III. Metaphase spreads from human–hamster hybrid GM10115 cells clonally expanded from a single cell surviving exposure to 5 Gy of X-rays.

A: Metaphase chromosomes from non-irradiated GM10115 cells. Using fluorescence in situ hybridization, the human chromosome 4 in these human–hamster hybrid cells is painted green, and the hamster chromosomes are stained red. B–H: Representative metaphase cells showing the chromosomal rearrangements observed in one colony of cells clonally expanded from a single cell. Radiation-induced instability stimulated the dynamic rearrangement of the genetic material, resulting in multiple subpopulations of cytogenetically rearranged cells within the clonally expanded population. Such cytogenetic rearrangements in GM10115 cells have been used by Morgan and colleagues [K9, L6, L9, L30, L31, M2] as a measure of genomic instability induced by both high- and low-LET radiation.



8. Instability is a frequent event in colonies of surviving cells. Kadhim et al. [K3] reported karyotypic abnormalities in 40–60% of murine stem cells exposed to doses of alpha particles that would produce about one hit per cell. Sabatier and colleagues [S44] observed late passage non-random chromosomal instability in >50% of metaphase cells from human dermal fibroblasts irradiated with a wide range of high-LET radiations (386 to 13,600 keV/μm). Likewise, Limoli et al. [L6] observed that X-rays induced chromosomal instability in ~3% of surviving human–hamster hybrid GM10115 clones per gray of radiation. This increased to ~4% Gy^{-1} after high-LET iron ion exposure [L12, L13]. This observed frequency of instability is grossly in excess of the reported frequency for gene mutations at similar doses. Therefore it is unlikely that mutation in a single gene or gene family is responsible for the unstable phenotype in unstable clones. Instead it is reasonable to suppose that factors contributing to maintaining genomic instability over time include critical pathways in DNA damage and repair [C10, H11, M48, Y3], chromosomal replication [B32], cellular homeostasis [B3, B6, M24, M48] and alterations in gene expression [S59, S60, S61, S62].

9. The high frequency of induced instability observed in different systems raises the intriguing question as to what is really being measured and of the significance of these observations. While the numerous in vitro studies summarized in table 1 have established the occurrence of radiation-induced genomic instability, many of the cell lines used were not "normal" initially, and in many cases they involved tumour-derived cell lines. Dugan and Bedford [D17] have pointed out that instability is sometimes not observed in apparently normal cells after irradiation. However, there are many reports of instability in the progeny of irradiated normal human and murine bone marrow cells [K1, K2] and in cultured human lymphocytes [B11, H7, H8, H9]. Some of this confusion may relate to the role of a functional *TP53* tumour suppressor gene. Both *TP53*-dependent [S9] and *TP53*-independent pathways have been proposed [K5, L8], and Moore et al. [M54] showed that genomic instability might differ both quantitatively and qualitatively as a consequence of altered *TP53* expression. Furthermore, differences in cellular proliferation patterns and susceptibility to mutation between cells and cell lines might also influence the reported results [C19]. Because the majority of reports indicate that irradiated normal primary cells readily demonstrate the instability phenotype (table 1), this implicates ionizing radiation as the causative agent. It also establishes induced instability as a phenotype associated with radiation exposure. As always, however, caution should be exercised when extrapolating from in vitro cell culture systems to the human situation in vivo.

10. When cells or tissues are directly exposed to ionizing radiation, biological effects are generally induced in a dose-dependent manner. One perplexing feature of radiation-induced genomic instability, and of non-targeted effects in general, is the lack of a well-defined dose response profile. Most investigators report that non-targeted effects are independent of dose. However, some investigators have observed a dose response at lower doses that tends to saturate at higher doses, and a few investigators have observed consistent dose-related effects. These data are summarized in table 1 and are reviewed in references [L6, M32]. Furthermore, radiation-induced instability appears independent of dose rate, although this has not been extensively investigated to date [L6]. In addition, there does not appear to be a significant LET effect for radiation-induced genomic instability, with both high- and low-LET radiations being effective [H2, K34, L12].

11. Many of the genomic changes described under the title of induced instability are changes of the same type as observed in human tumours. Radiation-induced cancers have no known molecular signature, and continued investigation aimed at understanding the processes and pathways by which radiation induces genomic alterations in the progeny of irradiated cells should provide insights into the mechanisms of radiation-induced transformation and carcinogenesis [M62]. Kennedy et al. [K10] demonstrated replication dependence of radiation-induced transformation of C3H 10T½ cells. A similar replication dependence was also reported for radiation-induced mammary cancers in rats, in which expression of epigenetic initiation required replication of irradiated mammary stem cells in the tissue microenvironment [K7].

12. While the multiple phenotypes associated with radiation-induced genomic instability are relatively well characterized, the molecular, biochemical and cellular events that initiate and perpetuate instability remain unknown. Directly induced DNA damage, e.g. induced DNA double-strand breaks, is probably not causative [M13]. Instead, deficiencies in cellular responses to DNA damage [C10, Y3], changes in gene expression [B6] or perturbations in cellular homeostasis [B3] are more likely to be involved, and provide a rational explanation as to why the unstable phenotype can persist. In the GM10115 cell system, clones of unstable cells continue to show the dynamic production of novel chromosomal rearrangements for over four years post-irradiation [N1]. Attempts to define the target for induced instability indicate that, while the nucleus may be the ultimate target [B6, K9], there is evidence for a persistent increase in reactive oxygen species (ROS) in cultures of cells showing radiation-induced genomic instability. A role for enhanced oxidative stress in perpetuating the unstable phenotype was first described by Clutton et al. [C5], and was later confirmed in studies by Limoli et al. [L7, L9, L10] and Redpath and Gutierrez [R3]. A persistent induction of ROS has also been shown to cause delayed reinduction of *TP53* in normal human fibroblasts [R6]. A study by Roy et al. [R5] revealed that hypoxia (2% oxygen) significantly reduced X-ray-induced delayed effects, specifically cell death, giant cell formation and chromosomal aberrations, compared with cells cultured under their "normal" 20% oxygen conditions. The role of ROS in radiation-induced genomic instability has been reviewed in detail by Mikhailov and colleagues [B45, M48]. It should be noted that oxygen tension in normal tissue shows a typical Gaussian distribution of values with a median between 40 and 60 mm Hg, and no values below 10 mm Hg [A13]. Tumours, on the other hand, invariably

show a distribution with much lower oxygen tension [B44]. As will be described later, a role for ROS in non-targeted radiation-induced bystander effects has also been described, suggesting a potential commonality in processes involved in these delayed effects of exposure to ionizing radiation.

13. It is well known that most mammalian cells do not divide indefinitely in vitro or in vivo, owing to a process termed replicative senescence. In human cells, replicative senescence can be caused by telomere shortening, but murine cells senesce despite having long, stable telomeres. Parrinello et al. [P19] showed that the phenotypes of senescent human fibroblasts and mouse embryonic fibroblasts (MEFs) differ under standard culture conditions that include 20% oxygen. The MEFs did not senesce in physiological (3%) oxygen levels, but underwent a spontaneous event that allowed indefinite proliferation in 20% oxygen. The proliferation and cytogenetic profiles of DNA repair-deficient MEFs suggested that DNA damage limits MEF proliferation in 20% oxygen. Indeed, MEFs accumulated more DNA damage in 20% oxygen than in 3% oxygen, and more damage than human fibroblasts in 20% oxygen. These results identify oxygen sensitivity as a critical difference between mouse and human cells, explaining their proliferative differences in culture, and possibly their different rates of cancer induction and ageing. Furthermore, they may contribute to explaining some of the differences between mouse and human studies described later in this annex.

14. There is emerging evidence implicating a role for extranuclear and even extracellular events in initiating and perpetuating radiation-induced chromosomal instability. Kadhim et al. [K3] analysed chromosomal instability in murine haemopoietic stem cells following alpha particle irradiation. Many of the surviving cells were those that were not traversed by an alpha particle during irradiation. Expanding these studies, Wright and colleagues used a protective metal grid to shield regions of the cell culture flask and lethally irradiated the non-shielded regions of the flask. They then cultured the non-irradiated, shielded cells and examined the clonal progeny for induced chromosomal instability. A high frequency of instability was observed in the progeny of cells that were not directly hit by radiation [L24]. Clearly, induced instability has an extracellular component, and signals from irradiated cells can stimulate chromosomal rearrangements in non-targeted cells within the radiation environment (reviewed in reference [M10]). These observations have implications for the fate of cells surviving radiation exposure in that some of these surviving cells may develop genomic instability. These observations also indicate that even cells outside the radiation field can manifest phenotypes similar to those of irradiated cells.

B. Induced genomic instability after in vivo irradiation followed by in vitro analysis

15. Weissenborn and Streffer were the first to describe induction of genomic instability after irradiation in vivo followed by analysis in vitro. They reported structural and numerical chromosomal anomalies as well as micronuclei at the first, second and third mitosis after in vivo irradiation of one- or two-cell mouse embryos with X-rays or neutrons [W6, W7]. These observations were extended by Ullrich and Davis [U18], who irradiated inbred BALB/c mice and at varying intervals after irradiation removed and cultured the mammary glands in vitro. Cytogenetic analysis indicated that instability could develop and persist in situ in a mature, fully differentiated tissue after in vivo irradiation. Furthermore, there was a dose-dependent increase in the frequency of delayed aberrations at low doses (0.1–1 Gy) that reached a plateau at higher doses [U18].

16. Cellular studies on radiation-induced murine mammary cancer demonstrated strain-dependent differences in susceptibility, presumably resulting from differences in sensitivity to neoplastic initiation [U17]. Similar strain susceptibility is apparent for in vivo irradiation followed by in vitro analysis of induced instability. Mammary cells from BALB/c mice are more susceptible to radiation-induced genomic instability than those from C57BL/6 or F_1 hybrid crosses of C57BL/6 and BALB/c mice [P9, U20]. Studies of DNA repair in the radiosensitive BALB/c mouse revealed inefficient end-joining of gamma-ray-induced double-strand breaks in DNA. This is apparently due to reduced expression of the DNA-PKcs protein and lowered DNA-PK activity in these mice. This may impair the animals' ability to appropriately respond to induced damage and may thus account for the increased instability [O4, Y3]. Most DNA repair processes have evolved to prevent genomic instability induced by endogenous lesions [L49] and induced DNA damage (for a comprehensive discussion, see the BEIR VII [C23] and French Academies [T8] reports).

C. Induced genomic instability after in vitro irradiation followed by in vivo analysis

17. Conversely, instability induced in vitro can be transmitted in vivo following transplantation of irradiated cells into recipient animals. Paquette and Little [P3] irradiated C3H 10T½ cells and cultured half in cell culture in vitro; the other half was transplanted into syngeneic and non-immunosuppressed C3H mice. Interestingly, a higher frequency of minisatellite instability was observed in those irradiated cells injected into mice than those cultured in vitro. Watson et al. [W3] reported the induction and long-term persistence of chromosomal instability after murine bone marrow cells were irradiated in vitro and then transplanted into female CBA/H mice that had received 10 Gy of X-irradiation less than two hours before to eradicate the host bone marrow. These studies were later extended to demonstrate that instability induced by X-ray or neutron irradiation in vitro can be transmitted in vivo [W4]. A recent analysis of a series of radiation-induced sarcomas [G10] showed a prevalence (53%) of somatic *TP53* mutations, which was significantly higher than that for sporadic sarcomas (16.8%).

The mutations were inactivating and associated with the loss of the other *TP53* allele. This loss of heterozygosity was due to the loss of a large fragment of the chromosome or of the whole chromosome, probably indicating a more general chromosomal instability similar to that previously described [L1].

18. Watson et al. [W2] have provided convincing evidence that the induction of genomic instability following in vitro irradiation and in vivo expression can result from a non-targeted bystander-like effect. That is, rather than resulting from the direct effect of radiation exposure being passed on to the progeny of that irradiated cell, instability might also result from soluble signals being passed from irradiated cells to non-irradiated cells. When non-irradiated cells were mixed with cells irradiated with 0.5 Gy of neutrons at 0.04 Gy/min and then transplanted into recipient CBA/H mice, instability was observed in the non-irradiated cell population [W2]. An elegantly conceived chromosomal marker system allowed the investigators to distinguish between the irradiated and non-irradiated transplanted cells and cells derived from the host mouse. Irradiated and non-irradiated cells were distinguished by using marrow from CBA/H mice (40XY cells) and the congenic CBA/H strain (40XY6T6 cells) homozygous for the stable T6 reciprocal translocation between chromosomes 14 and 15. Using this system, unambiguous evidence for non-clonal chromosomal aberrations was observed in clonal populations derived in vitro from neutron-irradiated bone marrow cells. Furthermore, after transplantation with neutron-irradiated cells, translocations and deletions were observed for a period of 3–13 months. Significantly, there was also a higher frequency of unstable aberrations in the bone marrow of the recipient mouse. These results implicate an in vivo bystander-like mechanism in the induction of chromosomal instability, and suggest that the instability observed in the non-irradiated cells is not an artefact of clonal selection. This result was confirmed by Xue et al. [X2], who injected nude mice with a mixture of human colon LS174T adenocarcinoma cells and LS174T-cells prelabelled with lethal doses of DNA-incorporated 5-[125I]iodo-2'-deoxyuridine (^{125}IUdR). A distinct inhibitory effect on the growth of the unlabelled LS174T tumour cells was observed. Because ^{125}IUdR is incorporated into DNA, almost all the electrons emitted during radioactive decay have a subcellular range of <0.5 μm. This led the authors to conclude that the inhibitory result was due to a bystander effect generated in vivo by factors present within and/or released by the ^{125}IUdR-labelled cells. However, it is also possible that debris and breakdown products from the heavily irradiated cells might affect bystander cells, and these non-labelled cells might even incorporate ^{125}I released from dying cells.

19. Currently the mechanisms underlying the induction and persistence of instability are not understood. The induction of chromosomal aberrations in vivo by a bystander-like mechanism might provide insights into the mechanisms as well as link instability to bystander effects. Bystander effects can be mediated by cell-to-cell gap junction communication and secretion of soluble factors. These secreted factors [S39] might include extracellular cytokine-like factors [L2, N8] that are able to increase intracellular levels of ROS in non-irradiated cells [L23, M10, M16]. Lorimore et al. recently proposed a potential mechanism for these in vivo radiation-induced bystander effects [L22]. They found persistent macrophage activation combined with neutrophil infiltration following 4 Gy whole-body irradiation of mice. The inflammatory nature of the observed responses may provide a mechanism for the long-term production of genetic damage by a bystander effect, ultimately contributing to radiation-induced instability and potentially leukaemogenesis. This will be discussed in more detail in the section on radiation-induced genomic instability and bystander effects.

D. Radiation-induced genomic instability in vivo

20. In reviewing the literature on in vivo non-targeted effects of ionizing radiation, it becomes obvious when considering the mouse studies that many of the observed effects are highly dependent upon the mouse strain used and the sex of the animal studied [M58]. Consequently, there has been an effort throughout this annex to identify the mouse strain used when comparing conflicting data. It is also apparent that even the same mouse strain can vary significantly when bred in different colonies in different laboratories. Differences due to sex might also exist, but not all of the studies provide adequate details on the sex of the animals used. While much has been learned from animal models [F7], caution should be exercised when extrapolating from the animal studies to the human situation.

21. The reports of radiation-induced genomic instability in vivo are summarized in table 2, which also lists the end point used to assay instability, the model system, the type of radiation used and whether or not genomic instability was observed. In this section the methods of analysing instability in vivo will be highlighted along with potential areas of conflict and associated caveats.

22. Nowell [N19] first proposed that genomic instability might be a driving force in tumorigenesis and a hallmark of many cancers [C7, L3]. There is accumulating evidence suggesting that instability may represent a critical step in the genesis of certain radiation-induced cancers [L14, S3, U20]. Implicit in this annex is the hypothesis that radiation-induced genomic instability provides relevant underlying mechanistic contributions to some radiation-induced cancers. While the precise relationship between radiation-induced genomic instability and radiation carcinogenesis remains to be determined, understanding the mechanisms of induced instability might provide valuable insights into health risks associated with radiation exposure and the carcinogenesis process in general.

Table 2 In vivo studies of radiation-induced genomic instability (RIGI) and transgenerational effects

Year	*End point*	*Cellular system*	*Radiation type*	*RIGI*[a]	*Reference*
1976	Graft versus host reactions; non-specific bystander activity	(PVGc × Wistar) F1 hybrids; PVGc spleen cells	X-rays	+	[J10]
1979	Chromosomal aberrations	Haemopoietic cells in atomic bombing survivors	Neutrons; gamma rays	–	[K21]
1979	Tumour induction	Mouse localized exposures	X-rays; neutrons	+	[U19]
1980	Tumour induction	Mouse localized exposures	X-rays; neutrons	+	[U16]
1982	Foetal deaths, malformations in F1 mice	Mouse whole-body irradiation	X-rays	+	[K13]
1982	Tumour induction in F1	Mouse whole-body irradiation	X-rays	+	[N17]
1984	Foetal deaths, malformations in F1 mice	Mouse whole-body irradiation	X-rays	+	[K12]
1985	Tumour induction	Mouse whole-body irradiation	X-rays	+	[C6]
1988	Chromosomal aberrations	Skin fibroblasts (mouse zygotes irradiated)	X-rays; neutrons	+	[P1]
1988	Chromosomal aberrations	Mouse embryo	X-rays; neutrons	+	[W14]
1988	Congenital abnormalities	Skin fibroblasts (mouse zygotes irradiated)	X-rays; neutrons	+	[P1]
1988	Micronucleus frequency	Mouse embryo	X-rays	+	[W14]
1989	Cell proliferation in F1 and F2 generations	Mouse whole-body irradiation	Gamma rays	+	[O2]
1989	Chromosomal aberrations	Mouse zygotes	X-rays	+	[P17]
1989	Chromosomal aberrations	Mouse embryo	X-rays	+	[W6]
1989	Micronucleus frequency	Mouse zygotes	X-rays	+	[P17]
1990	Cancer prevalence	Children of nuclear plant workers	X-rays; gamma rays	+	[G2]
1990	Foetal deaths, malformations	Mouse embryos	X-rays	+	[M26]
1991	Chromosomal aberrations	Blood lymphocytes of uranium miners after whole-body irradiation	Alpha particles	–	[M34]
1991	Sister chromatid exchanges	Blood lymphocytes of uranium miners after whole-body irradiation	Alpha particles	–	[M34]
1993	Chromosomal aberrations	Skin fibroblasts in atomic bombing survivors	Neutrons; gamma rays	–	[H13]
1993	Microsatellite instability in F0, F1 and F2 generations	Mice after whole-body irradiation	Neutrons; X-rays; gamma rays	+	[D10]
1994	Cancer prevalence	Children of nuclear plant workers	X-rays; gamma rays	–	[D6]
1994	Microsatellite instability in F0, F1 and F2 generations	Mice after whole-body irradiation	Neutrons; X-rays; gamma rays	+	[S1]
1994	Minisatellite instability	C3H 10T½ murine cells (irradiated in vitro, then injected into mice)	X-rays	+	[P3]
1995	Cancer prevalence	Progeny of cancer patients who had undergone radiation therapy	X-rays	–	[H4]
1995	Microsatellite instability in F0, F1 and F2 generations	Mice after whole-body irradiation	Neutrons; X-rays; gamma rays	+	[F2]
1995	Tumour induction in F1	Mouse whole-body irradiation	X-rays	–	[C4]
1996	Chromosomal aberrations	Murine bone marrow (in vitro irradiation)	Alpha particles	+	[W3]
1996	Minisatellite instability	Chernobyl survivors after whole-body irradiation	Gamma rays	+	[D11]
1996	Minisatellite instability	Haemopoietic cells in atomic bombing survivors	Neutrons; gamma rays	+/–	[S5]

Year	*End point*	*Cellular system*	*Radiation type*	*RIGI*[a]	*Reference*
1996	Neoplastic transformation	Epithelial cells of whole-body-irradiated mice	X-rays	+	[U17]
1997	Cancer prevalence	Children of nuclear plant workers	X-rays; gamma rays	–	[D7]
1997	Cell proliferation in F1 and F2 generations	Mouse whole-body irradiation	Gamma rays	+	[W10]
1997	Minisatellite instability	Chernobyl survivors after whole-body irradiation	Gamma rays	+	[D12]
1997	Mutation frequencies in F1	Mice after whole-body irradiation	Gamma rays	+	[L25]
1998	Chromosomal aberrations	Human lymphocytes after whole-body irradiation	Gamma rays	–	[S2]
1998	Chromosomal aberrations	Lymphocytes from uranium miners	Alpha particles (radon)	+/–	[S25]
1998	Chromosomal aberrations	Blood lymphocytes from plutonium workers	Gamma rays	–	[W9]
1998	Micronucleus frequency	Rat lung cells after partial-volume irradiation	Gamma rays	+	[K11]
1998	Micronucleus frequency	Lymphocytes from uranium miners	Alpha particles (radon)	+	[S25]
1998	Microsatellite instability in F0, F1 and F2 generations	Mice after whole-body irradiation	Neutrons; X-rays; gamma rays	+	[D15]
1998	Tumour induction in F1	Mouse whole-body irradiation	Gamma rays	+	[L20]
1999	Chromosomal aberrations	Lymphocytes of Chernobyl survivors after whole-body irradiation	Gamma rays	+	[G3]
1999	Chromosomal aberrations	Lymphocytes of Chernobyl recovery operations workers and workers from the nuclear power plant	Gamma rays	+/–	[L36]
1999	Chromosomal aberrations	Haemopoietic cells in atomic bombing survivors	Neutrons; gamma rays	–	[N6]
1999	Chromosomal aberrations	Mouse epithelial cells after whole-body irradiation	X-rays	+	[U18]
1999	Chromosomal aberrations	Mouse bone marrow cells after whole-body irradiation	X-rays	+	[X1]
1999	Prenatal mortality; developmental and skeletal defects in F2 mice	Mouse zygotes	X-rays	+	[P7]
1999	Sister chromatid exchanges	Lymphocytes of Chernobyl recovery operations workers and workers from the nuclear power plant	Gamma rays	+	[L36]
1999	Minisatellite instability	Children of Chernobyl recovery operations workers	Gamma rays	+/–	[L18]
2000	Chromosomal aberrations	Foetal haemopoietic cells of mice after foetal irradiation	Gamma rays	+	[D3]
2000	Chromosomal aberrations	C57BL/6 mice after whole-body irradiation	Gamma rays	+	[S20]
2000	Chromosomal aberrations	Blood lymphocytes of patients after whole-body irradiation for radiotherapy	X-rays	–	[T2]
2000	Chromosomal aberrations	Mixture of irradiated and non-irradiated murine bone marrow cells	Neutrons	+	[W2]
2000	Micronucleus frequency	CBA mice after prenatal irradiation	Gamma rays	–	[A1]
2000	Microsatellite instability in F0, F1 and F2 generations	Mice after whole-body irradiation	Neutrons; X-rays; gamma rays	+	[D16]
2000	Tumour induction in F1	Pregnant mice	Gamma rays	+	[U21]
2000	Minisatellite instability	Sperm from three seminoma patients	X-rays	–	[M5]

Year	*End point*	*Cellular system*	*Radiation type*	*RIGI*[a]	*Reference*
2001	Chromosomal aberrations	Mouse bone marrow cells after whole-body irradiation	Alpha particles (^{224}Ra); X-rays	–	[B12]
2001	Chromosomal aberrations	Mouse bone marrow cells after whole-body irradiation	X-rays	+	[M1]
2001	Chromosomal aberrations	Blood lymphocytes of uranium miners after whole-body irradiation	Alpha particles	–	[M35]
2001	Chromosomal aberrations	Blood lymphocytes of cancer patients after whole-body irradiation	Gamma rays	–	[V1]
2001	Chromosomal aberrations	Murine bone marrow; irradiated cells or whole-body irradiation	X-rays; alpha particles; neutrons	+	[W4]
2001	Chromosomal aberrations	Blood lymphocytes from plutonium workers	Gamma rays	–	[W8]
2001	Ductal dysplasia	Epithelial cells of ATM$^{+/-}$ mice	X-rays	+	[W15]
2001	Minisatellite instability	Children of Chernobyl recovery operations workers	Gamma rays	–	[L17]
2001	Minisatellite instability	Haemopoietic cells in atomic bombing survivors	Neutrons; gamma rays	+/–	[N7]
2001	Minisatellite instability	Children of Chernobyl recovery operations workers	Gamma rays	+	[W5]
2001	Mutation frequencies in F1	Mice after whole-body irradiation	Gamma rays	+	[N14]
2001	Signal kinase activity in F3	Mouse whole-body irradiation	Gamma rays	+	[B4]
2001	Sister chromatid exchanges	Blood lymphocytes of uranium miners after whole-body irradiation	Alpha particles	–	[M35]
2002	APRT, HPRT mutation frequency	Mouse T-lymphocytes after whole-body irradiation	X-rays	+	[L35]
2002	Cancer prevalence	Progeny of radiation workers	X-rays; gamma rays	+	[D20]
2002	Cell proliferation in F1 and F2	Mouse whole-body irradiation	Gamma rays	+	[B5]
2002	Chromosomal aberrations	Mouse T-lymphocytes after whole-body irradiation	X-rays	+	[L35]
2002	Microsatellite instability in F0, F1 and F2 generations	Mice after whole-body irradiation	Neutrons; X-rays; gamma rays	+	[B2]
2002	Microsatellite instability in F0, F1 and F2 generations	Mice after whole-body irradiation	Neutrons; X-rays; gamma rays	+	[D9]
2002	Minisatellite instability	Chernobyl survivors after whole-body irradiation	Gamma rays	+	[D19]
2002	Minisatellite instability	Children living in Semipalatinsk	^{131}I, ^{90}Sr and ^{137}Cs	+	[D8]
2002	Mutation frequencies in F1	Mice after whole-body irradiation	Gamma rays	+	[S26]
2002	Signal kinase activity in F3	Mouse whole-body irradiation	Gamma rays	+	[V2]
2003	ROS content; state of DNA structure	Bone marrow cells of male mice after whole-body irradiation	X-rays (1.5 Gy)	+	[M58]
2003	Minisatellite instability	Children of Chernobyl recovery operations workers	Gamma rays	–	[K37]
2004	DNA double-strand breaks; morula and gastrula formation	*Oryzias latipes* F3 embryos derived from male founders	Gamma rays	+	[A18]
2004	Epigenetic global genomic DNA methylation changes	Male and female mouse whole-body irradiation	Low-dose X-rays	+	[K30]
2004	Latency of cancer risk	Cohorts of underground miners exposed to radon	Radon	–	[L41]

Year	*End point*	*Cellular system*	*Radiation type*	*RIGI*[a]	*Reference*
2004	Chromosomal aberrations; transformation frequency; cell killing; DNA damage	Mouse embryonic fibroblasts from mice deficient in Hsp70.1 and Hsp70.3		+	[H26]
2004	Epigenetic global genomic DNA methylation changes	Male and female mouse whole-body irradiation	High-dose X-rays (5 Gy)	+	[P21]
2004	Somatic mutation assay	F1 and F2 progeny of *Oryzias latipes*	Gamma rays	+	[S42]
2004	Destruction of haematopoietic progenitor and stem cells in F1	Mice after sublethal irradiation		+	[Z8]
2004	Chromosomal aberrations; tumour incidence; blood counts	Pregnant mice; mice foetal liver and spleen cells	1 Gy irradiation	+	[U22]
2004	Apoptosis; cell proliferation and differentiation	Mouse limb bud cells	0.3 and 5 Gy	+	[W21]
2004	Micronuclei; number of ovules; male fertility	Pea plant seedlings for two generations	Gamma rays	+	[Z8]
2004	Mini- and microsatellite instability	Children of Chernobyl recovery operations workers	Gamma rays	–	[S55]
2005	Chromosomal aberrations	Pregnant Swiss albino mice	Gamma rays (1–1.5 Gy)	+	[U23]
2005	Initiation of intestinal adenoma	Apc mice	X-irradiation	–	[E20]
2005	Locus-specific mutations	*Oryzias latipes* spermatogonial stem cells	Gamma rays (0.03 cGy/min and 95 cGy/min)	+	[S43]
2005	Chromosomal instability	Congenic haematopoietic cells into irradiated mouse host	Gamma rays	+	[L47]
2005	Chromatid aberrations; chromosomal radiosensitivity	Prostrate patients with prostatic hyperplasia	Ionizing radiation	+	[H25]
2005	Clonogenic survival; apoptosis	Bladder explants from C57BL6 and CBA/Ca mice after whole-body irradiation	Low-dose irradiation (0.5 Gy)	+	[H25]
2005	Chromosomal aberrations	Clonal T-cells from atomic bombing survivors	Gamma rays	–	[K29]
2005	Chromosomal instability	Haemopoietic stem cells	Gamma rays	+	[L47]
2005	Tumour formation	Female B6C3 F1 mice	Gamma rays (1.9 Gy)	+	[S66]
2005	Microsatellite instability	Children of Chernobyl recovery operations workers	Gamma rays	–	[F15]

[a] +: genomic instability was observed; –: genomic instability was not detected.

1. Mouse models for radiation-induced genomic instability in vivo

23. As described above, transmissible genomic instability has been observed after irradiation in vivo followed by culture in vitro and vice versa. The picture following irradiation in vivo and subsequent expression of instability in vivo is less clear and more controversial. Following irradiation of bone marrow cells from 12 week-old CBA/H mice with 0.5 Gy of alpha particles, Watson et al. [W3] observed a constant frequency (10–13.4%) of cells with stable chromosomal aberrations for up to 17½ months. This increased to 49.8% at 24 months in pooled samples from three CBA/H mice. They noted significant variation between individuals, with a few animals exhibiting little or no induced instability despite CBA/H being an inbred mouse strain. In contrast, protracted whole-body gamma irradiation of prenatal CBA-Ca mice at either 44, 99 or 265 mGy/day (to a total dose of 0.7, 1.6 or 4.2 Gy) did not induce damage in erythroid stem cells that could be detected as persistent or delayed chromosomal aberrations as measured by micronucleated erythrocytes at 35 days after irradiation [A1]. Bouffler et al. [B12] also failed to find evidence of transmissible chromosomal instability 50 or 100 days after in vivo exposure of CBA/H mice either to alpha particles from the bone-seeking radionuclide ^{224}Ra or to X-rays. Likewise, chromosomal instability was not detected in peripheral blood lymphocytes from C57BL/6 mice for up to 30 days after whole-body gamma radiation [S21] or up to 21 months [S20]. These last results are consistent with in vitro studies indicating that induced instability was not observed in C57BL/6 mice [P9, W1]. Furthermore, no instability was reported in bone marrow

cells from Swiss mice up to 100 days after exposure to 3 Gy of X-rays [X1]. These results were initially presumed to indicate that the Swiss mouse strain, like the C57BL/6 mouse strain, is refractory to radiation-induced instability. However, this does not appear to be the case. When Swiss albino mice were exposed to 0.25–1.5 Gy of gamma radiation on day 14 or 17 of gestation, significant dose-dependent increases in chromosomal aberrations, micronuclei and/or changes in ploidy were observed in the bone marrow at 12 months of age [D3]. The investigators concluded that radiation-induced genomic instability in the foetal haemopoietic cells of the mouse persisted post-natally [D3]. These data are summarized in table 2.

24. Although not designed to specifically investigate radiation-induced genomic instability, a number of studies have examined the persistence of cytogenetic rearrangements in animals at delayed times after irradiation. Hande et al. [H18, H19, H20, H21] used female Swiss mice to study the induction and persistence of dicentrics and translocations in splenocytes up to 112 days after exposure to 2 Gy of whole-body X irradiation. The frequencies of dicentrics decreased exponentially with time, while the frequencies of translocations were constant in the period 0–7 days and then decreased linearly or exponentially. No new chromosomal rearrangements were observed, suggesting that there was no delayed cytogenetic instability in these animals. Similar studies using other mouse models have reported similar results [T5].

25. In attempting to reconcile the apparently conflicting results described above, Bouffler et al. [B12] have noted the sensitivity of mouse bone marrow cells to perturbations through transplantation and culture, and emphasized the need for sound control experiments to be performed concurrently. For instance, some of the radiation-induced transmissible chromosomal instability reported by Watson et al. [W3] could be attributed to the low background frequency of aberrations observed in the control repopulating cells. This is in contrast to the higher background described by Bouffler et al. [B12]. It is also possible that the disparate literature on radiation-induced genomic instability in vivo reflects the inherent variability between the inbred mouse strains used and differences due to sex within the animal strains used.

26. To investigate the in vivo non-targeted effects of low-LET radiation, Lorimore et al. [L47] used the same congenic sex-mismatch bone marrow transplantation protocol as used by Watson et al. [W2] to repopulate the haemopoietic system from a mixture of gamma-irradiated and non-irradiated haemopoietic stem cells such that host-, irradiated donor- and non-irradiated donor-derived cells could be distinguished. Chromosomal instability in the progeny of irradiated haemopoietic stem cells accompanied by a reduction in their contribution to the repopulated haemopoietic system was observed and is consistent with a delayed genomic instability phenotype being expressed in vivo. However, chromosomal instability was also shown in the progeny of the non-irradiated haemopoietic stem cells, implicating a bystander-like mechanism. Studies of the influence of irradiated recipient stromal microenvironment and experiments replacing irradiated cells with irradiated cell-conditioned medium revealed the source of the in vivo bystander effect to be the descendants of irradiated cells rather than the irradiated cells themselves. Lorimore et al. [L47] speculated that it is possible that a radiation-induced genomic instability phenotype in vivo need not necessarily be a reflection of intrinsically unstable cells but the response to ongoing production of inflammatory-type damaging signals [L22] as a long-term unexpected consequence of the initial radiation.

27. While the literature is replete with apparently contradictory reports of radiation-induced instability in mouse model systems, these results clearly indicate that genetic factors can play a major role in the instability phenotype and that analysis of radiation-induced genomic instability in vivo is significantly more complicated than in vitro. Critical analysis of radiation-induced genomic instability in vivo is not a trivial undertaking. In any animal model there is likely to be some inherent genomic instability that complicates the selection of appropriate control populations. Such experiments generally involve inbred strains of mice, and even in radiation-sensitive populations only a small percentage, generally <50%, will exhibit an instability phenotype. Furthermore, extrapolating such results to other mouse strains or outbred populations is difficult at best. Until a careful study involving sound and relevant controls as well as statistically relevant numbers of animals exposed to a homogeneous quality of radiation is carried out, the induction of radiation-induced genomic instability in vivo will remain controversial.

2. Human studies

28. Radiation therapy has improved over recent years, and many of the cancer patients treated with radiation are surviving longer than did those in the past. Second cancers occurring in the irradiated field have been reported in some of these patients, suggesting a direct role of the radiation exposure [B37, B38]. Data on second cancers occurring in children irradiated for cancer indicate that some genetic predisposition to cancer may also predispose them to radiotherapy-related second cancers [D22, E19, F14]. Nevertheless, it is still difficult to identify the radiation-induced lesions initiating the second malignancy. At the time of diagnosis, multiple genomic alterations are present in the tumours, and the majority are likely to represent secondary events occurring during tumour evolution and subsequent selection. This underscores that caution must be applied to analysis of radiation-induced genomic instability and its role in human carcinogenesis. The subsequent discussion in this section highlights the controversies and contradictions inherent in the human studies. To this end it is reasonable to expect that analysis of normal, healthy populations of individuals would not provide evidence of instability regardless of the individuals' radiation history. Indeed, the majority of studies investigating instability in radiation-exposed populations have analysed samples from normal, healthy individuals and did not find evidence of instability [T2, T4]. It is also reasonable

to expect that analysis of instability in individuals manifesting phenotypic effects of radiation exposure, e.g. cancer or leukaemia, might well show evidence of induced instability. Once again, limited studies indicate that this is the case [N6, N7]. Whether or not the observed instability is a direct or non-targeted effect of radiation exposure, or a secondary selective effect of disease evolution, cannot be definitively determined at present. Furthermore, this question is unlikely to be resolved in the foreseeable future. This caveat should be kept in mind in the following discussion.

29. As has been described utilizing the mouse as a model system, both induction and lack of induction of transmissible radiation-induced genomic instability have been reported in humans, and once again genetic factors appear to play a role in the observed instability [K4]. Induced chromosomal instability has been described in long-term cultures of human lymphocytes following irradiation and culture in vitro [H9]. Using the same lymphocyte culture protocol, chromosomal instability was reported in blood samples from individuals exposed during the radiation accident in Estonia in 1994 [S2]. Radiation exposure was variable, protracted and not precisely determined. Furthermore, blood samples were taken well after radiation exposure. No dose response was apparent, and contrary to previous studies from the Lambert laboratory, chromosomal instability was also observed in long-term cultures from non-exposed controls [S2]. In contrast, cytogenetic analysis of 18 individuals who had received between 35 and 80 Gy of fractionated radiation therapy for different cancers showed no increase in aberrant cell types as a function of time after completing therapy. Thus no cytogenetic evidence that fractionated radiotherapy induced a persistent or late-manifesting state of genomic instability was found [T2]. It should be stressed that the majority of patients treated for different malignancies received localized, partial-body irradiation with emphasis on minimizing damage to normal tissue. Consequently, different proportions of bone marrow stem cell populations and peripheral blood lymphocytes would have been exposed to the radiation. It is likely that more cells than the number actually analysed (<200 per patient), would have to be interrogated before evidence of persistent transmissible chromosomal instability would be observed in these individuals, if it indeed existed [T2].

30. The availability of cultured lymphocyte preparations from radiation workers with internal deposits of plutonium has provided the opportunity to examine whether protracted irradiation of bone marrow cells had induced a transmissible genomic instability in descendant cells in the peripheral blood [W8]. Bone marrow dose calculations provided individual cumulative estimates at the time of sampling ranging up to 1.8 Sv. Chromosome analysis revealed no significant differences, either in comparisons between the total group of plutonium workers and controls for comparable periods or when the comparisons were restricted to a group of plutonium workers with initial bone marrow plutonium doses of greater than 0.25 Sv. There was therefore no evidence from this study for the induction of persistent transmissible genomic instability in the bone marrow of radiation workers with internal deposits of plutonium [W8]. Likewise, clonally expanded T-cell lymphocyte populations did not demonstrate increased chromosomal instability using either G-band analysis or multicolour fluorescence in situ hybridization [K29].

31. The long-term effect of radiation exposure on uranium miners employed by the Wismut uranium mining company in the former German Democratic Republic was investigated by scoring the frequency and percentage of micronuclei with and without a centromere. Kryscio et al. [K38] reported that genomic instability had occurred in the lymphocytes of miners, especially those with cancer.

32. A number of investigators have studied the alpha radiation risks in patients who received injections of Thorotrast, an X-ray contrast medium used in Europe, Japan and the United States from the late 1920s to 1955. Thorotrast was composed of thorium dioxide and contained ^{232}Th, a naturally occurring radionuclide. Because the physical half-life of ^{232}Th is 14 billion years and Thorotrast is not appreciably eliminated from the body, the tissues in which it was deposited are irradiated by alpha rays for the entire lifetime of the subject. The major causes of death among the Thorotrast patients are liver cancer, liver cirrhosis, leukaemia and other cancers. Mutation analyses of the *TP53* gene and loss of heterozygosity (LOH) studies at the 17p locus were performed by Ishikawa et al. [I2] to characterize the genetic changes in Thorotrast-induced liver tumours. LOH was not frequent; most mutations were transitions, suggesting that genetic changes in Thorotrast-induced cancers were mainly delayed mutations and not the result of the direct effects of radiation.

33. Likewise Iwamoto et al. [I3] analysed mutations in *TP53* from 20 Thorotrast recipients who developed cancer, mostly of hepatic bile duct and blood vessel origin. Of the 20 cases, 19 had *TP53* point mutations. Moreover, the accompanying non-tumour tissues from these patients also had *TP53* mutations, albeit at lower frequency. The distribution pattern of the point mutations was significantly different between the non-tumour and tumour tissues, with most mutations in malignant tissues located in the highly conserved domains of the *TP53* gene. These results support the idea that *TP53* mutations are important in the genesis of Thorotrast-induced tumours but that these point mutations are a secondary outcome of genomic instability induced by the irradiation. A similar result was reported by Kamikawa et al. [K24], who investigated mutations of the *RAS* and the *TP53* genes in archival sections of liver cancers induced by Thorotrast. These investigators were unable to rule out the possibility that genetic insults occurred indirectly in the proliferating cells adjacent to the necrosis rather than being a direct effect of alpha particles.

34. Wada et al. [W17] also investigated genetic changes in the *TP53* gene in 19 autopsy cases of liver malignancies. LOH at the 17p13 locus and mutations in *TP53* were analysed. A number of cases were informative: four cases

showed LOH and eight contained mutations. The direct action of alpha particles was thought to result in relatively large deletions, such as those detected by LOH. Therefore the low frequency of such changes (27%) compared with point mutations (47%) suggests that the genetic changes in the *TP53* gene in the liver tumours related to Thorotrast were not caused mainly by direct actions of alpha particles but rather by indirect effects that may have been due to cycles of necrosis and regeneration. This study was recently expanded to compare Thorotrast-induced liver cancers to those not associated with Thorotrast exposure. LOH at 37 loci was investigated. Liu et al. [L46] found frequent LOH at microsatellite markers D4S1538, D16S2624 and D17S1303 to be common to all the subtypes of liver cancer, independent of the specific carcinogenic agent. In contrast, LOH at marker D4S1652 was generally not observed in Thorotrast-induced cancers. LOH analysis revealed that Thorotrast-induced cancers share some LOH features with cancers not induced by Thorotrast, and Liu and colleagues concluded that induced LOH is not simply due to direct insult to DNA by alpha particles, but can occur through complex mechanisms, including bystander effects [L46]. Such a conclusion is reasonable given the analysis of Goto et al. [G18], who used imaging plate autoradiography to examine the microdistribution of alpha particles in pathological sections of tissues from Thorotrast patients. They found that the amount of thorium deposited in tumour tissue was correlated with that in non-tumour tissue, and that Thorotrast deposition was not associated with DNA damage determined by histochemistry. Goto et al. [G18] concluded that radioactive thorium always migrates in macrophages within the deposited organs, and that the organs are evenly exposed to alpha particles.

35. In an evaluation of Thorotrast-induced genomic instability, Liu et al. [L45] analysed microsatellite instability in Thorotrast-induced liver cancers. The frequency of microsatellite instability cases was 62.5% in Thorotrast-induced cancers, whereas it was 22.7% in non-Thorotrast induced cancers. Liu and colleagues suggested that microsatellite instability induced by exposure to Thorotrast mainly reflects clonal expansion of cancer cells and is partly due to inactivation of the DNA mismatch repair gene hMLH1 by hypermethylation. A recent finding also suggests that methylation changes in DNA can be associated with radiation-induced genomic instability [K35].

36. Littlefield et al. [L16] examined the cumulative genetic damage in a 72-year-old man who was treated with a 32 mL bolus of Thorotrast during cerebral angiography performed more than 40 years earlier. Peripheral T-lymphocytes were cultured to quantify the frequencies and cellular distributions of asymmetrical and symmetrical types of chromosomal aberrations. Assays of glycophorin A (GPA) mutations in red blood cells were also performed. Their results revealed that approximately 30% of the lymphocytes in this patient contained one or more chromosomal aberrations, the majority of which were of the “stable” type. About one third of the lymphocytes with chromosome damage carried multiple aberrations, suggesting that significant numbers of stem cells survived exposures to alpha particle radiation that induced complex genomic alterations. Increased frequencies of GPA mutations were observed, demonstrating that genomic damage was also induced in erythroid progenitors. Despite the relatively severe burden of somatic cell damage induced by 40 years of internal alpha particle irradiation, the patient remained free of any serious illness. Furthermore, these results provided no in vivo evidence for the continued expression of genomic instability. A similar observation was reported by Hande et al. [H3], using a fluorescence in situ hybridization technique that made possible the detection of intrachromosomal rearrangements and deletions. They described the quantification of stable intrachromosomal aberrations in lymphocytes of healthy former nuclear weapons workers who were exposed to plutonium. Even many years after occupational exposure, more than half the blood cells of the healthy plutonium workers contained large (>6 Mb) intrachromosomal rearrangements. The yield of these aberrations was highly correlated with plutonium dose to the bone marrow. It is significant that, despite the relatively high frequency of intrachromosomal aberrations, there was no evidence of transmissible chromosomal instability and no obvious detrimental health consequences in the populations sampled.

37. Nevertheless, a role for radiation-induced genomic instability has been described for solid tumours developing after radiotherapy for bilateral retinoblastoma [L1]. Genome alterations of second tumours (five osteosarcomas, one malignant peripheral sheath nerve tumour, one leiomyosarcoma) occurring in the field of irradiation of seven patients treated for bilateral retinoblastoma were studied. Because of a germ line mutation in the retinoblastoma gene (RB1), these patients were predisposed to develop radiation-induced tumours. In all radiation-induced tumours analysed, the normal RB1 allele was lost, whereas the germ line mutated allele was retained and the two *TP53* alleles were inactivated. A comparison of these tumours with the non-radiation-induced tumours led Lefevre et al. [L1] to conclude that this loss was due to the radiation-induced chromosomal instability rather than a direct effect of ionizing radiation. A similar observation was reported by Ryabchenko and colleagues [R12] after analysis of chromosomal aberrations in peripheral lymphocytes taken from Hodgkin’s disease patients after prolonged (up to 31 years) remission periods. The mean frequency and patterns of aberrations in remission patients were significantly different from comparison groups (healthy donors and primary Hodgkin’s disease patients). New cancer cases were diagnosed in a number of the remission patients, leading the investigators to suggest that the tumorigenic potential of radiochemotherapy is mediated via induction of genomic instability in exposed cells. Long after the therapy, the instability may become an initiating event in the development of new malignancies in affected tissues, whereas the instability induced in haemopoietic stem cells may reveal itself in peripheral lymphocytes derived from previously exposed precursor cells. The caveat, of course, is that these individuals were cancer patients and may have been inherently predisposed to second cancers.

E. Genomic instability and radiation-induced leukaemia

1. Mouse models

38. Plumb et al. [P8] have reviewed the relationship between radiation-induced genomic instability and radiation-induced leukaemia. They presented evidence that genomic instability plays a role during radiation leukaemogenesis. However, with the exception of a high incidence of non-clonal chromatid-type cytogenetic aberrations in neutron-induced acute myeloid leukaemia in mice [B26], the genetic lesions described (including non-clonal chromosomal aberrations, LOH and minisatellite/microsatellite mutations) were similar to those detected in de novo leukaemias and cancers. This damage was not transmissible and the authors interpreted these observations as evidence of apoptosis or other cell death. Nevertheless, this radiation-induced damage in vivo was indistinguishable from de novo multistage leukaemogenesis. Thus it is not yet possible to define a type of genomic instability in radiation-induced leukaemias in mice that demonstrates a specific characteristic of immediate or delayed effects of the initiating exposure to ionizing radiation. Evidence for radiation-induced genomic instability in mouse leukaemia and haemopoietic stem cells led MacDonald et al. [M1] to conclude that the induced instability contributed significantly to the induced leukaemia. A similar conclusion was reached by Ban et al. [B1], who suggested that loss of *TP53* function triggers the tumorigenic process leading to stem cell leukaemia through the induction of chromosomal instability. These authors also pointed out that the aetiology of stem cell leukaemia is likely to differ from that of myeloid leukaemia, because different results were observed in acute myeloid leukaemia (AML) [B1].

39. Interestingly, however, susceptibility to radiation-induced leukaemia is genetically separable from sensitivity to radiation-induced genomic instability [B13]. A series of matings, backcrosses and intercrosses between CBA/H mice susceptible to radiation-induced acute myeloid leukaemia and radiation-resistant C57BL/6 mice was carried out, and acute myeloid leukaemia and thymic lymphoma susceptibility was analysed. No simple genetic relationship between susceptibility to radiation-induced leukaemia and the sensitivity of the haemopoietic stem cells to induced instability was found.

2. Human studies

40. Cytogenetic analysis of leukaemia patients among the survivors of the atomic bombings in Japan revealed that patients exposed to >2 Gy exhibited a higher incidence of chromosomal aberrations and more complex chromosomal rearrangements than did patients exposed to lower radiation doses or unexposed patients [T1]. A more recent cytogenetic analysis of the heavily exposed patients with acute myelocytic leukaemia or myelodysplastic syndrome in the cohort of bombing survivors indicated persistent chromosomal instability [N6]. These cytogenetic observations are supported by studies demonstrating high frequencies of microsatellite instability in those bombing survivors with acute myelocytic leukaemia and a history of high exposure [N7]. These investigators concluded that this persistent instability might strongly influence the development of leukaemia in humans exposed to ionizing radiation. This study stimulated two letters to the editor of the journal in which it was published. The first, by Little [L40], claimed that although there was evidence that the microsatellite instability rate was higher in the AML cases among the bombing survivors than in the control group, the evidence that this higher rate was related to the radiation dose these cases received was weak. Little went on to show that the number of loci for which microsatellite mutation data were not detectable was higher in the bombing survivor cases than in the control group. The second letter, by Cox and Edwards [C15], raised the issue of the statistical strength of the dose-related association between expression of genomic instability in AML and the probability of causation by radiation. In response to these letters, Plumb [P18] pointed out that, from a biological perspective, the striking similarities in the leukaemias that arose in the bombing survivors and in therapy patients indicated that a significant proportion of the AML cases among the bombing survivors described by Nakanishi et al. [N6, N7] could indeed have been induced by the radiation. Reconciling mathematical models [L40] and statistical analysis [C15] with the biological observations will always be difficult, however, particularly with the small number of AMLs observed in this unique population.

41. Mazurik and colleagues [M63] investigated molecular, biochemical and cytogenetic parameters in blood samples from 17 radiation accident victims who between 1.7 and 43.8 years previously had suffered acute radiation sickness ranging in severity from grade I to grade IV. All patients showed ~25–30% reduction in oxidative status and increased levels of both stable and unstable chromosomal aberrations that correlated with the severity of the acute radiation sickness. These data were interpreted as evidence for delayed genomic instability in these radiation exposed individuals.

F. Role of telomeres and telomerase in radiation-induced genomic instability

42. Telomeres are specialized DNA–protein complexes at the ends of linear chromosomes. They are composed of a repetitive DNA sequence and associated proteins. Telomere alterations, caused by replication-mediated shortening, direct damage or defective telomere-associated proteins, can generate chromosomal instability, which can be observed in senescence and during the immortalization process (reviewed in reference [M47]). Telomeres are essential for proper maintenance of chromosomes and may play a role in ageing and cancer [G13, G14]. Telomere length can be maintained by telomerase [B25], or by the alternative mechanism of telomere lengthening which is telomerase-independent [M7, S50]. Telomere length abnormalities observed in

radiosensitive cells suggest that, in some human cells, short telomeres might correlate with radiation sensitivity [M49]. This may or may not be the case in mouse cells, as both hypersensitivity [G19] and the absence of an effect have been described [M49]. To complicate the matter further, long but dysfunctional telomeres have been found to correlate with chromosomal radiosensitivity in a mouse AML cell line 7926 [F13].

43. Another role for telomerase appears to be the de novo formation of telomeres, or chromosome healing to stabilize broken chromosomes [M41, S35]. Telomere loss results in sister chromatid fusion and prolonged breakage–fusion–bridge cycles leading to extensive DNA amplification and large deletions (reviewed in reference [M37]). Significantly, the loss of a single telomere can result in the instability of multiple chromosomes [S46] and generate many of the types of cytogenetic rearrangements commonly associated with human cancer. Telomere dysfunction can also trigger extensive DNA fragmentation and the evolution of complex chromosomal abnormalities in tumours in mice [A10] and humans [G11, M37]. This also appears to involve repeated cycles of dicentric chromosome formation, anaphase bridging, subsequent breakage and refusion events [G12]. In addition, telomere dysfunction can result in increased mutation rates and genomic instability (reviewed in references [F9, M37]).

44. It is noteworthy that exposure to ionizing radiation can induce telomerase activity both in vitro [H23, N27] and in vivo [H22]. This appears to be *TP53*-dependent [N26] and has been suggested as a measure for monitoring the radiocurability of tumour cells [S49]. Radiation-induced telomerase activation depends on dose rate, is not related to cell cycle redistribution or to the induction of cell death, and is likely to be the consequence of specific regulatory responses to ionizing radiation [P20].

45. Telomere repeat-like sequences are also seen as discrete bands at distinct intrachromosomal sites in a number of vertebrate species [M42]. These interstitial telomere sites probably represent ancestral telomere fusion events or amplification of the repeat sequences in ancestral karyotypes as latent telomeres [L39, M42]. There is compelling evidence that some of these interstitial telomere sites may be hot spots for both spontaneous [A11, H15] and radiation-induced chromosome damage [A9, B27, S36, S57, S58]. While it is clear that interstitial telomere sequences can influence the radiation sensitivity of chromosomes, many of these studies were performed in hamster cells where the interstitial telomeres are located at or near pericentromeric heterochromatin. Analyses of radiation sensitivity in a naturally occurring short interstitial telomere in a human chromosome (2q31, [A16]) and of a transfected 800bp telomeric repeat in human chromosome 4q indicate that human interstitial telomere sequences might not be prone to spontaneous [D24] or radiation-induced [D25] breakage.

46. The involvement of telomeric repeats in radiation-induced chromosomal instability was first described by Sabatier et al. [S44] in high-LET-irradiated human fibroblasts. They demonstrated that instability acquired by human chromosomes recurrently involved telomeric associations. This was not due to drastic telomere shortening [S45]. A role for recombination involving interstitial telomere-like repeat sequences in inducing chromosomal instability was later demonstrated by Day et al. [D1] in an in vitro Chinese human–hamster hybrid model system. In this instance it was apparent that rearrangements involving unstable chromosomes occurred preferentially at the sites of interstitial telomere sequences rather than at the true terminal telomeres as observed by Sabatier [S45]. This difference is likely to be due to the telomeres on transformed hamster chromosomes being very small relative to human telomeres [S56]. Ojima et al. [O6] have reported that telomeres are destabilized several generations after X-irradiation in normal human fibroblasts. Their data suggest X-irradiation might not affect telomeres directly, but rather by inducing a delayed instability.

47. Initially it was thought that an interstitial telomere-like repeat sequence on chromosome 2 played an important role in the deletions in somatic haemopoietic cells that characterized the earliest phases of radiation-induced AML in the CBA/H mouse [B28]. Subsequent detailed molecular analysis of break points suggested that, while telomere sequences were located close to regions frequently involved, break points were not exactly coincident with telomere sequences [F10]. The regions of frequent breakage appeared to have properties expected of matrix/scaffold attachment regions [F10]. At this stage one can only speculate on whether telomeric changes are a cause, an effect or a combination of both in contributing to genomic instability independent of radiation exposure.

G. Conclusions

48. Radiation-induced genomic instability is now well established in a number of normal and transformed cell lines in vitro. Instability can manifest as multiple different end points and can result from both targeted and non-targeted events. The results of studies of induced instability in vivo are more complex and contradictory. In mice, observation of the instability phenotype appears to be dependent upon the mouse strain used and the power of the investigation in terms of the numbers of animals investigated. The evidence for induced instability in exposed human populations is controversial, with both positive and negative effects being reported. It is possible that radiation-induced genomic instability provides the impetus for those genomic alterations associated with radiation carcinogenesis. This view should be tempered by the high frequency with which instability is observed both in vitro and in vivo, and the general lack of a dose–response curve. Overall, a specific role for induced instability in the genesis of radiation-induced cancers has yet to be definitively demonstrated.

II. BYSTANDER EFFECTS AND RADIATION EXPOSURE

49. There has been a resurgence of interest in radiation-induced bystander effects, largely because of the development of single-cell charged-particle irradiators. The term "bystander effect" was adopted from the gene therapy literature, where it usually refers to the killing of several subpopulations of tumour cells by targeting only one "type" of cell within a heterogeneous population [F6]. For the purposes of this annex, the definition proposed by Djordjevic will be used [D5]. That is, "bystander effect" describes the ability of cells affected by an agent to convey manifestations of damage to other cells not directly targeted by the agent or not necessarily susceptible to it per se. Thus radiation-induced bystander effects are effects manifesting in cells that were non-irradiated neighbours of irradiated cells or that received factors secreted or shed by irradiated cells. It is implicit in this review that in vitro bystander effects are the result of a signal generated by an irradiated cell interacting with a non-irradiated cell [C22] and are not the result of radiation-induced changes in the culture medium [Z2], or due to experimental variables such as the cell culture environment [M50]. This underscores the critical role of adequate and appropriate controls. A historical perspective describing key events in the study of radiation-induced bystander effects has been presented by Mothersill and Seymour [M45].

A. Bystander effects in vitro

50. Radiation-induced bystander effects in vitro embrace a number of different non-targeted experimental effects, some of which are likely to be detrimental to the cell, whereas others are not. Different effects are observed in different cell types, and depend on the cell type producing the bystander signal after irradiation and the cell type receiving the bystander signal (summarized in table 3). Consequently, no rigid rules can be applied to the multitude of responses occurring in cells not targeted by radiation. For convenience, bystander effects have been divided into four, not necessarily mutually exclusive, subcategories.

Table 3 In vitro studies of the bystander effect

Year	*Origin of bystander effect*	*End point*	*Cell type*	*Radiation type*	*Reference*
1992	Low-fluence alpha particle irradiation	Sister chromatid exchanges	Human fibroblasts and epithelial cells	Alpha particles	[N4]
1996	Low-fluence alpha particle irradiation	Sister chromatid exchanges	Human lung fibroblasts	Alpha particles	[D2]
1997	Medium transfer	Sister chromatid exchanges	Human lung fibroblasts	Alpha particles	[L2]
1997	Medium transfer	Clonogenic survival	Human epithelial cells; human fibroblasts	Gamma rays	[M18]
1997	Medium transfer	Clonogenic survival	Human keratinocytes	Gamma rays	[S12]
1997	Microbeam irradiation	Si- mutants induced	A_L human–hamster hybrid cells	Alpha particles	[H14]
1998	Low-fluence alpha particle irradiation	p53, p21, MDM2, CDC2, RAD5 1 protein levels	Human fibroblasts	Alpha particles	[A3]
1998	Low-fluence alpha particle irradiation	Plating efficiency; chromosomal aberrations	Murine bone marrow cells	Alpha particles	[L24]
1998	Medium transfer	Clonogenic survival	Human keratinocytes	Gamma rays	[M22]
1998	Microbeam irradiation	Micronucleus frequency; apoptosis	Human fibroblasts	Alpha particles	[P10]
1999	Cytoplasmic irradiation by microbeam	Clonogenic survival; CD59 mutation frequency	A_L human–hamster hybrid cells	Alpha particles	[W13]
1999	Low-fluence alpha particle irradiation	HPRT mutations	Chinese hamster ovary cells	Alpha particles	[N5]

Year	*Origin of bystander effect*	*End point*	*Cell type*	*Radiation type*	*Reference*
2000	Cytoplasmic irradiation by microbeam	Clonogenic survival; CD59 mutation frequency	A_L human–hamster hybrid cells	Alpha particles	[Z1]
2000	Low-fluence alpha particle irradiation	GI checkpoint	Human fibroblasts	Alpha particles	[A8]
2000	Medium transfer	Clonogenic survival; AP-endonuclease; *TP53*; ROS	Human lung fibroblasts (HFL-I)	Alpha particles	[I10]
2000	Medium transfer	Clonogenic survival; intracellular calcium levels; mitochondrial membrane potential; ROS levels	Human keratinocytes	Gamma rays	[L28]
2000	Medium transfer	Clonogenic survival	Human keratinocytes	Gamma rays	[S13]
2001	Co-culture	Clonogenic survival	V79 Chinese hamster cells (3-D tissue culture model)	^{3}H beta particles	[B24]
2001	Co-culture	p53, HSP72 protein levels	A-172 human glioblastoma cells	X-rays	[M3]
2001	Co-culture	Enhanced plating efficiency; micronucleus frequency	Human salivary gland cells	X-rays or carbon beam	[S30]
2001	Low-fluence alpha particle irradiation	Changes in gene expression; induction of DNA damage	Human fibroblasts and epithelial cells	Alpha particles	[A4]
2001	Medium transfer	Clonogenic survival; apoptosis; transformation frequency	CGLI human HeLa × skin fibroblast hybrid cells	X-rays	[L5]
2001	Medium transfer	Clonogenic survival; intracellular calcium levels; mitochondrial membrane potential; ROS levels	Human keratinocytes	Gamma rays	[L38]
2001	Medium transfer	Clonogenic survival	Human urothelium cells	Gamma rays	[M39]
2001	Microbeam irradiation	Micronucleus frequency	Primary human fibroblasts	Alpha particles	[B9]
2001	Microbeam irradiation	Transformation frequency	C3H 10T½ murine fibroblasts	Alpha particles	[S6]
2001	Microbeam irradiation	Clonogenic survival	C3H I0T½ murine fibroblasts	Alpha particles	[A7]
2002	Co-culture	Enhanced plating efficiency and proliferation	Human salivary gland cells	Carbon beam	[S15]
2002	Co-culture on double Mylar dishes	Clonogenic survival; CD59 mutation frequency	A_L human–hamster hybrid cells	Alpha particles	[Z4]
2002	Low-fluence alpha particle irradiation	Micronucleus frequency	Human fibroblasts	Alpha particles	[L43]
2002	Low-fluence alpha particle irradiation	HPRT mutations; sister chromatid exchanges	Human fibroblasts and epithelial cells	Alpha particles	[L37]
2002	Low-fluence alpha particle irradiation	Chromosomal aberrations	Chinese hamster ovary cells	Alpha particles	[N3]
2002	Low-fluence alpha particle irradiation	HPRT mutations; sister chromatid exchanges	Chinese hamster ovary cells	Alpha particles	[N21]
2002	Medium transfer	Clonogenic survival; AP-endonuclease; *TP53*; ROS	Human lung fibroblasts (HFL-I)	Alpha particles	[I1, I5]
2002	Medium transfer	Clonogenic survival; intracellular calcium levels; mitochondrial membrane potential; ROS levels	Human keratinocytes	Gamma rays	[L26, L27]
2002	Medium transfer	Clonogenic survival	Thirteen cell lines: human epithelial carcinoma cells, SW48 human colon carcinoma cells	Gamma rays	[M21, M38]
2002	Microbeam irradiation	Micronucleus frequency; apoptosis	Sections of human and porcine ureter	Alpha particles	[B7]

Year	*Origin of bystander effect*	*End point*	*Cell type*	*Radiation type*	*Reference*
2002	Microbeam irradiation	Clonogenic survival	V79 Chinese hamster cells	Alpha particles	[S27]
2003	Co-culture	Micronucleus frequency	Human fibroblasts	Carbon-ion beam: beta particles	[S28]
2003	Co-culture	Enhanced plating efficiency and proliferation	Human salivary gland cells	Carbon beams	[S29]
2003	Low-fluence alpha particle irradiation	HPRT mutations	Chinese hamster ovary cells	Alpha particles	[N20]
2003	Medium transfer	Clonogenic survival	Human keratinocytes	Gamma rays	[M27]
2003	Microbeam irradiation	Micronucleus frequency; apoptosis	Sections of human and porcine ureter	Alpha particles	[B8]
2003	Medium transfer	Apoptosis; micronuclei	Human–hamster hybrid cells (GM10115; Fe10-3; LS12)	X-rays	[N1, N2]
2003	Carbon ion beam	Micronuclei; gap junctions; ROS	Human fibroblasts	Carbon ion beam	[S28]
2003	Helium ion microbeam	Micronuclei; nitric oxide	T98G cell nuclei from human glioblastoma	Helium ion beam	[S16]
2003	Microbeam irradiation	A_L cell mutagenic assay	A_L cells	Charged particle microbeam; alpha particles; X-rays	[Z6]
2004	Medium transfer	RPA expression	Primary human fibroblasts	Gamma irradiation	[B42]
2004	Medium transfer	Micronucleus frequency	Chinese hamster ovary cells	Ultrasoft X-ray microprobe	[K31]
2004	Medium transfer	Transformation frequency	HeLa × skin fibroblast hybrid cells	Low-dose X-rays	[K32]
2004	Co-culture	Cell proliferation using ^{3}H-TdR incorporation	Antigen presenting cells (J774A.1) and T-lymphocytes (EL-4)	Low-dose irradiation	[L44]
2004	Chronic and acute irradiation	iNOS accumulation	WTP53 cells	Chronic gamma rays; acute X-rays	[M59]
2004	Co-culture and medium transfer	Oncogenic transformation frequency	C3H 10T½ cells	High- and low-dose X-rays	[M51]
2004	Cell–cell contact during irradiation	Oncogenic transformation frequency	C3H 10T½ cells	X-rays	[M43]
2004	Co-culture and medium transfer	Cloning efficiency; cell numbers	Repair-deficient human cell lines	Gamma rays	[M44]
2004	Co-culture	Chromosomal instability	Human fibroblast BJ1-htert	Alpha irradiation	[P22]
2004	Co-culture	Apoptosis; necrosis	LY (L5178Y) suspension cells; human salivary gland cells	Carbon ions; X-rays	[S31]
2004	Co-culture	Micronuclei; nitric oxide	Glioma cells; primary human fibroblasts	He ion particles	[S32]
2004	Co-culture	A_L cell mutagenic assay	A_L cells	Low-dose X-rays	[Z10]
2004	Microbeam irradiation	Micronuclei and cell cycle delays	Human fibroblasts	90 keV/μm alpha particles	[P6]
2005	Co-culture	Transposition of chromosomal loci	Human lymphocytes	X-rays (10 cGy)	[T4]
2005	Medium transfer	Micronucleus frequency; apoptosis		X-rays	[K33]
2005	Medium transfer	Mutation and deletion in mitochondrial DNA	Human keratinocytes (HPV-G)	Gamma irradiation	[M60]
2005	Medium transfer	Micronucleus frequency; HPRT mutation frequency	GM10115 human–hamster hybrid cells	X-rays	[N30]
2005	Co-culture	Sister chromatid exchanges and chromosomal aberrations	Hamster cell lines (V3 and irs3)	Low-fluence alpha particles	[N25]

Year	*Origin of bystander effect*	*End point*	*Cell type*	*Radiation type*	*Reference*
2005	Co-culture	Micronuclei; nitric oxide; ROS	Glioma cells (T98G) and fibroblasts (AG01522)	Helium particles	[S23]
2005	Medium transfer	Micronuclei; induction of $p21^{waf1}$ protein; gamma H2AX foci; ROS; clonogenic survival	Human fibroblasts	X-rays	[Y5]
2005	Bystander assay	COX-2 signalling involving mitogen-activated protein kinases		Charged particle beam	[Z5]
2005	Co-culture	Micronuclei; apoptosis	Mouse embryonic stem cells	Alpha particles	[Z7]
2006	Apoptosis from bystander effect	Iodine incorporation for apoptosis; gap junctions; connexin-43 expression	Non-small-cell lung cancer	Ionizing radiation	[Z9]

1. Bystander effects after cytoplasmic irradiation

51. The most convincing demonstration of the bystander effect has come from studies using charged-particle microbeams [F3, F4, F5, R1]. The microbeam is capable of putting an exact number of particles through a specific subcellular compartment of a defined number of cells in a particular radiation environment.

52. Using the microbeam at the Radiological Research Accelerator Facility of Columbia University in the United States [R1], Wu et al. [W13] targeted and irradiated the cytoplasm of human–hamster (A_L) cells. They observed a significant increase in mutations at the CD59 (S1) nuclear gene locus while causing minimal cytotoxicity. Cytoplasmic irradiation with a single alpha particle doubled the spontaneous mutation frequency, while a two- to threefold increase was observed after four cytoplasmic traversals. The mutation spectrum was similar to the spontaneous, non-irradiated mutation spectrum, but different from that observed after targeted nuclear irradiation. The addition of the free radical scavenger dimethyl sulphoxide or the intracellular glutathione inhibitor buthionine-S-R-sulfoximine indicated that the mutagenicity of cytoplasmic irradiation depended on the generation of ROS (figure IV). Shao et al. [S32] also used a charged-particle microbeam, at the Gray Cancer Institute in the United Kingdom, to target individual glioma cells cultured alone or in co-culture with primary human fibroblasts. They found that even when only a single cell within the glioma cell population was precisely traversed through its cytoplasm with one helium ion, bystander responses were induced in the neighbouring non-irradiated glioma cells or fibroblasts. Significantly, the yield of bystander-induced micronuclei was similar when the cytoplasm or nucleus of a cell was targeted, indicating that direct DNA damage is not required for switching on cell-signalling mechanisms after low-dose irradiation. Two important conclusions can be reached from these experiments. First, because bystander effects are observed after cytoplasmic irradiation, the target for genetic effects of radiation is larger than the nucleus. Secondly, cytoplasmic traversal by alpha particles may be more deleterious in the long term than nuclear traversal. This is because, as the number of nuclear traversals increases, the probability of cell killing increases, whereas after cytoplasmic irradiation the increased mutagenicity occurs where there is negligible killing of the irradiated cells [W13].

2. Bystander effects after low fluences of alpha particle irradiation

53. It is implicit in the evaluation of bystander effects that bystander cells were not hit by the radiation but received signals from an irradiated cell that generated a response in the bystander cell. Broad-beam irradiation with low fluences of alpha particles does not traverse every cell in the radiation environment, and data suggest that a sizeable portion of the damage observed after exposure to low fluences of alpha particles results from responses occurring in cells that were not actually traversed by an alpha particle.

54. Nagasawa and Little [N4] observed small increases in sister chromatid exchange frequency in ~30% of cells analysed, even though <1% of the cell nuclei were actually traversed by an alpha particle. This observation was later confirmed and extended by Lehnert and co-workers [D2, L2]. The induced sister chromatid exchanges could be inhibited by superoxide dismutase, once again indicating a role for ROS [L2, N8, N9]. The alpha-particle-induced increase in ROS appears to be temporally linked to enhanced production of tumour necrosis factor alpha and interleukin 1, which in turn operate in an autocrine manner to up-regulate interleukin 8 [N9]. Low fluences of alpha particles can also increase mutation yield [N5] and cause accumulation of the tumour suppressor protein *TP53* in a higher percentage of the exposed population than calculated to receive a nuclear traversal by one or more alpha particles [H5]. Whether or not mutation induction or induced gene expression is mediated by ROS is uncertain, but whatever the mechanism, it involves gap-junction-mediated intercellular communication in the transmission of damage signals from irradiated to non-irradiated cells (figure V[A]). By examining changes in gene expression after low-fluence alpha particle irradiation, Azzam et al. [A3, A4] demonstrated the involvement of connexin-43-mediated intercellular communication in the transmission

of damage signals to non-irradiated cells. In gap-junction-competent cells, induction of $p21^{Waf1}$ protein far exceeded the fraction of cells whose nuclei had been traversed, and correlated with the induction of micronuclei as a measure of DNA damage as well as with increased Ser-15 phosphorylation of *TP53* (reviewed in reference [A5]).

Figure IV. Induced S1 mutations in human–hamster A_L cells per 10^5 survivors.

AL cells were irradiated with four alpha particles through the nucleus (1) or the cytoplasm (2). Dimethyl sulphoxide (8%) from 10 minutes before until 10 minutes after cytoplasmic irradiation with four alpha particles significantly suppressed mutation yield (3). Treatment with d-dimethyl sulphoxide alone did not increase mutation frequency (4). In contrast, pretreatment of A_L cells with a 10 μM dose of buthionine-S-R-sulfoximine for 18 hours, which reduced the intracellular glutathione content to <5% of control levels, increased the mutagenicity of cytoplasmic irradiation with four alpha particles by four- to fivefold (5). Treatment with buthionine-S-R-sulfoximine alone had no significant effect on mutation frequency (6). These data strongly implicate ROS as being the mediator of the mutagenic response of cytoplasmic irradiation (adapted from reference [W13]). The insert represents a schematic of the irradiation protocol.

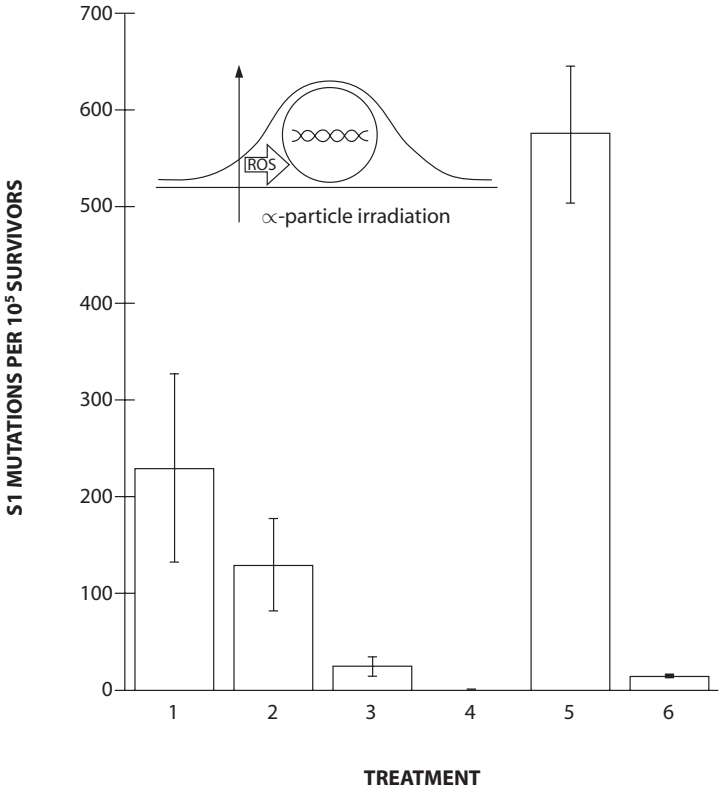
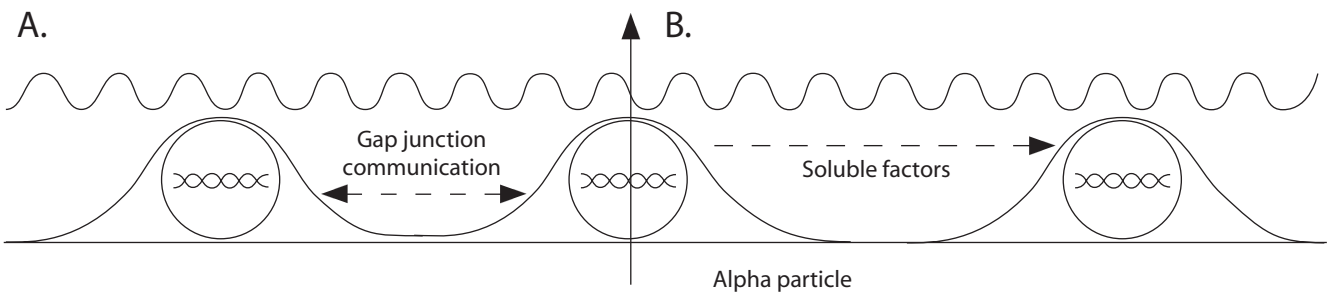


Figure V. Bystander effects are those effects occurring in cells that were not directly subjected to the deposition of energy by radiation but were in contact with irradiated cells or received a signal from an irradiated cell.

A cell is irradiated through the nucleus with an alpha particle (vertical arrow). This irradiated cell then communicates a signal to a non-irradiated bystander cell by intercellular cell-to-cell gap junction communication (A) or the transmission of soluble factors from the irradiated cell to the non-irradiated cell via the cell medium (B).

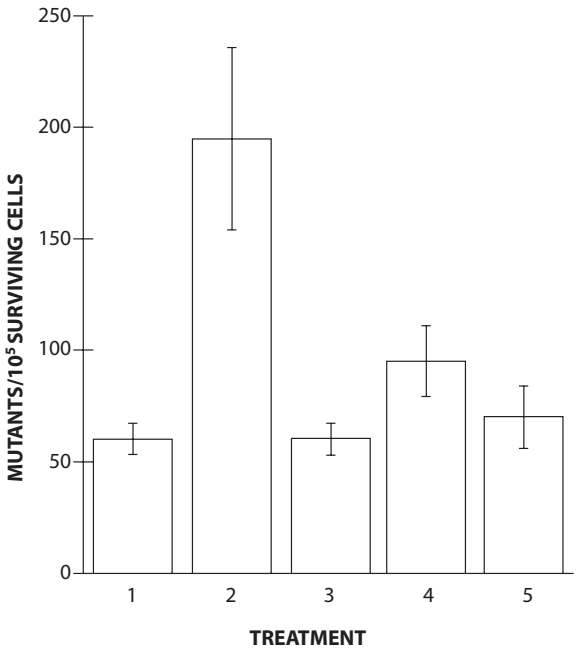


55. A bystander effect for chromosomal aberrations induced by low fluences of alpha particles in wild-type and repair-deficient (xrs-5) Chinese hamster ovary cells has been described [N3]. Gross chromosomal aberrations are generally associated with DNA double-strand breaks [M15], suggesting that the factors responsible for the bystander effect are also ultimately capable of cleaving the double helix. As an explanation of their data, Nagasawa and Little [N3] proposed that the relatively small bystander effect in wild-type cells was due to proficient double-strand break repair as compared with the significantly enhanced bystander effect, manifesting as dramatic increases in aberration yields in repair-deficient xrs-5 cells. This study was expanded to examine potential bystander effects for sister chromatid exchanges and chromosomal aberrations in hamster cell lines deficient in either DNA-PKcs (V3 cells, deficient in non-homologous DNA end joining) or RAD51C (irs3 cells, deficient in homologous recombination). Cells were irradiated with very low fluences of alpha particles such that <1% of the nuclei were traversed by an alpha particle. Wild-type cells showed a prominent bystander response for sister chromatid exchange induction; an even greater effect was observed in V3 cells. On the other hand, no significant induction of sister chromatid exchanges was observed in the irs3 RAD51C-deficient bystander cells. In contrast, a marked bystander effect for chromosomal aberrations occurred in V3 cells, and the induction of chromosomal aberrations in irs3 bystander cells was minimal and similar to that of wild-type cells. On the basis of these findings, Nagasawa et al. [N25] hypothesized that homologous recombination is essential for the induction of sister chromatid exchange in bystander cells; but homologous recombination is unable to repair the damage induced in non-homologous end-joining-deficient bystander cells that leads to either sister chromatid exchange or chromosomal aberrations.

3. Bystander effects after irradiation with a charged-particle microbeam

56. Using human–hamster hybrid AL cells, Zhou et al. [Z1] located all cells in the radiation environment and exposed 20% of these to 20 alpha particles using the Columbia University microbeam. This dose of radiation allowed less than 1% of the irradiated cells to survive. They then assayed surviving cells for mutations in the target human chromosome and found a mutation frequency four times that of background (figure VI). Since the irradiated cells were exposed to lethal doses of radiation, these mutations must have arisen in non-exposed bystander cells. Furthermore, the mutation spectrum observed in bystander cells was significantly different from the spontaneous spectrum and from that observed after cytoplasmic irradiation, suggesting that different mutagenic mechanisms were involved in the two processes [Z1].

Figure VI. CD59 mutants per 10^5 surviving A_L cells.
(1) Non-irradiated cells. (2) Observed mutation yield when 20% of A_L cells were exposed to a lethal dose of 20 alpha particles per cell. (All cells irradiated with 20 alpha particles should have been killed.) (3) Expected mutation yield when 20% of A_L cells were exposed to a lethal dose of 20 alpha particles per cell. (4) Effect of two hours of pretreatment and 72 hours of post-treatment with 40 μM Lindane when 20% of A_L cells were exposed to a lethal dose of 20 alpha particles per cell. (5) Effect of 40 μM Lindane alone on A_L cells (adapted from reference [Z1]).



57. Unlike cytoplasmic irradiation [W13] or the bystander effect reported by Lehnert et al. after low fluences of alpha particle irradiation [L2, N8, N9], the bystander effect described by Zhou et al. [Z1] was not modulated by addition of the free radical scavenger dimethyl sulphoxide. Instead, when A_L cells were treated with the cell-to-cell gap junction communication inhibitor Lindane, the bystander effect was significantly reduced but not eliminated (figure VI).

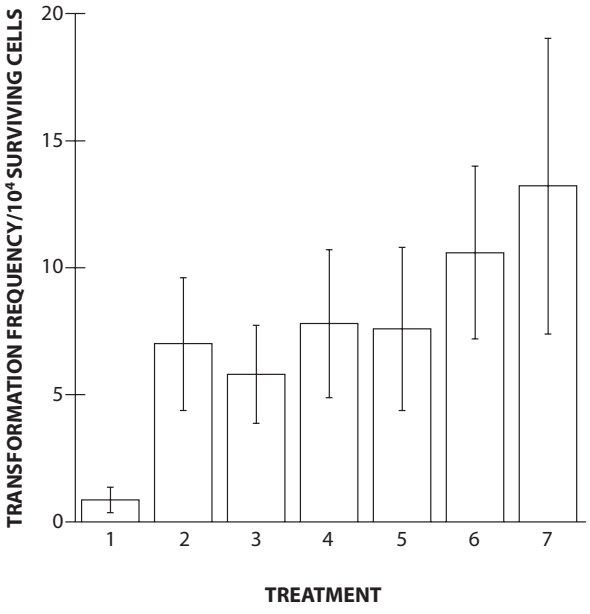
58. In a subsequent study, Zhou et al. [Z3] used the Columbia University microbeam to deliver exactly one alpha particle through the nuclei of 5%, 10%, 20% or 100% of the A_L cells. Their results indicated that the frequencies of induced mutations and chromosomal changes in populations where known fractions of nuclei were hit were consistent with non-hit cells contributing significantly to the response. When 10% of a confluent mammalian cell population was irradiated with a single alpha particle, the mutation yield was similar to that observed when 100% of the cells were irradiated. This effect was significantly reduced in cells pretreated with octanol, which inhibits gap-junction-mediated intercellular communication, or in cells carrying a dominant negative connexin-43 vector. These results indicate that a single alpha particle can induce genomic damage in cells that were not irradiated. Since a cell cannot receive a

lower dose of radiation after exposure to alpha particles than a single traversal, these data suggest that at the very lowest radiation dose of alpha particles, i.e. a single particle, the genotoxic risk for high-LET radiation may be underestimated. It should be noted that traversal of a single alpha particle results in a dose of 0.074–0.17 Gy, depending upon the nuclear cross-sections of a given cell type and assuming an average LET of 90 keV/μm [R1].

59. The precision of intracellular irradiation and the high throughput available at the microbeam facility at Columbia University have also enabled studies of oncogenic transformation to be carried out. Sawant et al. [S6] utilized sparsely populated monolayers of mouse C3H 10T½ cells and irradiated the nucleus of every cell, or every tenth cell at random, with either two or four alpha particles. The yield of transformed foci was determined morphologically, and the frequency of transformation was similar whether 100% or only 10% of the cells were irradiated (figure VII).

Figure VII. Transformation frequency in C3H 10T½ cells in vitro.

(1) Non-irradiated controls. (2) 10% of the cells received two alpha particles. (3) 100% of the cells received two alpha particles. (4) 10% of the cells received four alpha particles. (5) 100% of the cells received four alpha particles. (6) 10% of the cells received eight alpha particles. (7) 100% of the cells received eight alpha particles (adapted from reference [S6]).

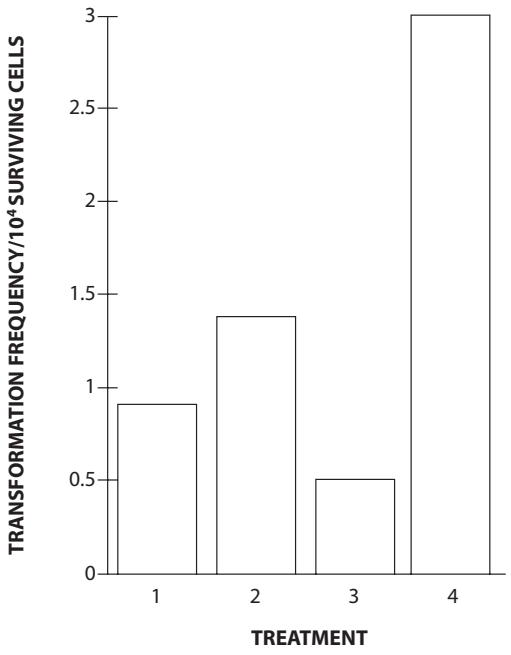


Furthermore, Lindane (the cell-to-cell communication gap junction inhibitor) reduced but did not eliminate this bystander effect. In subsequent studies from the Columbia University group, a role for secreted factors or factors released from irradiated cells into the culture medium has also been identified. However, it is clear from studies of bystander-induced cell killing and oncogenic transformation that cell-to-cell gap junction communication is the most important mediator of the bystander effect in C3H 10T½ cells after microbeam irradiation [M43].

60. Related studies using microbeam-generated alpha particles have important implications for evaluating potential hazards associated with radiation exposure [M6]. One alpha particle per nucleus, the lowest possible dose of high-LET radiation, effectively elicits a bystander response in different cell types [B9, Z3]. By comparing the biological effectiveness of exposure to exactly one alpha particle per cell with that expected from normal broad-beam irradiation similar to that used in the experiments described above (using a mean of one particle per cell), it is clear that exactly one particle per cell is less biologically effective than a mean of one particle per cell. Miller et al. [M6] found that the transformation frequency after irradiation with exactly one alpha particle per nucleus did not differ significantly from that observed in non-irradiated cells (figure VIII). This suggested that the increased transformation frequency observed after broad-beam alpha irradiation, where a mean of one alpha particle traversal per cell results in a Poisson distribution of particles per cell, is a consequence of the minority of cells subjected to multiple (≥2) alpha particle traversals.

Figure VIII. Transformation frequency in C3H 10T½ cells in vitro.

(1) Non-irradiated controls for the microbeam experiment. (2) Cells receiving exactly one alpha particle per nucleus from the Columbia University microbeam. (3) Non-irradiated controls for the broad-beam experiment. (4) Cells receiving a mean of one alpha particle per nucleus after broad-beam irradiation. Note that the transformation frequency in cells receiving exactly one alpha particle per nucleus is only slightly different from that in the non-irradiated controls (adapted from reference [M6]).



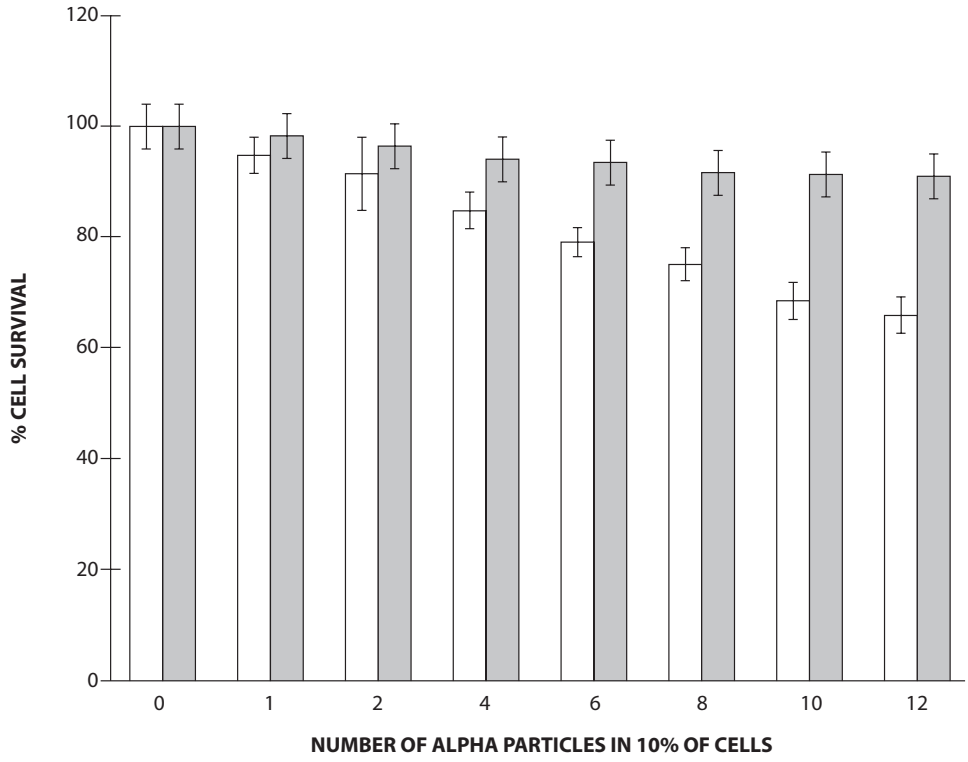
61. An important advantage of using low fluences of alpha particles or a charged-particle irradiator is that it represents environmentally relevant exposure conditions, where most cells in a tissue would not be traversed by an alpha particle. It should be stressed, however, that to irradiate cells in vitro with a high-energy alpha particle, target cells must be grown as monolayers on a Mylar substrate. Under these culture conditions, cells by necessity are more flattened and more elongated that would reasonably be expected in a three-dimensional in vivo situation. Consequently, the energy deposited per traversal is much less biologically effective. To address this issue, three-dimensional model tissue culture systems are being developed. Any evaluation of potential hazards associated with these types of high-LET radiation must consider these unique cell culture conditions.

62. There is also a microbeam at the Gray Cancer Institute, where ^{3}He ions with an LET of 100 keV/μm have been used for targeted cellular irradiation [F5]. In addition, the Gray Cancer Institute microbeam has recently been adapted so that the effects of ultrasoft X-rays can also be investigated [F3]. Using this microbeam, evidence for a bystander effect in primary human fibroblasts, as measured by micronucleus formation and cell killing, revealed significantly more damage than expected on the basis of direct effects of radiation [B7, B8, B9, P11, P12]. The bystander effects observed using the Gray Cancer Institute microbeam can occur when cells are considerable distances apart from one another and not in contact, thus eliminating potential cell-to-cell gap junction communication [B9, P10]. This indicates that radiation-induced soluble factors may be released into the culture medium and affect non-irradiated cells (figure V[B]), and suggests that there are at least two different types of bystander effect, those effects mediated by cell-to-cell gap junction communication and those induced by secreted soluble factors (figure V). Alternatively, there are at least two mechanisms for the same biological effect. Intriguing data from Sawant et al. [S7] indicated that, as the number of alpha particles through a fixed (10%) number of cells increases, more bystander-induced cell killing occurs (figure IX). These observations suggested that increasing induced damage in 10% of the cells in the culture produced more cell-to-cell gap junction communication and/or increased secretion of cytotoxic substances into the culture environment. A strategy combining medium transfer experiments and gap junction inhibitor studies would enable investigators to determine the relative contributions of these bystander-mediated cytotoxic effects and ultimately this would aid in identification of the secreted factors.

63. Interestingly, some novel insights into the mechanisms of radiation-induced bystander effects in vitro have been revealed using the single-cell microbeam at the Gray Cancer Institute. When human glioblastoma T98G cell nuclei were individually irradiated with an exact number of helium ions, it was found that, when only one cell in a population of approximately 1,200 cells was targeted, cellular damage

Figure IX. Bystander effect for cell survival.

Ten per cent of the cells, selected randomly, were exposed to increasing numbers of alpha particles from 2 to 12, and the percentage of cell survival was determined by reduction in plating efficiency. Open bars: experimentally determined survival. Filled bars: expected survival if only irradiated cells were killed (adapted from reference [S7]).



measured as induced micronuclei was increased by 20% [S16]. When the percentage of cells individually targeted was increased from 1% to 20%, the yield of micronuclei in the population greatly exceeded that predicted on the basis of the yield when all of the cells were targeted assuming no bystander effect. However, when 2-(4-carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO), a nitric-oxide-specific scavenger, was present in the culture medium, the micronucleus yields decreased to the predicted values. This indicates that nitric oxide contributed to the bystander effect. Moreover, the medium harvested from cells targeted with helium ions showed a cytotoxic effect by inducing micronuclei in non-irradiated T98G cells, and this bystander response was also inhibited by c-PTIO treatment. The induction of micronuclei in the population could also be decreased by c-PTIO treatment when 100% of cells were individually targeted by one or two helium ions, indicating a complex interaction of direct irradiation and bystander signals. A role for nitric oxide in the medium-mediated bystander effect has now been described in a number of studies [S15, S16, S31, S32]. Furthermore, the secretion of nitric oxide as a bystander effect has been linked to the induction of radioresistance in recipient cells [M3, M4]. However, it is not clear whether nitric-oxide-mediated bystander effects occur in conjunction with, or independent of, increased levels of ROS, and how this might affect the characterization of subsequent radiation sensitivity.

64. Microbeam experiments using high-Z elements (460 MeV ^{40}Ar, 1,260 keV/µm and 260 MeV ^{20}Ne, 380 keV/µm) have also been performed at the Japan Atomic Energy Research Institute [S33]. Confluent normal human fibroblasts were targeted and the induced micronuclei evaluated after replating the cells. Even when only a single cell was hit, a 1.4-fold increase in the frequency of micronuclei was observed, indicative of a bystander effect. The observed increase in micronucleus frequency saturated when four cells were targeted and could be suppressed when dimethyl sulphoxide (a scavenger of ROS) or PMA (an inhibitor of gap junction communication) was present at the time of irradiation. Thus a role for nitric oxide, ROS and cell-to-cell gap junctions has been invoked in bystander responses.

65. It is likely, however, that there are multiple bystander pathways. By using the Columbia University charged particle beam in conjunction with a strip dish design, Zhou et al. [Z5] show that the cyclooxygenase-2 (COX-2, also known as prostaglandin endoperoxide synthase-2) signalling cascade plays an essential role in the bystander process. Treatment of bystander cells with NS-398, which suppresses COX-2 activity, significantly reduced the bystander effect. This provided evidence that the COX-2-related pathway, which is essential in mediating cellular inflammatory response, is a critical signalling link for the bystander phenomenon in this assay system. Furthermore, any signalling pathway likely to be involved is complicated by the genotype of the exposed organism. For example, Zhu et al. [Z7] examined the ability of mouse embryonic stem cells differing in the status of the DNA repair gene, Rad9, to express a bystander effect after exposure to alpha particles. All populations, when confluent, demonstrated a dose-independent bystander effect with respect to cell killing and apoptosis. Minimal alpha particle induction of micronuclei in bystander cells was observed, except for the Rad9$^{-/-}$ mutant, where a significant increase above background was detected. Therefore the Rad9 null mutation selectively sensitizes mouse embryonic stem cells to spontaneous and high-LET-radiation-induced bystander apoptosis and micronucleus formation, but it has much less impact on cell killing by direct or bystander alpha particle exposure.

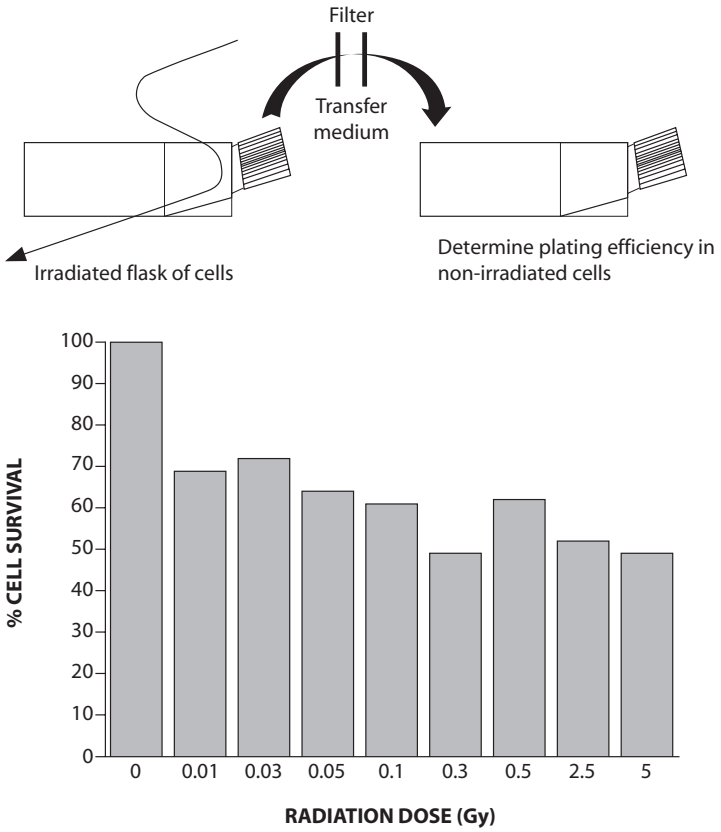
66. At present there is no detailed information on potential bystander effects occurring after cellular exposure to low-LET radiation in non-irradiated cells subsequently cultured in the same physical proximity. This is because the development of focused low-LET radiation sources has lagged well behind that of high-LET charged particle microbeams, owing to practical and technical complications. Gamma rays interact with matter in a number of ways, including photoelectric absorption, Compton scattering and pair production. Since the electrons will undergo scattering as they exit the vacuum system, it is not practical at present to irradiate single cells or subcellular structures, as is possible with the charged particle microbeams described above. However, by using pulsed electron beams, a low-LET microbeam can be constructed that will mimic many important aspects of the interaction of gamma rays with cells. Such devices are currently available at the University of Maryland [S37] and Texas A&M University [W16] in the United States. Consequently, it is anticipated that in the foreseeable future many of the studies that have been performed with high-LET alpha particles or intermediate-LET ultrasoft X-rays can be replicated using these new low-LET microbeams. In addition, the ability to experimentally vary the LET distribution by changing the incident beam energy will allow investigation of the relative biological importance of various parts of the energetic electrons' track and hence evaluate how this might modulate bystander responses [S38].

4. Bystander effects after transfer of medium from irradiated cells

67. Mothersill and Seymour [M18] demonstrated a significant reduction in plating efficiency in non-irradiated cultures of human epithelial cells (but not fibroblasts) that received culture medium from irradiated cultures. These observations indicated that irradiated cells secreted a toxic substance, a "bystander factor", into the culture medium that can kill non-irradiated cells (figure X). Medium irradiated in the absence of cells had no effect on survival when transferred to non-irradiated cells. Not all cells are capable of producing the toxic bystander factor, nor are all cells capable of receiving and responding to the secreted signal [M17, M18, M22, M25]. The effect was dependent on the cell number at the time of irradiation, could be observed as early as 30 minutes post-irradiation, and was still effective when medium transfer occurred 60 hours after irradiation.

Figure X. Bystander effects in an immortalized human keratinocyte cell line as demonstrated by medium transfer experiments.

Upper portion: schematic of the experimental protocol. A flask of cells is irradiated, and as a function of time post-irradiation, medium is removed from the irradiated cells, filtered and transferred to non-irradiated cells. Clonogenic survival is then investigated in these non-irradiated cells. Bystander effects are indicated by a reduction in plating efficiency in the non-irradiated cells that were cultured in medium from the irradiated cells. Lower portion: plating efficiency expressed as percentage of survival after transfer of medium from cells exposed to increasing doses of ionizing radiation (adapted from reference [S13]).



68. Whether this is the same bystander phenomenon as described above for bystander effects after low fluences of alpha particles or irradiation with a charged particle microbeam has yet to be determined. If it is not an identical phenomenon, it is likely to be similar to those bystander effects defined above that involve the secretion of soluble factors [M43, S64, Z2]. However, one caveat in these studies should be mentioned. When A_L cells were plated on either one or both sides of double Mylar dishes before irradiation, and one side (with or without cells) was irradiated with alpha particles, different effects on different cellular end points were observed for both survival and mutation [Z2]. When the side with cells was irradiated, the surviving fraction among the non-irradiated cells was significantly lower than that of the controls after 48 hours co-culture. However, such a change was not detected after 1 hour co-culture or when medium alone was irradiated. Furthermore, co-cultivation with irradiated cells had no significant effect on the spontaneous mutagenic yield of non-irradiated cells collected from the other half of the double Mylar dishes. These results suggested that the irradiated cells released certain cytotoxic factors into the culture medium that killed the non-irradiated cells. Importantly, such factors had little effect on mutation induction, indicating that different bystander end points may involve different mechanisms with different cell types. This is supported by the study by Wang and Coderre [W18], who used a co-culture system to examine bystander effects transmitted through the medium from the directly targeted cells to tumour cells growing on an insert well beyond the range of the alpha particles. Alpha particle doses of 0.1–6.0 Gy to the targeted cells on the Mylar membrane, followed by a 2 hour co-incubation of the cells on the insert in the irradiated medium above the irradiated cells, all caused an approximately 50% increase in micronucleus formation in the non-targeted co-cultured cells. Addition of the radical scavenger dimethyl sulphoxide to the medium during the irradiation and the 2 hour post-irradiation incubation period completely blocked the bystander effect, whereas addition of a nitric oxide scavenger had no effect.

69. Medium transfer experiments demonstrated that irradiation can lead to secretion of a factor or factors by irradiated cells that can reduce cloning efficiency, predominantly

by stimulating apoptosis [L26, L27, L28, S12], increasing neoplastic transformation [L5] or inducing genomic instability [S12] in non-irradiated cells. The first detectable effect on recipient cells after transfer of medium containing the bystander factor from irradiated cells was a rapid calcium pulse (1–2 minutes) followed 30–120 minutes later by changes in mitochondrial membrane permeability and the induction of ROS [L28, L38, M24]. Cell-to-cell contact during irradiation was not required to induce killing of bystander cells, but medium from cell cultures irradiated at high densities resulted in the greatest amount of bystander-induced cell death [M22]. Furthermore, the use of apoptosis inhibitors or medium from lactate dehydrogenase or glucose-6-phosphate dehydrogenase mutant cells reduced or prevented the bystander effect [M24]. Treatment with the antioxidants L-lactate and L-deprenyl prevented bystander-factor-associated cell killing [M24], suggesting that energy/redox metabolism may be involved in the medium-mediated bystander response.

70. Mothersill and co-workers [M44] showed that repair-deficient human cell lines produced a moderate to severe amount of bystander-induced cell death after medium transfer to autologous cells or to a reporter cell line. Normal "repair-proficient" lines have much less severe, or no, bystander-induced effects on cloning efficiency. These results are in agreement with the observations of Little and colleagues [L42, N3]. Mothersill et al. [M44] interpreted these data as supporting the hypothesis that bystander effects play a protective role in biological systems by terminating division in cells containing DNA damage. Thus the repair-deficient cells, irrespective of the actual repair defect, may respond to the occurrence of DNA damage in the population by removing large numbers of cells from the proliferating pool. It should be noted that repair-deficient cell lines tend to show an increased frequency of induced genomic instability, again suggesting a commonality in the mechanism of radiation-induced genomic instability and bystander effects.

71. The majority of medium transfer experiments reported have utilized low-LET radiation to induce the bystander effect and have come primarily from a single laboratory (reviewed in references [M19, M20]). A fascinating observation from these studies, but one that is difficult to reconcile with those of other studies, concerns the radiation doses required to elicit a bystander response. In human keratinocytes, low-LET ^{60}Co gamma ray doses of 0.01–0.5 Gy reduced clonogenic survival after transfer of irradiated medium. This was entirely due to bystander effects. The magnitude of cell killing was relatively constant and appeared to saturate at doses in the range 0.03–0.05 Gy [S13]. At doses of greater than 0.5 Gy, cell killing was the result of the direct effects of radiation as well as the dose-independent bystander effect [S13]. These observations are difficult to explain in terms of the classical cell survival curve, where there is very little if any cell killing observed at radiation doses of a few milligrays in directly irradiated cells.

72. In contrast to the cell-killing effects reported by Mothersill and Seymour after medium transfer from gamma-irradiated cultures, Iyer and Lehnert [I4] have observed quite different cellular responses in human fibroblast cells cultured in supernatants from alpha-irradiated fibroblasts. They observed decreases in basal levels of *TP53* and CDKN1AQ in non-irradiated cells, rather than increases as described by others (e.g. [A4, H5]). These decreases were accompanied by increases in proliferating cell nuclear antigen and CDC2, apparently mediated by TGF-beta 1 and the induction of intracellular ROS [N8]. In contrast to the detrimental effects on cell well-being characteristic of bystander effects so far described, Iyer and Lehnert [I4] showed that their decreased *TP53*/CDKN1A bystander effect correlated with enhanced cell proliferation.

73. Attempting to reconcile these conflicting results raises a number of questions. While the quality of radiation and the cell types under investigation are different, these studies highlight the family of responses characterizing the bystander effect. Mechanistically, factors transferred via cell-to-cell gap junction communication or secreted into the culture medium may interact with those non-irradiated cells in an antagonistic manner, ultimately killing the non-irradiated cells (reviewed in reference [S39]), but the reasons why cultured cells should secrete cytotoxic factors and why no such dramatic reduction in plating efficiency is observed when populations of cells are irradiated with low doses of low-LET radiation are not immediately obvious. Likewise, the pro-mitogenic response reported by Lehnert et al. [L2, L48] after medium transfer is contradictory to those direct effects observed in irradiated cells where irradiation can inhibit cell growth. Cell proliferation following irradiation appears contrary to the long-term well-being of the cell, tissue, organ or organism, whereas cell death would be likely to protect against the possibility of detrimental mutations, chromosomal rearrangements, and so on. Clearly bystander effects can modify cellular responses to radiation, and it remains to be determined whether these effects characterized in non-irradiated cells in vitro have a major role in the response of irradiated cells in vitro or in irradiated and non-irradiated cells in vivo.

B. Bystander effects in vivo

74. Bystander effects have been observed predominantly by using single-cell in vitro systems that do not have realistic multicellular morphology. Given that the bystander phenomenon must involve cell-to-cell interactions, the relevance of such single-cell in vitro studies is questionable. However, Belyakov et al. [B36] have described bystander responses in a three-dimensional, normal human tissue system. While not a true in vivo assay, this model skin system does provide some semblance of multicellular interactions. End points were induction of micronucleated and apoptotic cells. Non-irradiated cells up to 1 mm distant from irradiated cells showed a significant enhancement in the effect over background levels, with an average increase in effect of 1.7-fold for micronuclei and 2.8-fold for apoptosis. The surprisingly long range of bystander signals in a human

tissue model system suggests that bystander responses may be important in extrapolating potential radiation effects from epidemiologically relevant doses down to very low doses (<200 mGy), where non-hit bystander cells would likely predominate [M36].

75. Compared with the number of in vitro studies on bystander effects, there are relatively few studies on bystander effects in vivo, and these are summarized in table 4. Many of these studies appear to have been performed not to look specifically at non-targeted effects of radiation but for other purposes, so that critically evaluating bystander effects in vivo is premature at present. It should be mentioned at this stage that investigators are beginning to move from single-cell systems to multicellular systems using primary explant techniques [B8, B36] as well as three-dimensional model systems [P11]. Consequently, it is anticipated that as more information from these model systems becomes available and more focused studies in vivo are undertaken, it will be possible to critically re-evaluate bystander effects in vivo.

Table 4 In vivo studies of the bystander effect

End point	*Cellular system*	*Radiation type*	*Reference*
Chromosomal aberrations	Chinese hamster ovary and liver	$^{239}PuO_2$ particles; alpha particles	[B16, B17, M40]
	Mixture of irradiated and non-irradiated mice bone marrow cells	Neutrons	[W2]
Micronucleus frequency	Rat lung	Gamma rays	[K11, K22]
Calcium mobilization; alkaline phosphatase levels; embryonic development	Rat incisor, thyroid and abdomen	X-rays	[C8, H1]
Regenerative capacity	Earthworm	X-rays	[M8]
Macrophage activation; respiratory burst; NO activation; neutrophil infiltration	*TP53*$^{-/-}$ mice	Gamma rays	[L22]
Growth of tumour	Mixture of human colon LS174T adenocarcinoma cells and ^{125}I-labelled LS174T cells	^{125}I beta particles	[X2]
	C57BL/6 mice	Gamma rays	[C1]

76. Despite the caveats outlined above, there are studies indicating a bystander effect in vivo. Chinese hamsters were injected with different sized particles of the internally deposited alpha emitter plutonium. The radioactive particles concentrate in the liver and produce chronic low-dose radiation exposure, with the dose and dose rate being highest to cells located closest to the largest particles. However, analysis of induced chromosome damage in these livers revealed increased cytogenetic damage that was not directly related to the local dose distribution [B17]. These observations were interpreted as indicating that all the cells in the liver were at the same risk of induced chromosome damage despite only a small fraction of the total liver being exposed to the radiation. The cumulative incidence of liver cancer as a function of time after plutonium injection and total dose was also determined. Neither the time of tumour onset nor the tumour incidence varied with particle size, indicating that the number of cells hit by alpha particles was not a factor in tumour induction in irradiated livers [B16].

77. These two studies suggest that radiation-induced genetic damage and ultimately tumour induction are related to the total dose to the organ, i.e. the whole liver, rather than the dose to individual cells or the number of cells traversed by an alpha particle [B16, B17]. Furthermore, these data raise the intriguing possibility that the target for induced bystander effects may actually be limited to the specific organ irradiated and that adjacent non-irradiated organs are not targets for bystander effects. It is certainly not unexpected that multicellular organs function in response to genotoxic stress in a coordinated fashion [G24], and recently Barcellos-Hoff and Brooks have hypothesized that multicellular responses through extracellular signalling are integral components of predicting cancer risk after radiation exposure [B3]. To this end, there is evidence from in vivo studies of radiation-induced genomic instability that delayed instability has a significant bystander component [W2, X2]. Nevertheless, a recent report of the International Commission on Radiological Protection (ICRP) [I11] suggests that early initiating cellular and molecular events are the major determinants of risk at low doses, rather than cell-, tissue- and host-modifying factors.

78. The evidence for in vivo bystander effects has been reviewed in detail [B43, K25] and shows that these effects probably involve a genetic component [M61]. In addition to damage directly induced by the deposition of energy in the irradiated cell, consideration must now be given to

these indirect effects of radiation, and a model quantifying these considerations has been proposed [B14]. An irradiated cell can send out a signal and induce a response in a cell whose nucleus was not hit by radiation. Thus a detrimental bystander effect, e.g. chromosomal aberrations, in essence "modifies" the biological effectiveness of a given radiation dose by increasing the number of cells that experience adverse effects over that directly exposed to the radiation. Significantly, these bystander effects appear to be limited to the organ irradiated, i.e. are organ-specific [B43]. Thus, at the present state of our knowledge, it is reasonable to assume that any bystander effect induced in vivo is accounted for in models of organ risk evaluation. As a result, it is unlikely that the resurgence of interest in these non-targeted radiation effects will substantially alter risk estimates as discussed in detail in the BEIR VII report [C23]. Nevertheless, it cannot be excluded that increasing the knowledge basis for in vivo bystander effects at low doses and low dose rates in specific organs may affect current organ risk estimates.

III. RELATIONSHIP BETWEEN RADIATION-INDUCED GENOMIC INSTABILITY AND BYSTANDER EFFECTS

79. The evidence for effects occurring in cells that themselves were not irradiated but are the progeny of irradiated cells (radiation-induced genomic instability), and non-targeted cellular effects usually associated with direct exposure to ionizing radiation occurring in non-irradiated cells (bystander effects), has been reviewed in the previous paragraphs. Many of the end points associated with these two phenomena are the same: induced chromosomal rearrangements, micronuclei, increased mutation, increased transformation and cell killing. So what is the relationship, if any, between induced instability and bystander effects? Chromosomal instability in haemopoietic cells can be induced by an indirect, non-targeted bystander type of mechanism [K3, L24]. Persistently increased levels of intracellular ROS have been reported in chromosomally unstable cells [C5, L7, L8, L9, L10, R3, R6], and provide a plausible mechanism for perpetuating instability over time (reviewed in references [M10, M16]). Considerable experimental support for this hypothesis comes from Wright and co-workers [L22, W2, W4], and this has recently been reviewed in detail [L23]. The resultant intercellular signalling cascades, cytokine production, nitric oxide production and persistent free radicals all have the potential to mediate both instability and bystander effects (reviewed in references [L23, M10]).

80. To critically evaluate the hypothesis that chromosomally unstable GM10115 clones perpetuate instability by secreting a bystander-like factor into the culture medium, thus driving the delayed production of chromosomal rearrangements, Nagar et al. took medium from a chromosomally unstable clone of GM10115 human–hamster hybrid cells, filtered it and cultured non-irradiated GM10115 cells in this medium. None of the non-irradiated GM10115 cells were able to survive and form colonies in medium from the unstable clone. Nagar et al. called this novel effect, by which cells cultured in medium from chromosomally unstable GM10115 cells die, the death-inducing effect [N1]. The unstable clones showing the death-inducing effect also showed increased numbers of apoptotic cells and elevated levels of intracellular ROS [N2], either or both of which might contribute factors to the culture medium responsible for the death-inducing effect. Furthermore, Nagar et al. [N1] have interpreted this observation as indicating that unstable clones of cells do secrete factors that, while generally not toxic to the unstable clone, most likely contribute to the perpetuation of the unstable phenotype. It should be stressed that the death-inducing effect is separate from the bystander effect observed after transferring medium from irradiated cells. Nagar et al. did not observe a reduction in plating efficiency when medium from irradiated GM10115 cells was transferred to non-irradiated GM10115 cells. This indicates that GM10115 cells either do not secrete a cytotoxic bystander factor or are not susceptible to a bystander factor, and that the death-inducing effect is not the same as the bystander effect described by Mothersill and co-workers. Likewise, chromosomal instability was not detected in GM10115 cells after transferring medium from irradiated cells as described by Mothersill and Seymour [M18, M21] for induced bystander effects.

81. It should be noted that Mothersill et al. [M44] have also reported that they do not find a bystander response in some Chinese hamster cell lines after medium transfer. This implies that, like the human–hamster hybrid GM10115 cell line, CHOK1 hamster cells may be deficient in producing a bystander signal or in responding to that signal. It is interesting that when medium from irradiated CHOK1 cells was added to either non-irradiated CHOK1 cells or repair-deficient XR1 hamster cells, the plating efficiency actually increased rather than decreased [M44]. However, significantly increased bystander effects after cellular exposure to low fluences of alpha particles have been described in repair-deficient Chinese hamster cells compared with wild-type hamster cells [N3, N20, N25].

82. Evidence increasingly suggests that induced instability and bystander effects are linked (reviewed in references [L23, M10, M16]), and it is likely, given the commonality of the end points observed, that both phenomena could be manifestations of the same non-targeted processes [M10, M16]. Furthermore, a significant contribution from bystander-like factors could help explain the high frequency of radiation-induced instability reported, for example, in references [K3, L6, M2].

83. The Committee continues to hold the view that mechanistic information is important for its recommendations on radiation-induced health effects at doses of below ~200 mSv and for risk assessment for individuals. The latter aspect is important because epidemiology always refers to populations, and genomic instability, for example, varies among individuals.

A. Relationship between radiation hypersensitivity at low doses and bystander effects

84. To date, investigation of the relationship between radiation hypersensitivity at low doses and bystander effects has been limited to a single study. Joiner et al. [J4] described radiation hypersensitivity at radiation doses where

the in vitro bystander effect would be expected to predominate. Interestingly, an investigation into the relationship between radiation-induced low-dose hypersensitivity and the bystander effect indicated a weak inverse correlation between these two low-dose phenomena. Specifically, those cells exhibiting a large bystander effect did not show radiation hypersensitivity [M23]. Should these results be confirmed, they would suggest that, at very low radiation doses, bystander effects might dominate the overall cellular response. Furthermore, such bystander effects at doses of a few milligrays could have some bearing on the apparent elimination of damaged cells and the absence of repair of radiation-induced DNA double-strand breaks in the very low dose range (1.2 mGy) in X-irradiated normal human fibroblasts as reported by Rothkamm and Lobrich [R14].

B. Relationship between radiation adaptive response and bystander effects

85. The radiation adaptive response refers to the phenomenon by which cells irradiated with a sublethal dose of ionizing radiation (an "adaptive" dose of a few centigrays) become less susceptible to subsequent exposure to high doses of radiation (a "challenge" dose of several grays). There is a vast literature on "adaptive responses", and this section is not intended to be an exhaustive literature review. Instead, the goal is to provide examples of the main evidence for low doses of radiation protecting against a subsequent high-dose radiation challenge. The adaptive response to radiation was first described as a reduction in chromosomal aberration frequency in stimulated human lymphocytes [O7]. Subsequent reported adaptive responses include reduction of cell killing [I6], micronucleus formation and sister chromatid exchange [I7, I8], mutation [K26, R11, U25] and transformation [A17, R8]. An adaptive response has also been described after clinical [M53], environmental [G16] or occupational [B34] exposures to radiation. The mechanism for this radioadaptation is thought to be that low radiation doses enhance DNA repair ability and antioxidant activity, resulting in more proficient cellular responses to the subsequent challenge [G21, I9, S48].

86. Reports of the adaptive response to radiation are conflicting, however, because radioadaptation is not consistently a robust effect in all cell systems [A14, B35, B40, H16]. The variation among different studies could be related to a number of factors, including cell type [R7], cell culture conditions, cell cycle effects, types of radiation used, doses and dose rates, as well as time interval between irradiations [S54]. Current uncertainties in interpreting experimental data from both adaptive response and bystander investigations do not permit any firm conclusions regarding the relationships between these two low-dose phenomena to be reached at present.

87. Nevertheless, radiation-induced bystander effects can be considered a competing phenomenon with respect to an adaptive response [S7]. Using the Columbia University charged particle microbeam and the A_L cell mutagenic assay, Zhou et al. [Z6] showed that pretreatment of cells with a low dose of X-rays four hours before alpha particle irradiation significantly decreased this bystander mutagenic response. Furthermore, bystander cells showed an increase in sensitivity after a subsequent challenging dose of X-rays. Using the same irradiation system, Mitchell et al. [M51] found that an adaptive dose of 2 cGy of X-rays cancelled out the majority of the bystander effect produced by alpha particles. For oncogenic transformation, but not cell survival, radioadaptation could occur in non-irradiated cells via a transmissible signal.

C. Conclusions

88. Ionizing-radiation-induced bystander effects are those effects occurring in cells that were not traversed by radiation but were induced by signals from irradiated cells. Mechanistically, the signal is passed from cell to cell by gap junction communication or is secreted into the culture medium where it can be transferred to non-irradiated cells. Both positive effects for the cell (e.g. increased cell proliferation or an induced radioprotective adaptive response) and negative effects (e.g. cytogenetic damage or cytotoxic bystander responses) have been described. Bystander effects induced by high-LET radiation have been described in a number of different cell types in studies using either low radiation fluences of alpha particles or charged particle microbeams. A bystander effect induced by low-LET radiation is less well established and to date has only been demonstrated after medium transfer experiments. Experimental verification in different laboratories and the use of newly developed low-LET microbeams will extend these observations. To date, low-LET bystander effects appear to be a low-dose phenomenon, and reconciling low-dose bystander cytotoxicity with the lack of directly induced cell killing at these same doses is perplexing.

IV. ABSCOPAL EFFECTS OF RADIATION

A. Review

89. An abscopal effect may be defined as a significant tissue response to irradiation in tissues definitively separate from the region exposed to radiation. The response must be measurable, and the distance separating the responding tissues and the portal(s) of irradiation must be great enough to rule out any possible effect of scattered radiation [N15]. Originally described by Mole [M52] in 1953, the word abscopal comes from the Latin ab (position away from) and scopus (mark or target). The mechanism of the abscopal effect is unknown, although a variety of underlying biological events can be hypothesized, including a possible role for the immune system [M46, U24].

90. An example illustrating radiation-induced abscopal effects is the study of early DNA damage induced in rat lung cells following single-dose, partial-volume (lung base and lung apex) irradiation [K11]. When the lungs were removed at 16–18 hours after whole-lung irradiation, an average of ~0.85 micronuclei per binucleate cell were observed in the irradiated animals, compared with 0.02 micronuclei per binucleate cell in the lungs from control animals. When only the lung base was irradiated, the frequency of micronuclei in cells from the irradiated field was 0.85. However, non-irradiated cells from the out-of-field lung apex also showed a significant increase in the frequency of micronuclei, 0.43 per binucleate cell, significantly higher than the non-irradiated control value. Cells from the lungs of rats injected with superoxide dismutase within one hour prior to irradiation of the lung base and processed 16–18 hours after irradiation showed a reduction in the number of micronuclei in the shielded lung apex, indicating the potential involvement of oxygen radicals [K11].

91. Ohba et al. [O3] described the case of a 76-year-old Japanese man with hepatocellular carcinoma that regressed after radiotherapy for thoracic vertebral bone metastasis. Serum levels of tumour necrosis factor alpha increased after radiotherapy, and the investigators suggested that such abscopal-related regression might be associated with host immune response, involving cytokines such as tumour necrosis factor alpha. To understand the potential mechanisms, Camphausen et al. [C1] examined whether the abscopal effect was mediated through *TP53*. Non-tumour-bearing legs of C57BL/6 (wild-type *TP53*) and *TP53* null B6.129S2-Trp53(tm1Tyj) mice were irradiated to determine whether an abscopal effect could be observed against Lewis lung carcinoma and T241 (fibrosarcoma) implanted at a distant site. In the *TP53* wild-type mice, both the Lewis lung carcinoma and T241 tumour cells implanted into the midline dorsum grew at a significantly slower rate when the leg of the animal was exposed to five 10 Gy fractions of radiation compared with sham-irradiated animals. This suggests that the abscopal effect is not tumour-specific. When the radiation dose to the leg was reduced (12 × 2 Gy), the inhibition of Lewis lung carcinoma tumour growth was decreased, indicating a radiation dose dependency for the abscopal effect. In contrast, when the legs of *TP53* null animals or wild-type *TP53* mice treated with pifithrin-alpha (a *TP53* blocker) were irradiated (5 ×10 Gy), tumour growth was not delayed. These data implicate *TP53* as a key mediator of the radiation-induced abscopal effect and suggest that pathways downstream of *TP53* are important in eliciting this response.

92. Ionizing radiation can reduce tumour growth outside the field of radiation [A15, E16, K27, R9, R10], but this abscopal effect remains a rare and poorly understood event. Ionizing radiation generates inflammatory signals and in principle could provide both tumour-specific antigens from dying cells and maturation stimuli that are necessary for dendritic cells to activate tumour-specific T-cells. Demaria et al. [D23] tested the hypothesis that the abscopal effect elicited by radiation is immune-mediated. Mice bearing a syngeneic mammary carcinoma, 67NR, in both flanks were treated with growth factor Flt3-Ligand daily for 10 days after local radiation therapy to only one of the two tumours at a single dose of 2 or 6 Gy. The second, non-irradiated tumour was used as indicator of the abscopal effect. Radiation alone led to growth delay exclusively of the irradiated 67NR tumour. However, growth of the non-irradiated tumour was also impaired by the combination of radiation and ligand. Importantly, the abscopal effect was shown to be tumour-specific, because growth of a non-irradiated A20 lymphoma in the same mice containing a treated 67NR tumour was not affected. Moreover, no growth delay of non-irradiated 67NR tumours was observed when T-cell-deficient (nude) mice were treated with the combination of radiation and ligand. These results demonstrate that in this cell system the abscopal effect is in part immune-mediated and that T-cells are required to mediate distant tumour inhibition induced by radiation.

93. Abscopal effects after partial-body irradiation have also been described in earthworms [M8], White Leghorn cockerels [M9] and rats [C8, H1]. Abscopal reactions have also been described in patients with chronic leukaemias, wherein irradiation of an enlarged spleen or liver will induce a generalized remission with return of the bone marrow, white blood cell count and peripheral blood cells to normal ranges [N15]. In fact, there are a number of well-recognized effects

described by radiation therapists that occur beyond the radiation field. Goldberg and Lehnert [G7] have recently reviewed these bystander-like effects but concluded that the clinical literature does not provide strong evidence for or against the existence of radiation bystander effects, and by extension abscopal effects, in vivo. They argue that many studies can be interpreted as suggesting non-targeted effects in vivo, and recommend that prospective clinical trials be carried out that involve detailed field and dose information combined with well-documented patient risk factors in order to investigate potential bystander effects after radiation therapy [G7].

B. Conclusions

94. The few studies discussed in this section that describe the potential abscopal effects of ionizing radiation are generally descriptive in nature and provide little or no interpretation in terms of the mechanism underlying the response. This together with the lack of confirmatory studies means that definitive conclusions on the impact of any potential abscopal effects are not possible. Additional focused research involving well-designed prospective clinical trials could clarify this issue.

V. CLASTOGENIC FACTORS INDUCED BY IONIZING RADIATION

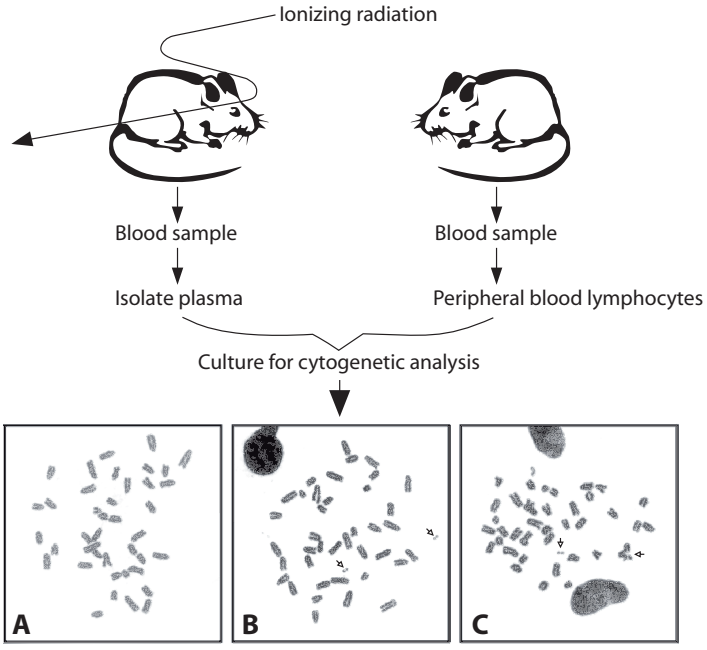
A. Review

95. Following whole-body irradiation, blood plasma from some animals and humans can contain factors that can cause chromosome damage, hence the name clastogenic factors. This indicates the persistence of soluble factors induced by radiation that are capable of causing effects in non-irradiated cells. As such, clastogenic factors are not necessarily classical bystander effect factors but are included here to support the concept of a role for secreted and released soluble factors in delayed effects associated with radiation exposure. Strictly, under the definitions presented in this review, clastogenic factors, like abscopal effects, could well be considered bystander effects. However, for historical reasons, both abscopal effects and clastogenic factors are considered separately. In reality, however, no mechanistic distinctions are implied between these non-targeted effects of exposure to ionizing radiation.

96. There is a large body of evidence demonstrating that plasma from irradiated animals and humans can contain factors capable of inducing detrimental effects in unexposed cells. These "clastogenic factors" (or clastogenic plasma factors) were first described by Parsons et al. [P5], who observed bone marrow damage in the sternum of children with chronic granulocytic leukaemia whose spleens had been irradiated. This report was corroborated by Souto [S19], who showed that rats injected with plasma from irradiated animals developed mammary tumours at a significantly higher rate than rats exposed to plasma from non-irradiated animals. A number of reports eventually followed that demonstrated that culturing normal human peripheral blood lymphocytes in medium containing plasma obtained from accidentally [G5] or therapeutically [H6, L15] irradiated individuals resulted in significantly more chromosomal aberrations than culturing lymphocytes in medium with plasma from non-irradiated individuals. These observations led to the suggestion that, after in vivo irradiation, exposed individuals can possess clastogenic factors in their blood plasma that, when transferred to cell cultures from unexposed individuals, can induce chromosome damage (figure XI).

Figure XI. Theoretical schematic for identifying clastogenic factors.

Plasma isolated from blood from an irradiated mouse is mixed with blood from a non-irradiated mouse and cultured for cytogenetic analysis. Clastogenic factors in the plasma from the irradiated mouse can cause chromosomal aberrations in the peripheral blood lymphocytes of the non-irradiated mouse. A: metaphase chromosomes from a peripheral blood sample from a non-irradiated mouse cultured in media containing isolated plasma from a non-irradiated mouse. No chromosomal aberrations are observed. B and C: metaphase chromosomes from a peripheral blood sample from a non-irradiated mouse cultured in isolated plasma from an irradiated mouse (B: chromatid deletions (arrows); C: chromatid exchange and deletion (arrows)).



97. Clastogenic factors have been described in plasma from survivors of the atomic bombings in Japan [P2], personnel involved in salvage operations after the Chernobyl accident [E8, E10] and children exposed as a consequence of the Chernobyl accident [E11, G3]. In addition, clastogenic factors have been reported in human [G1, L19, S10] and rat blood plasma [F1] after in vitro irradiation. Clastogenic factors can be induced within 15 minutes of irradiation [F1] and appear to be very persistent or continuously regenerated: times of 10 weeks post-irradiation have been reported for rats [F1], 7–10½ years for irradiated humans [E8, G5, G6] and >30 years for the atomic bombing survivors [P2]. Clastogenic factors reflect neither radiation-induced depletion of protective factors nor radiation-induced changes in normal plasma components, but rather represent products secreted or excreted by cellular elements as a result of irradiation [F1]. In vitro induction of clastogenic factors does not appear to be related to the dose [S10] or quality of radiation [G1]; however, this might not be the case in vivo [E8].

98. Emerit et al. [E8] investigated clastogenic factors in the plasma of 32 civil workers from Armenia who had been engaged as emergency workers around the Chernobyl atomic power station in 1986. They also included 15 emergency workers who had emigrated from the former Soviet Union to Israel. Reference plasma samples were obtained from 41 blood donors from the Armenian Blood Center in Yerevan. The samples were tested for their clastogenic activity in blood cultures from healthy donors. The samples from the first Armenian group, with the higher average radiation dose (0.6 ± 0.6 Gy), were more clastogenic than those from the second group, which had been exposed to 0.2 ± 0.2 Gy. The samples from the Israeli emergency workers also induced significantly increased aberration rates (14.0 ± 3.9% aberrant cells). The clastogenic activity described above could be inhibited by superoxide dismutase [E8], indicating that the chromosome-damaging effects of radiation-induced clastogenic factors are exerted via the intermediation of superoxide radicals, as is known for clastogenic factors of different origin [E18, F12].

99. It should be stressed that there is variability between individuals in their ability to produce clastogenic factors [E11, G3], and not all irradiated individuals exhibit this effect [L4]. Indeed it is difficult to evaluate how common is the induction of clastogenic factors in the human population. On the one hand, blood samples from irradiated individuals are a valuable commodity, usually studied for more conventional biomarkers of radiation exposure. On the other hand, it is perhaps not surprising that there are very few reports failing to detect clastogenic factors, as such negative results are generally less likely to be published.

100. The precise nature of clastogenic factors is unknown, but endogenous viruses and compounds that interfere with DNA repair and/or increase the production of free radicals have all been implicated [E1, E2, E3]. On the basis of a number of inhibitor studies, the bulk of evidence suggests that the mechanism of action of clastogenic factors is probably mediated by free radicals. Free radical scavengers such as superoxide dismutase, penicillamine, cysteine and various antioxidant plant extracts all reduce or eliminate clastogenic factor activity [E1, E2, E3]. The molecular mechanisms for this effect and the specific nature of the factors capable of persisting, or of being regenerated over protracted time intervals (>30 years in the case of some of the atomic bombing survivors [P2]), remain unknown. Nevertheless, it is tempting to speculate on the potential relationship between clastogenic factors and factors involved in the bystander effect. Both can be induced by ionizing radiation and the factors produced can cause genetic damage in non-irradiated cells. Given the current interest in bystander effects resulting from secreted factors produced after cellular irradiation [S39], it may be an appropriate time to revisit clastogenic factors and evaluate the biological significance and nature of these radiation-induced secreted factors.

101. At present, the biological significance of clastogenic factors remains unclear [H11]. Furthermore, it would be misleading to imply that clastogenic factors are unique to radiation exposure. Transferable clastogenic effects have been described in blood plasma after whole-body stresses as diverse as asbestos exposure [E7] and ischaemia reperfusion injury [E6], and occur spontaneously in patients with HIV-1 [E1], hepatitis C [E12], Crohn's disease [E5] and scleroderma [A2]. The reports of diffusible clastogenic factors induced by irradiation also resemble the reports of clastogenic activity in the plasma of patients with certain inherited disorders, including Bloom's syndrome [E4], ataxia-telangiectasia [S14] and Fanconi's anaemia [E9]. Individuals with these chromosome breakage syndromes show an increased incidence of cancer, which begs the question as to the role of clastogenic factors in creating a cellular environment predisposed to increased genomic instability and ultimately neoplastic transformation [H11, W11].

102. Emerit and colleagues have carried out many of the studies investigating clastogenic factors. These investigators have described these factors in plasma from individuals exposed to different types of radiation under a variety of exposure conditions. In addition, Emerit's group has described clastogenic factors after exposure to other DNA-damaging agents, as well as in individuals with a number of medical conditions and various genetic diseases. It would be misleading to imply that Emerit and colleagues are the only ones to describe clastogenic factors in various disease states. Reports from other laboratories lend support to these observations (e.g. [B10, G1, S14]), but the biological significance and potential health hazards associated with clastogenic factors remain to be determined.

103. Nevertheless, the presence of clastogenic factors in peripheral blood samples from some irradiated individuals raises intriguing questions concerning the role of chromosomal rearrangements as dosimeters of radiation exposure [S10]. Cytogenetic analysis of first division metaphase

cells from irradiated individuals reveals both asymmetrical (dicentric and polycentric chromosomes as well as ring chromosomes) and symmetrical (reciprocal translocations) exchange-type aberrations, insertions, inversions and chromosomal breaks (reviewed in reference [C9]). The asymmetrical exchange-type aberrations generally lead to proliferative cell death in subsequent mitoses and decline over time. Symmetrical translocations, on the other hand, are generally stable over time and can persist, although they also appear to decline, albeit at a much slower rate than asymmetrical aberrations [S20]. Since clastogenic factors from some irradiated individuals can induce chromosome damage, a role for these factors in the well-described persistence of chromosomal rearrangements in blood samples from irradiated individuals is possible but unlikely.

B. Conclusions

104. It remains difficult to establish a clear description of the relevance of clastogenic factors to overall cellular responses to ionizing radiation, particularly at low doses. In part, this is due to the paucity of data on the nature of the factors and on their mechanism of action. Furthermore, how such clastogenic factors might influence the dose–response curve at low doses is not possible to discern at this time.

VI. IMPACT OF NON-TARGETED AND DELAYED EFFECTS OF RADIATION ON FUTURE GENERATIONS

105. It is important to consider whether non-targeted effects of radiation influence our consideration of the consequences of irradiation of a parent on end points in offspring. The following sections summarize the data from non-human and human studies and consider them in the context of the Committee's current position on heritable effects of irradiation. Several diverse studies have provided a somewhat confusing picture of the potential non-targeted or delayed effects of ionizing radiation in humans, mice and other organisms. Many of the studies present technical difficulties and ambiguities in interpretation, for example, with respect to uncertain radiation doses in human studies, potential strain dependency of responses in mouse studies, and poorly defined criteria used to define effects in some molecular studies. These complicating factors will be highlighted throughout this section.

106. Many of the studies examine effects in the F_1 offspring of irradiated parents, while other studies consider F_2 and later generations. It is only these studies of F_2 and later generations that can be unambiguously considered transgenerational. This is because the F_1 studies may be revealing mutations that occur during parental germ cell development. Thus F_1 studies address "heritable effects", while F_2 and later generation studies address "transgenerational effects".

A. Studies in non-mammalian species

107. The first demonstrated transmission of genomic instability to subsequent generations was in *Drosophila* treated with ionizing radiation and mustard gas. However, the end point used was lethality, which revealed little about the nature or the mechanisms of the processes involved [A12].

108. Shima and colleagues have developed a "specific locus" test system using the Japanese medaka fish, *Oryzias latipes* [S40]. The genetic end points available are dominant lethal mutations, total "specific locus" mutations and viable "specific locus" mutations. The medaka has a transparent egg membrane and embryo body, and both visible mosaics and whole-body mutations can be detected during development at an early-expressed pigmentation locus [S40]. When wild-type +/+ males were gamma-irradiated and then mated with wl/wl females, the frequency of F_1 embryos with both wild-type orange leucophores (wl/+) and mutant-type white leucophores (wl/wl*) (mosaic mutants) was ~5.7×10^{-3} Gy^{-1}. The frequency of embryos with only white leucophores (whole-body mutants) was ~1.3×10^{-3} Gy^{-1}. These results suggest that delayed mutations arise frequently in medaka fish embryos that have been fertilized with irradiated sperm [S41].

109. There was also a significant dose-rate effect for this type of mutation. Shimada et al. [S43] determined the frequency of "specific locus" mutations at five pigmentation loci in medaka spermatogonial stem cells after gamma irradiation at 0.03 cGy/min and 95 cGy/min. At each total dose, the mutation frequency was significantly lower in the 0.03 cGy/min group than in the 95 cGy/min group. The ratio of the induced mutation frequency at 0.03 cGy/min to that at 95 cGy/min was approximately 0.42 for doses of less than 1.9 cGy and approximately 0.33 for doses of 1.9–4.75 Gy [S43]. There was some specificity as to when such mutation events can be induced during spermatogenesis. When sperm and late spermatids were irradiated, the mutation frequency within non-irradiated maternally derived alleles was approximately three times higher than in the control group. In the F_2 generation, however, no increase in mutation frequency was observed. Similarly, there was no significant increase in the F_1 mutation frequency when stem cell spermatogonia were irradiated. These data suggest that irradiation of sperm and late spermatids can induce indirect mutations in F_1 somatic cells, supporting the idea that genomic instability arises during F_1 embryonic development. Moreover, such instability apparently arises most frequently when eggs are fertilized just after the sperm are irradiated [S42]. It should be noted that dose-rate effects and germ-cell-stage specificities for mutational response were previously demonstrated for "specific locus" mutations in mice, and such findings have been factored into past decisions of the Committee regarding the estimation of the risk of hereditary effects. Although interesting, these new findings in fish provide no reason to modify those estimates.

110. Microsatellite mutations have also been studied in plants grown in heavily contaminated areas near Chernobyl [K15, K16]. Kovalchuk et al. [K15] investigated the mutation rates of 13 microsatellite loci in wheat plants grown in a contaminated (900 Ci/km^2) versus a control (<1 Ci/km^2) area, and found a 3.6-fold increase in germinal mutation rate in the contaminated versus the control plot. Ellegren et al. [E17] reported an increased frequency of partial albinism, a morphological aberration associated with loss of fitness, among barn swallows, *Hirundo rustica*, breeding close to Chernobyl. Heritability estimates indicate that mutations causing albinism were at least partly of germ line origin. Furthermore, evidence for an increased germ line mutation rate was obtained from segregation analysis at two hypervariable microsatellite loci, indicating that mutation events in barn swallows from Chernobyl were two- to tenfold higher than in birds from control areas in Ukraine and Italy.

B. Mouse studies

1. Irradiation of the mouse zygote

111. A long-held belief regarding radiation teratogenesis was that developmental defects are only inducible when the conceptus is irradiated during organogenesis. In contradiction to this, Streffer et al. [M26, P1, P7] demonstrated that irradiation of a single cell, the zygote, can induce developmental abnormalities, particularly gastroschisis, in the resulting animal. Indeed, when irradiated during early embryogenesis, induced teratogenesis is suppressed in a *TP53*-dependent manner, where apoptotic cell death plays a critical role [K23, N23, N24].

112. Streffer's studies used the Heiligenberger (now the HLG/Zte) mouse, which has a spontaneous frequency of gastroschisis of ~3%. One gray of X-rays to the zygote increased this to ~11%, leading the investigators to conclude that ionizing radiation enhances latent damage already present in this predisposed mouse strain [H27, S34]. Similar findings have been made by other investigators [G4, G20, J7] after exposure to chemical mutagens [G15]. Streffer and colleagues extended these studies to demonstrate congenital malformations in the 19-day-old foetuses after paternal irradiation (2.8 Gy of ^{137}Cs gamma rays) [M64]. This increased lethality occurred after exposure of all stages of spermatogenesis with the exception of early spermatogonia.

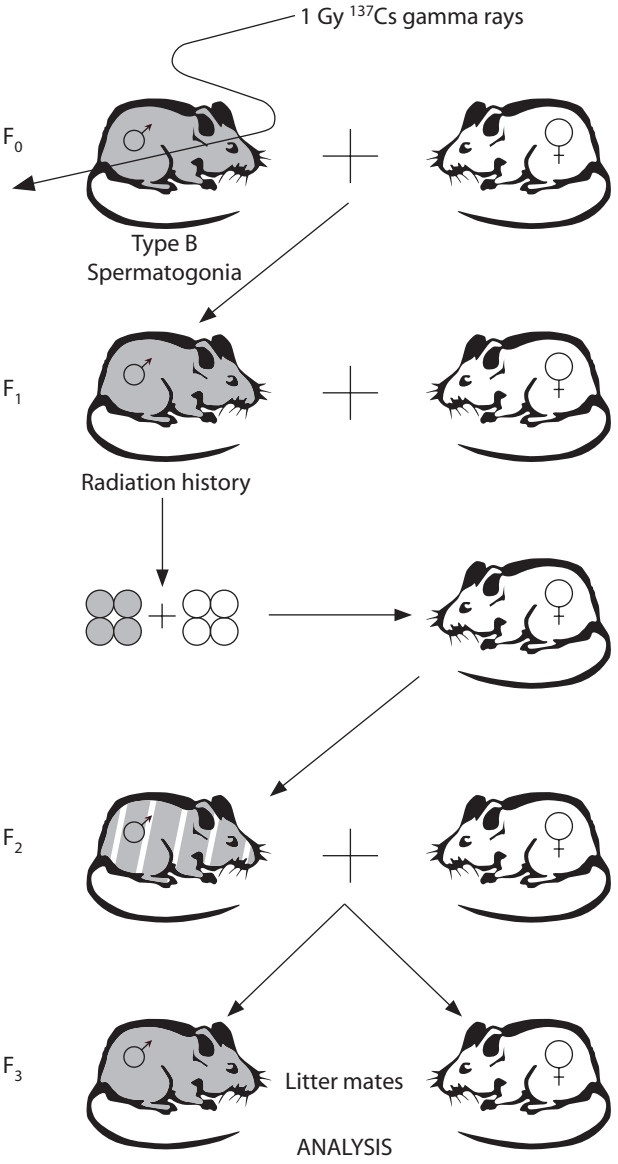
113. One possible interpretation of the gastroschisis results is that this mouse strain has a peculiarly high susceptibility to this type of gross abnormality and that the radiation treatments induced many dominant lethal mutations in germ cells or in the pronucleus that greatly exacerbated this extreme inherent susceptibility. While it is important to be aware that such situations exist, there does not seem to be any practical way to apply data on a strain of mice with a strong predisposition to a serious anomaly to the estimation of hereditary risk in human populations.

114. The effect of in utero exposure to ionizing radiation during the early phases of pregnancy has previously been reviewed [J7, P1, S34]. The risk of congenital malformations, the role of genomic instability after irradiation during the pre-implantation phase [S63], genetic susceptibility [J6] and health consequences [H17] have been reviewed in detail and will not be further considered here.

2. Pre-implantation embryo chimera assay

115. Wiley et al. have used a pre-implantation embryo chimera assay to demonstrate adverse effects in embryos after acute whole-body paternal irradiation (figure XII). They measured the competitive cell proliferation disadvantage of an embryo with a radiation history after challenge by direct cell-to-cell contact with a normal embryo in an aggregation chimera [O1, O2, W10]. The F_1 embryos conceived 6–7 weeks after paternal F_0 irradiation were most likely to display the phenotype, indicating that the type B spermatogonia were the most sensitive [W10]. Recently, Baulch et al. [B4, B5] have evaluated F_3 mouse offspring from F_0 paternal mice exposed to 1 Gy of ^{137}Cs gamma rays for gene products that can modulate cell proliferation rate, including receptor tyrosine kinase, protein kinase C and MAP kinases, and

Figure XII. Pre-implantation embryo chimera assay.
F_0 CD1 male mice were exposed to 1 Gy of ^{137}Cs gamma rays and mated to non-irradiated CD1 females six weeks after F_0 paternal irradiation. F_1 animals with a radiation history were mated with non-irradiated females to obtain F_2 embryos with a paternal F_0 radiation history. These four-cell embryos were paired with non-irradiated CD1 control four-cell embryos, and the resulting chimeras were cultured to the blastocyst stage and transferred to a foster mother. The pups resulting from the transfer were screened, and those with and without a paternal F_0 radiation history were identified and bred with normal females. Sex-matched pairs could then be evaluated for competitive cell proliferation disadvantage, protein kinase C, MAP kinase and GST activities, as well as p21^{waf1} and TP53 protein levels.



protein levels of nuclear TP53 and $p21^{waf1}$. All three-protein kinase activities were altered, and nuclear levels of TP53 and $p21^{waf1}$ protein were higher in F_3 offspring with a paternal F_0 radiation history than in non-irradiated litter-mates. While there is clear evidence of effects, it is unclear how this rather novel and artificial phenotype can be related to clinically important hereditary effects, and thus no attempt will be made to apply these results to hereditary risk estimation in humans.

3. Mouse mutation assays

116. To investigate whether preconception paternal irradiation can lead to the heritable transmission of genomic instability in mice, Luke et al. [L25] measured mutation frequency in a transgenic mouse model tagged with a lambda shuttle vector. This assay system allowed mutations in the *lacI* gene from the shuttle vector to be analysed in vitro after the animal had been irradiated in vivo. The results indicated that, as parental dose increased, there was a trend towards higher mutation frequency in vectors recovered from DNA from the bone marrow of F_1 progeny. These data demonstrate heritable transmission of factors leading to genomic instability in F_1 progeny following paternal pre-conception irradiation, although the results with a lambda shuttle vector would be complicated to apply in quantitative risk estimation for humans because they do not involve mammalian genes.

117. An increase in micronucleus frequency in bone marrow erythrocytes from the F_1 progeny of male BALB/c mice exposed to chronic low-dose gamma irradiation was observed by Fomenko et al. [F11]. Mice were irradiated with 10, 25 or 50 cGy at dose rates of 1, 5 and 15 cGy/day, and were mated with non-irradiated females on day 15 after irradiation. The obtained offspring had an elevated micronucleus frequency in bone marrow erythrocytes at the age of two months. This suggests the transmission of genomic instability from damaged germ line cells of irradiated male parents to somatic cells of the progeny. It is unclear, however, if the effects on micronuclei represent chromosome damage of consequence to clinical diseases beyond those already covered by the Committee's current methods to estimate hereditary risk.

118. The p^{un} mouse background allows visual detection of ~70 kb DNA deletions in the pink-eyed unstable (p^{un}) locus in developing mouse embryos. These are scored as black spots on the light gray fur or black cells on the transparent retinal epithelium of the offspring [R2]. In the fur spot assay, 10-day-old pups are observed for black spots on the light gray fur, and the number of animals with fur spots is counted. In the eye-spot assay, mice are sacrificed at ~20 days, the eyes are removed and the number of black cells in whole mounts of the unpigmented retinal pigment epithelium are determined [R2]. The C57BL/6Jp^{un}/p^{un} mouse strain contains a 70 kb tandem duplication of the pink-eyed dilution (p) gene [B29], the pun mutation. The p^{un} mutation is autosomal recessive and results in a dilute, light grey coat colour and pink eyes. Intrachromosomal homologous recombination between the 70 kb repeats that delete one copy of a duplicated 70 kb DNA fragment at the p^{un} locus restores the p gene and produces black pigment in the hair and retinal epithelium in wild-type mice. On the C57BL/6Jp^{un}/p^{un} inbred background, 5–10% of the mice spontaneously display fur spots and from four to six eye-spots per unpigmented retinal pigment epithelium.

119. The p^{un} fur spot assay was used to demonstrate that exposure of the parental germ line to ionizing radiation results in induction of delayed DNA deletions in mouse offspring [C16, S17]. Male p^{un} assay/p^{un} assay mice were irradiated with 1 Gy of X-rays and mated 28 days later with non-irradiated p^{un} assay/p^{un} assay females. The offspring showed a higher frequency of large fur spots. Since deletions occurring early in embryogenesis should yield larger spots than events occurring later, the large spots indicated deletion events occurring early in embryo development and many cell divisions after irradiation [C16]. Shiraishi et al. [S17] irradiated male mice with 6 Gy and observed an increase in p^{un} assay reversions resulting in eye-spots, in the irradiated paternal p^{un} assay allele as well as in the non-irradiated maternal p^{un} assay allele, indicating untargeted recombination in the offspring. The number of spots per retinal epithelium increased twofold when the male was irradiated at the spermatozoa stage [S17], but p^{un} assay instability was not observed when radiation was delivered either to spermatogonial stem cells or to late spermatids. It is noteworthy that radiation-induced instability of the p^{un} assay allele has been observed in F_1 mice but not in the F_2 generation (reported in reference [N22]).

120. Estimates by the Committee of hereditary risk for exposures of males have been based on damage to spermatogonial stem cells because they are the only germ cells in males that can accumulate appreciable doses under low-dose-rate exposure conditions. The finding that there is no induced p^{un} instability in spermatogonial stem cells suggests that there is no need to revise current risk estimates upward.

4. Alterations in tandem repeat DNA sequences

121. Alterations in tandem repeat DNA sequences, such as minisatellite DNA and expanded simple tandem repeats (ESTRs), in the genome have been used as markers of genetic change. Such loci have high spontaneous rates of mutation, which facilitates the measurement of induced mutation in relatively small numbers of samples. Alterations (mutations) are manifested as gains or losses in repeat units and are detected either by pedigree screening or by amplifications [N22, Y4]. Mutations in both minisatellite sequences and ESTRs appear to arise via indirect mechanisms rather than by direct damage to the repeat locus itself [Y4]. The significance of these DNA sequence changes is unknown. If they are genetically neutral they will not affect risk; nevertheless, the fact that they happen indicates that exposure to ionizing radiation can lead to genomic destabilization that may occur by a non-targeted mechanism.

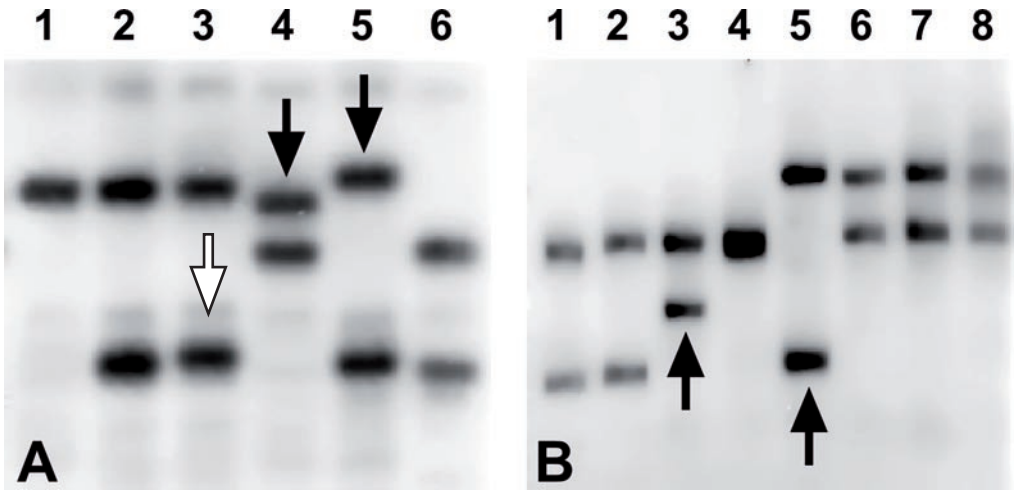
122. Minisatellites are tandem repeat loci, typically 0.5–30 kb long, with repeat units in the range 6–100 base pairs. Thousands of minisatellites exist in the genome and frequently they show variability in repeat copy number and therefore allele length [J1]. Some minisatellites are highly unstable and undergo a frequent length change mutation spontaneously in germ cells, sometimes as high as 10^{-1} per gamete [J2]. ESTRs were formerly classified as minisatellites but are now recognized as a fundamentally different entity. In contrast to minisatellites, ESTRs are composed of long arrays, up to 16 kb in length, of short (<10 bp) repeat units. Unstable ESTRs appear to be comprised almost exclusively of homogeneous arrays, with longer arrays exhibiting the highest rates of mutation [B30]. This is in contrast to the complex variant repeat distribution that makes up common minisatellite alleles. ESTRs exhibit high levels of somatic instability and also show different mechanisms of mutation from that observed in the highly mutable GC-rich minisatellites [Y4]. Examples of alterations (mutations) in an ESTR in mice are presented in figure XIII.

Figure XIII. Mutation analysis at two mouse ESTR loci.

A: Ms6-hm loci. Lanes: 1: father; 2: normal offspring; 3: offspring with a maternal mutation (open arrow); 4 and 5: offspring with two different paternal mutations (dark arrows); 6: mother.

B: Hm-2 loci. Lanes: 1: father; 2, 4, 6 and 7: normal offspring; 3 and 5: offspring with different paternal mutations (dark arrows); 8: mother.

[Figure kindly provided by Y. Dubrova, University of Leicester, United Kingdom.]



123. By analysing DNA fingerprints of the offspring of ^{60}Co gamma-irradiated mice, Dubrova et al. [D10] have shown that tandem repeat loci mutations appear to be induced in spermatogonia by low doses of ionizing radiation, with an estimated doubling dose of 0.5 Gy. This estimated doubling dose was subsequently revised downwards to 0.33 Gy [D14], which is similar to that reported earlier in the "specific locus" test in mice [R4]. This is an assay that has been relied upon by the Committee to a large extent in estimating hereditary risk.

124. Dubrova [D21] has since compared the spectra and dose response for mutations at ESTR loci in the germ line of male mice acutely exposed to low-LET X- or gamma rays at pre-meiotic stages of spermatogenesis in five strains of laboratory mice. He found that most mutation events involved the gain or loss of a relatively small number of repeat units, and the distributions of the observed length changes were indistinguishable between the exposed and the control males. Overall a significant bias toward gains of DNA repeats was detected, with approximately 60% of mutants showing gains. The values for ESTR mutation induction produced doubling doses of 0.44–0.98 Gy. Doubling dose estimations were also made by Niwa and his group, and values of 3.4, 0.893 and 4.0 Gy were reported for stem cells, spermatids and spermatozoa, respectively [F2, N14]. Inherent imprecision in the methodology used is likely to be the cause of these discrepancies [N22].

125. To date, most laboratory studies demonstrating heritable effects of radiation exposure have involved paternal irradiation [B4, B5, D14, D15, N14, S1, W10], and an approximately linear dose-response curve for paternal mutation induced at pre-meiotic stages was found [D14]. Germ line mutation in mouse tandem repeat loci appears to be a sensitive indicator of irradiation of pre-meiotic stage germ cells [D10, F2, S1], and an elevated paternal mutation rate was found after irradiation of mouse pre-meiotic spermatogonia. In contrast, post-meiotic irradiation of spermatids gave a result similar to that in control litters [D14]. However, both pre- and post-meiotic exposures were reported to increase mutation yield, suggesting that strains of mice may differ in stage susceptibility [F2, N14, S1].

126. Dubrova et al. [D14] also analysed the maternal mutation rate in mice after paternal irradiation at different stages of spermatogenesis, and found no difference in the frequency of maternal mutation. In contrast, Niwa et al. have described

a small but statistically significant increase in the maternally derived Ms6hm allele, in addition to the paternally derived allele, when only male parents were irradiated at the spermatozoa stage [F2, N14, S1]. Niwa and Kominami [N14] demonstrated a statistically significant increase in maternal allelic mutation rate in F_1 mice born to irradiated male parents. The authors concluded that, as a consequence of male (sperm) irradiation, genomic instability is triggered in the zygote, which then mutates the paternally derived allele in *cis* and the maternally derived allele in *trans*.

127. Mutation rates at two ESTR loci have been studied in the germ line of first- and second-generation offspring of inbred male CBA/H, C57BL/6 and BALB/c mice exposed to either neutrons or X-rays. Paternal CBA/H exposure resulted in increased mutation rates in the germ line of two subsequent generations. Comparable transgenerational effects were observed also in neutron-irradiated C57BL/6 and X-irradiated BALB/c mice. The spontaneous mutation rates and radiation-induced transgenerational instability varied between strains (BALB/c>CBA/H>C57BL/6). Pre- and post-meiotic paternal exposure resulted in similar increases in mutation rate in the germ line of both generations of CBA/H mice, which suggests that radiation-induced expanded simple tandem repeat instability is manifested in diploid cells after fertilization [B2]. Although there are some difficulties in interpreting these data [B41], they do suggest that excess transgenerational mutation at the unstable loci may be detected after irradiation of developing male germ cells.

128. Analysis of ESTR mutation induction in the mouse germ line suggests that mutations are likely to be due to pausing of the replication fork and subsequent polymerase slippage events [B31, D21]. The frequency of induced mutation appears to be significantly greater than the predicted frequency of radiation-induced DNA damage at a given tandem repeat locus. This has led many investigators to conclude that the increased mutation rates observed at these repeats are not necessarily the result of directly induced DNA damage at the "specific locus", but result from radiation-induced damage elsewhere in the genome or cell [D13, D14, D15, F2]. While alterations in tandem repeat DNA sequences may provide useful biomarkers of induced germ line effects, the biological significance of such mutations remains unknown. The similarity of the calculated doubling doses to those published for "specific-locus" mutations in mice supports the validity of the tandem repeat DNA sequence data, although the uncertainty in doubling doses in "specific-locus" experiments needs to be borne in mind [U1]. The comparative data between germ cell stages for mutations that affect tandem repeat DNA sequences remain of interest and potential importance.

5. Tumour induction in the offspring of irradiated parents

129. Nomura first reported a significant increase in lung tumours, mostly benign adenomas, in the F_1 offspring of X-irradiated ICR parental mice [N17, N18, N28]. This has been confirmed in some studies [H10, L20, L21, V3] but not all. For example, Cattanach et al. [C4], using the optimal experimental conditions defined by Nomura [N17], found that tumour incidence was no higher in the offspring of irradiated BALB/cJ mice than in the non-irradiated controls. They did find that the proportion of fertile females and mean litter size were affected by the radiation, showing a dose-dependent, dominant lethal response. In attempting to reconcile these differences, Cattanach et al. [C4] proposed that inconsistencies in the animal experiments may in part be due to lack of an appropriate concurrent control, whose periodic or cyclic variation in tumour incidence may have been out of phase with that in the treated animals. Alternatively, the reported differences could reflect strain differences in the mice used.

130. Selby and Priest [S52] reported no induced leukaemias when male CBA/Ca mice were injected with ^{239}Pu citrate solutions at nominal activities of 6 and 60 Bq/g, to give absorbed doses of approximately 0.3 and 4.0 cGy, and were mated to females of the same strain 54–68 days later. Nomura [N16] found no increase in leukaemia in the offspring of ICR mice derived from spermatogonia after acute irradiation. In contrast, when spermatogonia from the N5 mouse strain were irradiated, Nomura found a 10-fold-greater incidence of acute lymphocytic leukaemia in the offspring than in the non-irradiated controls. Once again, this may be explained by differences in genetic predisposition to leukaemia induction by radiation in these mouse strains.

131. Extending his original findings, Nomura hypothesized that, if radiation-induced mutations in the germ line led to heritable lung tumours in offspring, then all the cells in the lung should be at increased tumorigenic risk. Following a subsequent challenge with the carcinogen urethane, Nomura described significantly increased clusters of tumour nodules in the lung [N17, N18]. Vorobtsova and Kitaev [V3] reported similar findings, but Cattanach et al. [C21] were unable to replicate these results in C3H/HeH mice. Selby et al. [S53] suggested that the explanation for the surprisingly high rates of induction of dominant mutations that cause tumours suggested by the experiments of Nomura and others might result from the confounding effect of the radiation-induced dominant lethality that often occurs in such experiments. Other possible non-mutational explanations for the high mutation rates reported for dominant tumour mutations by Nomura have been suggested by Selby [S51] and Cattanach et al. [C4, C21].

132. Lord et al. have also investigated the heritable effects of pre-conception paternal irradiation from injected plutonium (^{239}Pu alpha particle irradiation), ^{137}Cs gamma rays or the Auger-electron-emitting radionuclide ^{55}Fe. They demonstrated perturbed haemopoiesis in offspring, as well as enhanced sensitivity to methylnitrosourea (MNU, 50 mg/kg) as a secondary carcinogenic insult [H10, L20, L21]. A major difference from the tumour experiments discussed above was that Lord et al. used much lower doses of radiation. As a result, there would have been little or no induced dominant

lethality, and the possibility that this was a confounding effect is therefore removed. The mutation rates calculated from these results suggest that Nomura's results actually underestimated the extent of this phenomenon. Since these studies rely on a secondary treatment with a carcinogen, there seems to be no way to apply their results to revise the Committee's estimates of hereditary risk, which are made for radiation alone.

C. Malformation induction in the offspring of irradiated parents

133. There are several studies addressing the question of malformation induction after irradiation of either female [K13, L50, M65, N17, N31, N32, N34, W22, W23] or male [K12, K36, M64, N17, N31, N32, N34, R15] mice and looking for malformations in the next or subsequent [L50, L51, N33, P7] generations. In all of these studies, conditions were found that resulted in transmission of radiation-induced germ cell effects in the form of malformations to the F_1 or subsequent generations. The mechanism for the development of these malformations is apparently different under these conditions from that which is responsible for the development of malformations induced by radiation exposures during major organogenesis [S34]. The following conclusions can be drawn from the experiments: comparatively high doses (>1 Gy) are required for transgenerational malformation effects to be detected. The effects seen after female exposure are not due to indirect effects because of radiation sickness of the mother, but must have, at least partly, a genetic background [L50, W22, W23]. This genetic background is quite obvious after radiation exposure of male mice. These transgenerational effects are not restricted to low-LET radiation [K36]. It seems unlikely that there is a direct relationship between chromosomal translocations and congenital anomalies [L50, N34], although some suspicion in that direction has been expressed [R15]. The basis of the malformations observed seems to be heterogeneous: some are due to genetic changes of high penetrance that are rapidly eliminated; some are due to modification of genes of low penetrance; and some are probably of non-genetic origin [L50]. There are strain-specific differences as to the extent of the transgenerational effect [R15]. There are indications that a major proportion of mutations are eliminated in the first generation and that only a minor proportion are transmitted to later generations [L50].

134. At present there are too many uncertainties about those data suggesting that dominant mutations and/or genomic instability cause tumours in progeny of irradiated mice to be able to apply such data in hereditary risk estimation.

D. Human studies

135. To date, no radiation-induced genetic, i.e. hereditary, diseases have been demonstrated in human populations exposed to ionizing radiation. Neither the offspring of individuals treated with chemotherapy and/or radiotherapy for cancer [B18] nor the offspring of women treated with radiation during infancy for haemangiomas [K6] demonstrated any significant effects attributable to parental exposure to chemicals or radiation [U1]. Furthermore, a number of studies involving the children of survivors of the Hiroshima and Nagasaki atomic bombings have failed to detect any transmitted genetic effects of radiation exposure [K14, N12, N13, S4, S5]. A cohort of 31,150 children born to parents who were within 2 kilometres of the hypocentre at the time of the bombing was compared with a control cohort of 41,066 children. During the children's early years, congenital defects, sexual development, physical development and survival were all investigated. Later, cytogenetic studies and the electrophoretic properties of a series of serum proteins or erythrocytic enzymes were analysed, in addition to a complete medical evaluation. None of these indicators was significantly modified by parental radiation exposure (reviewed in reference [N10]).

136. In addition, in a study that examined 50 families exposed after the Hiroshima and Nagasaki atomic bombings with 64 children, and 50 control families with 60 children [K14], minisatellite analyses revealed no genetic effects at six human tandem repeat. This study was later expanded to include analysis of mutations at eight hypervariable minisatellite loci in the offspring (61 from exposed families, in 60 of which only one parent was exposed, and 58 from unexposed parents) of atomic bombing survivors with mean doses of >1 Sv. They found 44 mutations in paternal alleles and 8 mutations in maternal alleles, with no indication that the high doses of acutely applied radiation had caused significant genetic effects [K28].

137. In contrast to these observations, Dubrova et al. [D11] described elevated mutation rates in DNA tandem repeat sequences in humans living in rural areas of the Mogilev district of Belarus, which was heavily contaminated with radionuclides from the Chernobyl reactor accident. The frequency of mutation was assayed both by DNA fingerprinting using one multilocus probe and by single-locus analysis using four probes, and this revealed mutation rates approximately twofold higher in the offspring of exposed parents when compared with an unexposed population from the United Kingdom. These initial observations were expanded to include analysis of families from rural areas in the Kiev and Zhytomir regions of Ukraine, which were heavily contaminated by radionuclides after the Chernobyl accident. A statistically significant 1.6-fold increase in mutation rate was found in the germ line of exposed fathers, whereas the maternal germ line mutation rate in the exposed families was not elevated [D9].

138. The initial report by Dubrova et al. [D11] generated commentary centred largely on the selection of a non-exposed population from the United Kingdom for comparison with exposed parents from Belarus [S4], the failure to exclude other contaminants such as pollutants and viral infections [N11], and questions regarding the biological significance of

increased mutations in hypervariable tandem repeat alleles [N10]. These criticisms initially appeared to detract from the significance of the findings of Dubrova et al. However, in a subsequent report, Dubrova et al. [D12] recruited more families from the affected region and used five additional minisatellite probes, and once again their data indicated a twofold higher mutation rate in exposed families than in non-irradiated families from the United Kingdom. They also used individual radiation doses for external and internal chronic exposure to ^{137}Cs as an indicator of long-term population exposure and found a significant positive correlation between radiation dose and mutation rate over multiple loci with the exposed families. Significantly, there were no obvious differences in the mutation spectrum observed between the exposed and the control families. In subsequent studies, children born to parents who participated in recovery operations after the Chernobyl accident also showed an elevated mutation rate at some loci [L18, W5], as did children born to parents living around the Semipalatinsk nuclear test site in Kazakhstan [D8].

139. It should be stressed that the analysis of tandem repeat loci mutation rate demands sophisticated molecular biology, and it is important that observed mutants be validated. Jeffreys and Dubrova [J3] suggested that technical artefacts might explain the sevenfold increase in mutation rate in children of Chernobyl recovery operations workers described by Weinberg et al. [W5]. Questions of valid paternity, sample mix-up, variation between DNA samples and the demands of the required technology are all potential sources of variability and could explain differences in results between different investigators.

140. Nevertheless, other studies have failed to reproduce these positive findings [F15, L17, S55]. A way of reconciling these apparently contradictory results was offered by Livshits et al. [L17]. They measured the frequency of inherited mutant alleles at seven hypermutable minisatellite loci in 183 children born to Chernobyl recovery operations workers and in 163 children born to control families living in non-irradiated areas of Ukraine. No significant difference in the frequency of inherited mutant alleles was found between the exposed and the control groups. The exposed group was then divided into two subgroups according to the time at which the children were conceived in relation to the fathers' work at the power plant. Eighty-eight children were conceived either while their fathers were employed at the facility or up to 2 months later (subgroup 1). The other 95 children were conceived at least 4 months after their fathers had stopped working at the Chernobyl site (subgroup 2). The frequencies of mutant alleles were higher for the majority of loci in subgroup 1 than in subgroup 2, suggesting that the timing of irradiation during spermatogenesis had affected its mutagenic potential.

141. Given the frequency of mutations in these hypervariable alleles and the lack of evidence for significant differences in the mutation spectrum between control and exposed families [D12], it is unlikely that the minisatellite loci themselves are the direct targets of the radiation. If the increased mutation rate is not caused by DNA damage directly, it might well result from non-targeted events associated with radiation-induced genomic instability [D13, D16, F2, N14, S1].

142. The reasons for the discrepancy between the data collected from the children of survivors of the Hiroshima and Nagasaki bombings and the induction of human germ line mutation in the majority of studies involving the offspring of parents living in radiation-contaminated environments are not easily reconciled. The types of radiation to which individuals were exposed differed between the two populations, and there are uncertainties associated with the doses. The bombings resulted in a single acute exposure to predominantly gamma radiation and a small amount of neutron radiation, whereas contamination from Chernobyl resulted in chronic exposures to internalized ^{131}I. However, this is unlikely to be the only reason for the discrepancy, because there are also negative results for the children of recovery operations workers exposed to low-dose-rate external/internal exposures [F15, S55].

143. The controversy surrounding the induction of mutations in tandem repeat sequences is far from resolved. May et al. [M5] examined the mutation frequency at hypervariable tandem repeats in sperm from three seminoma patients following hemipelvic radiotherapy. Scattered radiation doses to the testicles were monitored, and the mutation rates in pre-treatment sperm DNA were compared with sperm derived from irradiated meiotic and post-meiotic cells. No evidence for radiation-induced germ line mutation at these hypervariable loci was observed even though the patients were monitored for a period of 1 to 11 months.

144. Of all the reported studies, it is only those of the populations living in the Semipalatinsk region of Kazakhstan that permit comment on the potential transgenerational effects (i.e. F_2+ generations). In one such study, the F_2 offspring of those parents that received the highest external and internal doses did not show any elevation of minisatellite mutation frequency [D8].

145. These and related studies have been summarized in table 2 and have recently been subject to critical review [B41]. To summarize the conclusions of Bouffler and the expert review panel [B41]: only limited data are available indicating that germ line mutation of minisatellites can be detected in irradiated human populations. The data are inconsistent and show only limited evidence of dose dependence, and the panel found that additional work would be necessary to establish the radiation responsiveness of these loci. Furthermore, the data on mutation of human tandemly repeated DNA loci do not warrant a dramatic revision of germ line or cancer risk estimates for radiation at present [B41].

E. Cancer incidence in the offspring of irradiated humans

146. The risk of cancer in the offspring of humans irradiated prior to conception is also controversial. No excess

cancer incidence has been reported in children born to parents exposed to ionizing radiation by the atomic bombings in Japan [Y1, Y2] or in the offspring of cancer patients treated with radiotherapy [H4].

147. A major event early in this controversy was the conclusion by Gardner et al. [G2] from a case-control study that the increased incidence of leukaemia and non-Hodgkin's lymphoma among children living near the nuclear reprocessing plant in Sellafield in the United Kingdom was associated with paternal employment and the recorded external dose of whole-body radiation during work at the plant before conception. This conclusion was controversial [D6] and was not supported by the excess of childhood leukaemia observed at nearby Seascale [P4], or by an extensive study of radiation workers and childhood cancers [D7]. Nevertheless, Dickinson and Parker [D4] published results of a cohort study that supports the initial association with paternal radiation dose, which suggests that it still remains a possible explanation [B15], although population mixing [G17] is regarded as a potentially important factor in this particular cluster.

148. The United Kingdom Committee on Medical Aspects of Radiation in the Environment (COMARE), in their seventh report, reviewed the evidence concerning the incidence of childhood leukaemia and other cancers in the offspring of parents occupationally exposed to radiation prior to conception [C17]. The COMARE agreed that, while a link between parental exposure and such effects in the offspring was possible in principle, the epidemiological evidence from the offspring of radiation workers in the United Kingdom, the United States and Germany failed to support an increased rate of solid tumours in children. Furthermore, COMARE concluded that the balance of evidence indicated that the likelihood of developing childhood leukaemia or non-Hodgkin's lymphoma was not related to radiation dose.

F. Pregnancy outcomes in the offspring of irradiated humans

149. In their eighth report, COMARE reviewed pregnancy outcomes following pre-conception exposure to ionizing radiation in humans [C18]. They found that the available epidemiological data were inadequate to allow definitive statements about the effect of pre-conception radiation exposure on pregnancy outcomes. This was due to difficulties in obtaining reliable figures for the end points of concern and to the possibility that the radiation exposures in most studies may have been too low to produce a detectable effect. The conclusion reached by COMARE was that, from all the epidemiological data examined, there was little evidence that adverse reproductive outcomes in general are related to parental radiation exposure. Similarly, the limited data available did not link miscarriage or neonatal death with parental irradiation. COMARE did point out, however, that almost all of the published studies on pregnancy outcome following parental exposure to radiation in human populations lack statistical power; this is probably due to the low doses to which the populations were exposed and to the small population sample size.

150. The Scientific Committee takes note of these conclusions by COMARE, which support its own view that the types of genetic data discussed in this annex imply no modification of its own estimates of hereditary risk. It has been the Scientific Committee's position that the mutation rates in experimental organisms upon which it has based its estimates of hereditary risk are sufficiently low as to make it unlikely that analyses of the radiation-exposed human populations available for study would show statistically significant increases in hereditary diseases.

G. Genetic damage and malformation induction in the offspring of irradiated parents

151. The densely populated costal regions of Kerala state in southwest India have deposits of radioactive monazite-bearing sand and provide a unique opportunity to investigate the effects of high levels of natural radiation on human populations [N35]. The background radiation levels range from ≤1.0 mGy to >35.0 mGy per year owing to naturally occurring thorium and its decay products. There is a comprehensive programme to assess the biological and health effects of this radiation exposure in humans, focusing mainly on constitutional chromosome abnormalities and the incidence of congenital malformations in newborns. To date, the data do not reveal any effect on cytogenetic aberrations in lymphocytes [C24] or the incidence of congenital malformations in newborns [J11] that can be associated with exposure to ionizing radiation.

H. Impact of non-targeted and delayed effects of radiation on future generations

152. Heritable effects are observed in first-generation offspring and/or in later generations after one or both parents have been irradiated prior to conception. Since it was established, the Committee has made estimates of the genetic effects of radiation in humans in offspring of irradiated parents based upon clear demonstrations that mutations can be induced in experimental organisms, including experimental mammals. The UNSCEAR 2001 Report on the hereditary effects of radiation emphasized that no radiation-induced genetic (i.e. hereditary) diseases have so far been demonstrated in human populations exposed to ionizing radiation [U1]. No demonstrable adverse reproductive outcomes were described for the survivors of the atomic bombings in Japan, or for women irradiated during infancy for skin haemangiomas. No demonstrable hereditary effects of radiation exposure resulting from the Chernobyl accident have been described [U2]. Likewise, no increase in cytogenetic abnormalities [W19] or genetic effects [B39] has been reported in survivors of childhood cancer exposed to ionizing radiation before reproduction.

153. Ionizing radiation is considered a universal mutagen. Experimental studies in plants and animals have demonstrated that radiation can induce hereditary effects, and humans are unlikely to be an exception in this regard. It was for this reason that the Committee estimated hereditary risk in humans in the absence of direct evidence in humans. This annex presents a re-evaluation of some of the controversial data, in view of newer findings, and also a review of some types of damage not considered in earlier UNSCEAR reports. Two assumptions are commonly made in the estimation of genetic risk: (1) that the seven "specific loci" in the mouse constitute a suitable basis for extrapolation to genetic disease in humans; and (2) that heritable mutations are induced by radiation damage (energy-loss events leading to double-strand damage) occurring within the genome and are induced linearly with dose, at least at low doses. The issues of main importance in this section are whether the information on the types of mutation considered below might (1) be used to improve the Committee's estimates of hereditary risk, and (2) indicate some type of genetic instability that could lead to modification of risk through subsequent generations.

154. Overall it is clear that irradiation of the parent can lead to some changes in the offspring, but it is likely that most of these are due to the manifestation of direct damage caused by radiation in the original germ cell. The high incidence of offspring with "mutations" in ESTRs and the detection of mutations in maternal alleles after paternal irradiation suggest that non-targeted instability may be induced in specific circumstances. There is only very limited evidence that instability is transmitted across into the F_2 generation, and human data are negative. Therefore the Committee considers that these data are insufficient to justify modification of current risk estimates for hereditary effects or cancer in humans.

VII. IMPLICATIONS OF NON-TARGETED AND DELAYED EFFECTS

155. A wealth of information has been reviewed that deals with possible radiation-induced non-targeted and delayed genetic effects, as well as genetic end points that can occur spontaneously. Most of the estimates of hereditary risk made by the Committee in the past have been based on classical mutation experiments. Such studies often require exceedingly large samples of offspring. For the reasons described above, it does not appear that the new findings necessitate changes in the Committee's estimates of hereditary risk. It is possible that certain types of genetic damage detected by some of the assays have no relationship to clinically important phenotypes. It is also possible that the methods of hereditary risk estimation used by the Committee in the past adequately incorporate any genetic risks of clinical relevance that might be associated with the damage detected by the various assays considered. While it is clear that some of the assays permit demonstration of effects of radiation using much smaller sample sizes than more classical methods, uncertainty remains as to whether these effects correlate with the rare types of mutation that cause clinically serious conditions. There is no convincing evidence of transgenerational instability in humans caused by radiation that would lead to the propagation of clinically important effects over succeeding generations. Because the Committee's current risk estimates already assume transmission of many of the effects found in the first generation to later generations, rare instances of such propagation would have little impact on total risk estimates.

156. The relevance of non-targeted and delayed effects to the development of cancer and hereditary effects is not yet clear. Carcinogenesis involves a progression of genetic events that are associated with specific stages of the malignant process. It is tempting to speculate that induced genomic instability can drive the progression of genetic changes and thus provide the impetus for acquiring those genomic alterations associated with carcinogenesis. Yet this must be tempered by the high frequency with which instability is observed both in vitro and in vivo, and the observation that instability generally tends to saturate at low doses of radiation. Nevertheless, if radiation exposure induces a transgenerational instability that could be passed through the germ line and increase a child's susceptibility to cancer or genetic effects, this would have health ramifications. Bystander effects could also have significant implications for human exposures, particularly to very low fluences of high-LET radiation, e.g. to radon, where only a small fraction of the cell population would be hit, i.e. subject to energy deposition events. However, bystander effects appear to be limited to the irradiated organ, and since risk estimates are to an organ and not a cell, bystander effects are essentially encompassed in current radiation risk estimates for carcinogenesis.

157. Radiation-induced instability and the existence of bystander effects are well established and incontrovertible. A common observation of these responses is that they dominate at low doses and saturate with increasing dose (reviewed in references [P13, S18]). In addition to damage directly induced by the deposition of energy in the nucleus of the irradiated cell, consideration must now be given to these indirect effects of radiation. An irradiated cell can send out a signal and induce a response in a cell whose nucleus was not hit by radiation. This might result in genetic damage, genomic instability or lethality in non-irradiated cells. These non-targeted effects in essence "amplify" the biological effectiveness of a given radiation dose by increasing the number of cells that experience effects over those directly exposed to the radiation.

158. Understanding of these non-targeted effects is still in its infancy, and much of the data to date have been obtained from in vitro studies. While the significance of these indirect effects for human health remains to be elucidated, it would seem prudent to consider the implications of non-targeted delayed effects of radiation exposure when considering models of radiation carcinogenesis, particularly at low doses.

159. In vivo non-targeted effects are not new and have previously been implicated in radiation-induced carcinogenesis [S22]. The recent revival of interest in these non-targeted effects and the subsequent influx of new data suggest that it is time to re-examine the concepts of radiation dose and target size. Many of the indirect effects described indicate that the tissue volume in which detrimental effects of radiation may be observed is larger than the precise volume irradiated. This issue may have important implications for human health. Life exists in a radiation environment, the use of radiation has become an integral part of modern life, and the applications of radiation in medicine and industry bring tremendous benefits to society. Over time, biological systems have demonstrated a remarkable ability to adapt to environments to which they are gradually exposed, and low doses of radiation are no exception. Higher doses of radiation can cause neoplasia. This presumably occurs through a combination of direct damage and non-targeted effects, and models of radiation-induced carcinogenesis should incorporate both direct and indirect effects when evaluating radiation risks. Ultimately, understanding the multitude of multicellular responses to radiation may provide a framework for evaluating health risks associated with radiation exposure and a logical means of intervening in the development of suspected radiation-induced cancers.

CONCLUDING REMARKS

160. This annex has reviewed a multitude of studies on non-targeted and delayed effects of exposure to ionizing radiation. In addition to damage directly induced by the deposition of energy in the nucleus of an irradiated cell, consideration should now be given to these indirect effects of radiation. An irradiated cell can send out signals and induce a response in a cell whose nucleus was not subject to energy deposition events following irradiation. These non-targeted effects in essence "amplify" the biological effectiveness of a given radiation dose by increasing the number of cells that experience effects over those directly exposed to the radiation.

161. In spite of the large body of new information available, considerable disagreement remains concerning any definitive relationship between these non-targeted effects and the observed health effects attributable to radiation.

162. The Committee stresses that direct epidemiological observations and associated quantification of the health effects of radiation incorporate all mechanistic elements, including the targeted (direct) effects of irradiation as well as the non-targeted and delayed effects described in this report.

163. A specific role for non-targeted effects in the observed health effects associated with radiation exposure cannot be determined directly. Such effects can provide mechanistic information at doses of below ~200 mGy that could be pertinent to evaluating health effects at these low doses. However, in ascribing a mechanism to a particular biological effect, the data in question should be independently replicated and show a strong coherence with the particular end point considered. The UNSCEAR 2000 Report considered the conventional view that the deposition of energy in the nucleus and the subsequent cellular processing of induced DNA damage were consistent with the observed cancer/heritable effects induced by ionizing radiation.

164. In light of these considerations, the overall view of the Committee is that the data currently available do not require changes in radiation risk coefficients for cancer and hereditary effects of radiation in humans. The Committee will maintain surveillance of developments in the area of non-targeted and delayed effects, and recommends that future research pay particular attention to study design emphasizing replication, low-dose responses and associations with health effects particularly in the human population. Ultimately, understanding the range and multitude of multicellular responses to radiation will provide mechanistic insights into how radiation induces its observed health effects.

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