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Chromosomal instability in haemopoietic cells of the foetus, mother and offspring after *in utero* irradiation of the CBA/Ca mouse

M. ROSEMANN‡*, A. MILNER† and B. E. LAMBERT†

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Abstract.
Purpose: The present study was conducted to test the susceptibility of the mouse foetus to transmit chromosomal instability to the haemopoietic stem cells of offspring after *in utero* X-or plutonium-239-irradiation.
Materials and methods: Pregnant CBA/Ca-mice were injected with 80 kBq/kg ^{239}Pu or X-irradiated with 1 Gy X-rays on days 13 or 14 of gestation. CFU-A cultures were grown from haemopoietic stem cells sampled from foetal liver and the bone marrow from the offspring and from the mother. Non-clonal, unstable chromosomal aberrations were scored in metaphases from individual stem cell colonies.
Results: The relative excess (RE) of unstable chromosomal aberrations in foetal liver cells irradiated with 1 Gy X-rays increased from 1.6 at day 2 up to 2.7 at day 4 after irradiation. In the bone marrow cells from the mother, this value was 1.8 (average from cells sampled at days 3 and 14 after irradiation). After injection of the pregnant mice with ^{239}Pu, the yield of unstable chromosomal aberrations per cell was 0.14 ± 0.03 (RE approximately 10) in descendants of bone marrow cells from the mother, 0.11 ± 0.02 (RE = 10) in descendants of foetal liver cells and 0.16 ± 0.05 (RE = 10) in descendants of bone marrow cells from the offspring.
Conclusions: From the numerical analysis of non-clonal, unstable aberrations in haemopoietic cells from the foetus, the mother and the offspring after *in utero* irradiation, it was concluded that *in utero* irradiation of the CBA/Ca mouse was not more efficient in inducing chromosomal instability in the offspring than in the foetus or the mother. All three cell populations exhibited a similar degree of unstable aberrations, both in terms of the absolute numbers of non-clonal aberrations and in terms of relative excess compared with unexposed controls.

1. Introduction

Controversial observations persist about leukaemogenic risks after *in utero* exposure to ionizing radiation in view of reports about increased incidences of childhood leukaemia in the neighbourhood of nuclear power plants in Germany (Schmitz-Feuerhacke *et al.* 1993) and reprocessing facilities in the UK. Also, the increased leukaemia incidence in northern Greece (Petridou *et al.* 1996) and Germany (Michaelis *et al.* 1997), and the increased mutation rates in microsatellite sequences in children born to parents residing in heavily contaminated areas of Belarus (Dubrova *et al.* 1996) have been attributed to the fallout from the Chernobyl accident. However, most studies have received serious criticism for insufficient statistical evidence, absence of appropriate control groups, and for neglect of potential causes other than radiation (Darby and Roman 1996, Satoh *et al.* 1996, Michaelis *et al.* 1997). Two investigations on infant leukaemia from Belarus and Germany both came to the conclusion that a causal relationship between an increased leukaemia incidence and *in utero* exposure after the Chernobyl accident was not supported (Ivanov *et al.* 1998, Steiner et al 1998, *see also* Michaelis 1998 for a review).

However, from a mechanistic point of view, there are good reasons to assume that at least in the case of childhood acute lymphoid leukaemia, some of the early molecular or cellular alterations have occurred at the foetal stage (Greaves 1988, 1991). The small number of haemopoietic stem cells, which would represent the target for leukaemogenic transformation in the developing embryo, makes it difficult to explain the observed frequency of childhood leukaemia after *in utero* irradiation. It has recently been suggested that *in utero* irradiation of the early foetus may induce genomic instability, leading to an increase of aberrant haemopoietic stem cells that seed the bone marrow and expand in haemopoietic tissues (Edwards 1997). Such a mechanism has been shown in foetal skin fibroblasts from mice X-irradiated at the zygote stage (Pampfer and Streffer 1989): an increased frequency of *de novo* chromosomal aberrations was observed, demonstrating that chromosomal instability may indeed be transmissible from the irradiated embryo to the mature organism.

In vitro studies on haemopoietic stem cells of the CBA/H mouse (Khadim *et al.* 1992) revealed that,

*Author for correspondence at ‡. e-mail: rosemann@gsf.de
†Department of Radiation Biology, St Bartholomew's and the Royal London School of Medicine and Dentistry, Charterhouse Square, London EC1M 6BQ, UK.
‡Institute of Pathology, GSF Forschungszentrum für Umwelt und Gesundheit, Ingolstädter Landstrasse 1, D-85764 Neuherberg, Germany.

particularly after α-irradiation, the clonal descendants of irradiated cells frequently developed unstable aberrations. This chromosomal instability is being transmitted over several cell divisions before leading to overt aberrations. The instability could also be seen after the irradiated bone marrow had been transplanted into a recipient, with *de novo*-appearing unstable aberrations detected up to 1 year post-irradiation (Watson *et al.* 1996).

Chromosomal alterations are a characteristic feature of malignant cells in spontaneous (Rowley 1990, Testa 1990) as well as in radiation-associated leukaemias (Kamada 1991). There is also evidence that chromosomal or genetic instability is a mechanism that contributes to the progression not only of solid tumours (Loeb 1991, Cheng and Loeb 1993, Paquette and Little 1994, Shibata *et al.* 1994, Lengauer *et al.* 1997), but also of some forms of leukaemia. A number of genetic instability disorders in man, such as Fancony's anaemia, Bloom's syndrome and ataxia telangiectasia, are characterized by an increased frequency of spontaneous chromosomal breakage. These syndromes are also associated with a high susceptibility for malignant tumours, in particular for leukaemia and lymphoma (Cohen and Levy 1989). In a recent study in the CBA/H mouse, Plumb and colleagues demonstrated that the progression of X-ray-induced acute myeloid leukaemia (AML) is accompanied by an increasing instability of the Y-chromosome (Plumb *et al.* 1997).

As part of a larger project on the leukaemogenic potential of *in utero* exposure of the foetus to plutonium-239 (^{239}Pu), the authors studied whether and to what degree chromosomal instability could be detected in haemopoietic cells of the offspring born to mothers who were X-irradiated or exposed to ^{239}Pu during pregnancy.

2. Materials and methods

2.1. *Animal husbandry and irradiation*

CBA/Ca mice (Charles River, UK Ltd., Margate, UK) aged between 8 and 12 weeks old were mated and the day of appearance of a vaginal plug was taken as day zero of gestation. Pregnant mice were caged in pairs. On days 13 and 14 post-conception (p.c.), the pregnant animals received whole-body X-irradiation (1 Gy, 250 kVp, 1.5 mm Al-filter, dose rate 0.12 Gy/min) or were injected intraperitonally (i.p.) with 80 kBq/kg ^{239}Pu (as plutonium-nitrate in sodium-citrate solution, specific activity 10 kBq/ml), delivering approximately 6 mGy per day whole-organ dose. These doses were chosen because they represent the maximum tolerated value in terms of miscarriage or major birth defects of the offspring. Control pregnant animals were either sham-irradiated or received a sodium-citrate injection. Offspring were caged with their mothers until weaning at 3 weeks of age and then kept in groups of five sex-matched animals per cage.

At the indicated time-points, mice were killed by cervical dislocation and haemopoietic tissues were removed from the bone marrow (BM) of the mother's femur, from the foetal liver (FL) and from the bone marrow of the offspring's femur.

All mice were kept and treated in accordance with the Animals (Scientific Procedures) Act 1986, UK.

2.2. *Clonal cell culture (CFU-A)*

Colonies of haemopoietic stem cells were cultured as described by Lorimore *et al.* (1990). Single-cell suspensions were prepared from foetal liver or bone marrow and cultured at an initial concentration of 10^4/ml on soft agar. The cells were grown for 7 days at 37°C and 5% CO_2 in semisolid media consisting of Eagle's MEM (Gibco Life Technologies Ltd., Paisley, UK), 0.3% soft agar (low melting point agarose; Sigma, St Louis, USA), supplemented with 10% foetal calf serum, 10% horse serum, 5% AF1-19T cell-free conditioned media and 10% L929 cell-free conditioned media.

2.3. *Chromosomal preparation*

For CFU-A cultures the method for obtaining metaphase preparations was as described by Khadim *et al.* (1992). The cells were arrested in metaphase using 0.2 μg/ml colcemid and individual colonies were selected. Following hypotonic spreading with 0.075 M KCl, the cells were fixed in 3:1 methanol/acetic acid. All chromosome preparations were aged for 7 days at room temperature and G-banded using standard methods (Seabright 1971).

3. Results

3.1. *Unstable chromosomal aberration in foetal liver and adult bone marrow cells following X-irradiation of the pregnant mother as analysed by CFU-A colonies*

A total of 23 foetuses were X-irradiated *in utero* and 19 foetuses were sham-irradiated on days 13 or 14 p.c. Foetal liver cells were sampled between days 15 and 19 p.c. and plated out in CFU-A cultures. From 57 colonies picked for metaphase spreads, chromosomal aberrations were scored in 2282 cells (1414 from irradiated foetuses, 868 from control foetuses). A total of 96 unstable aberrations were found in progenitors of irradiated cells compared

with 39 in progenitors of unirradiated cells, giving aberration frequencies per metaphase of 0.068 ± 0.020 and 0.045 ± 0.007, respectively. This increase is statistically significant ($p < 0.05$) if each individual experiment is analysed as a data pair from irradiated and unirradiated mice (paired *t*-test).

Most of the unstable aberrations found were of the chromatid type, showing either a chromatid break with an acentric fragment or chromatid gaps (table 1). In progenitors of irradiated cells some chromosomal type aberrations were also found, which were mainly chromosomal breaks with acentric fragments. None of the chromosomal type aberrations found in individual colonies was of clonal origin as determined by the G-banding technique. Nevertheless, the distribution of aberrant cells per colony revealed an overabundance in some colonies, with an excess of cells carrying unstable aberrations over that expected assuming a Poisson distribution (data not shown). No dicentric aberrations were found, and only a few Robertsonian translocations (equal frequency in progenitors of control and irradiated cells).

The relative excess of unstable chromosomal aberrations increased with the sampling time post-irradiation (p.i.) from 1.6 at day 2 to 2.1 at day 3, 2.7 at day 4 and 1.9 at day 5, and this increase was statistically significant ($p < 0.05$) using the ANOVA test (figure 1).

A total of 458 metaphases from descendants of adult BM cells were analysed after clonal CFU-A culture (360 cells from four mice X-irradiated at day 13 p.c., 98 cells from four sham-irradiated mice).

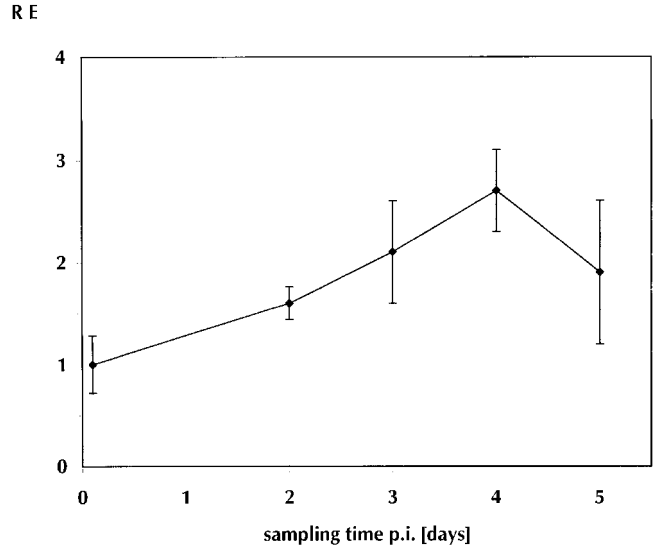

Figure 1. Relative excess of unstable aberrations in descendants of X-irradiated foetal liver cells sampled at different time-points after irradiation. Irradiation was carried out *in utero* on days 13 or 14 p.c. Foetal liver cells were sampled and seeded in CFU-A culture 2 to 5 days later. The relative excess (RE) is calculated as the ratio of unstable aberrations/metaphase in progenitors of X-irradiated and sham-irradiated cells. (Average value of up to three separate experiments ±SE of means.)

BM cells were removed from the femur between days 3 and 14 p.i. On average, 0.18 ± 0.09 unstable aberrations in the progenitors of irradiated BM cells were found compared with 0.10 ± 0.01 per unirradiated control cell. The difference between these mean values (four experiments) is statistically significant

Table 1. Different types of chromatid and chromosomal aberrations found in descendants of foetal liver cells and bone marrow cells from the mother and from the offspring after *in utero* irradiation to 1 Gy X-rays or injection of the mother with 80 kBq/kg ^{239}Pu.

	Irradiation	No. of cells analysed	Aberrant cells	Chromatid breaks and gaps	Chromosome breaks and acentric fragments	Dicentrics	Double minute	Fragmented chromatin
Foetal liver	X-ray	1414 (868)	75 (35)	80 (31)	12 (6)	0 (0)	3 (0)	1 (0)
Bone marrow (mother)	X-ray	360 (98)	n.d.	66 (10)[a]	[a]	[a]	[a]	[a]
Foetal liver	Pu	86 (29)	8 (0)	4 (0)	1 (0)	0 (0)	1 (0)	4 (0)
Bone marrow (mother)	Pu	133 (69)	15 (1)	15 (1)	4 (0)	0 (0)	3 (0)	1 (0)
Bone marrow (offspring)	Pu	223 (342)	29 (30)	36 (27)	5 (4)	0 (1)	0 (0)	5 (0)

Chromosomal preparations were made from cells of single CFU-A colonies derived from X-irradiated bone marrow cells from the mother (sampled between days 3 and 14 p.i.), ^{239}Pu-exposed bone marrow cells from the mother (sampled between days 32 and 42 p.p., equivalent to days 38 and 48 after injection), X-irradiated foetal liver cells (sampled between days 2 and 5 p.i.), ^{239}Pu-exposed foetal liver cells (sampled at day 3 after injection) and ^{239}Pu-exposed bone marrow cells from the offspring (sampled on days 22 and 55 p.p., equivalent to days 28 and 61 after injection).

Numbers in brackets refer to descendants of sham-irradiated cells.

n.d.: not determined.

[a]Not separated into different aberration types.

($p < 0.05$) using paired *t*-test. Due to the low number of analysed metaphases at some of the time-points no clear time-dependence of the aberration frequency could be determined.

3.2. *Unstable chromosomal aberrations in bone marrow cells from the mother and from the offspring after ^{239}Pu injection during pregnancy*

The transmission of unstable aberrations from the foetal liver to the bone marrow of offspring was studied following i.p. injection of ^{239}Pu into the pregnant mother. Plutonium was administered at day 14 p.c. and bone marrow cells were sampled from the mother between days 32 and 42 after birth. CFU-A cells derived from this bone marrow carried 0.14 ± 0.03 unstable aberrations/metaphase compared with only 0.014 ± 0.008 per sham-irradiated cell, giving a relative excess of 10. This confirms the high efficiency of incorporated ^{239}Pu in inducing unstable aberrations in haemopoietic cells *in vivo*.

Haemopoietic cells sampled from the foetal liver 3 days after ^{239}Pu injection and grown in CFU-A culture also exhibit a significant increase in the yield of unstable aberrations. In 86 metaphases scored in 10 CFU-A colonies from *in utero*-exposed foetal livers (sampled from five foetuses), four chromatid breaks or gaps, one double minute and four metaphases with fragmented chromatin were found. This yields 0.11 ± 0.02 unstable aberrations/cell compared with none found in 29 metaphases from unexposed foetal livers. Due to the low number of analysed metaphases from control cells, a reliable calculation of the relative excess cannot be given for ^{239}Pu-exposed foetal liver cells.

Bone marrow cells from the offspring with a history of *in utero* exposure to ^{239}Pu were sampled 22 and 55 days after birth and grown in CFU-A culture. Frequencies of unstable aberrations were, in general, higher than those observed in the two other cell populations. They ranged between 0.05 and 0.29 per cell from *in utero*-exposed mice, and between 0.09 and 0.26 per cell from unexposed offspring. In 223 descendants of exposed stem cells, 36 chromatid breaks and gaps and three chromosomal breaks with an acentric fragment were scored. Five metaphases had signs of completely fragmented chromatin. 342 cells derived from unexposed progenitors carried 30 chromatid-type and four chromosomal-type aberrations (table 1). The difference between the mean values of unstable aberrations/cell of 0.16 ± 0.05 (exposed group) and 0.11 ± 0.03 (control group) is statistically not significant ($p > 0.1$, *t*-test for paired samples; figure 2). A clearer demonstration of the effect of *in utero* administration of ^{239}Pu was found if the analysis was restricted to chromatid breaks only. Here the average yield increased from 0.019 in unexposed controls to 0.095 in descendants of *in utero*-exposed cells, giving a relative excess of approximately 5.

4. Discussion

Based on the observation that genomic instability increases with the number of cell divisions after radiation exposure (Marder and Morgan 1993), it has been suggested that a latent factor is transmitted from the exposed cell to its descendants. This would lead to overt genomic instability only after several cell divisions (Kennedy and Little 1984). This observation, together with many others on different cell types and phenotypic endpoints (Seymour and Mothersill 1989, Little *et al.* 1990, Chang and Little 1992, Khadim *et al.* 1992, Sabatier *et al.* 1992, Jamali and Trott 1996), was made *in vitro*. Consequently we cannot exclude the possibility that the forced and non-physiological cell proliferation contributes to the observed effects. Yet, a recent report showed that chromosomal instability could be detected *in vivo* up to 1 year after transplantation of α-irradiated haemopoietic stem cells (Watson *et al.* 1996). Also, there is evidence for the long-term persistence of genomic instability in skin fibroblasts from *in utero* X-irradiated mice (Pampfer and Streffer 1989).

The present study focused on the transmission of chromosomal instability from the *in utero*-irradiated foetus to the haemopoietic cells of the offspring. It was found that, following X-irradiation or injection of the mother with ^{239}Pu, the haemopoietic cells from the foetal liver showed a significant excess of unstable chromosomal aberrations.

The *de novo* appearance of these unstable cells from non-aberrant progenitor cells was suggested by the non-clonal nature of aberrant cells in single CFU-A colonies. The observation that the aberration frequency increased with the time period between X-irradiation and sampling of the cells (figure 1) also points to the underlying mechanism of delayed instability. The time-dependent increase of the relative yield of unstable aberrations in foetal liver cells sampled at different time-points after irradiation is consistent with chromosomal instability being a dynamic process requiring a number of cell divisions (Kennedy and Little 1984).

The observation that the distribution of aberrations in individual cells was overdispersed in favour of some heavily damaged cells is an indication of 'damage amplification' by the cell, a process that has been discussed in genetically unstable systems (Marder and Morgan 1993).

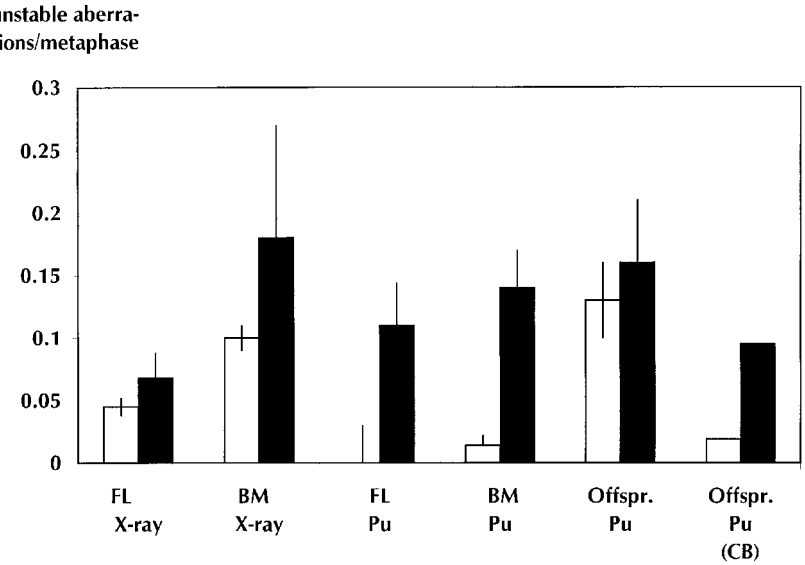

Figure 2. Unstable chromosomal aberrations/cell in CFU-A cultures derived from X- or ^{239}Pu α-irradiated cells (black bars) or from sham-irradiated cells (white bars). Foetal liver (FL) cells were sampled 2 to 5 days after X-irradiation or injection of the pregnant mother with ^{239}Pu, respectively. Bone marrow (BM) from the mother was sampled between days 3 and 14 after X-irradiation and between days 32 and 42 after birth (equivalent to days 38 and 48 after injection). Bone marrow from the offspring was sampled on days 22 and 55 after birth (equivalent to days 28 and 61 after ^{239}Pu exposure). Unstable aberrations include those listed in table 1. The last pair of columns reflect the exclusive appearance of chromatid breaks in the offspring's bone marrow. (Values are the means and error bars show standard error of the means.)

Chromosomal instability was also evident in bone marrow cells from the mother, both after X-irradiation or ^{239}Pu injection. Absolute yields of unstable aberrations in progenitors of bone marrow cells from the mother were 0.14 ± 0.03 after ^{239}Pu exposure and 0.18 ± 0.09 after X-irradiation. The accumulated organ dose delivered by incorporated ^{239}Pu during the 48 days between injection and sacrificing the animals is significantly below the 1 Gy as delivered by X-rays. The observation of a similar absolute yield of unstable aberrations after both radiation qualities would support a higher efficiency of incorporated ^{239}Pu for the induction of unstable aberrations. The relative excess of unstable aberrations (compared with unirradiated controls) also seems to be higher after plutonium exposure (relative excess = 10) than after X-irradiation (relative excess = 1.8), but this might be caused mostly by the higher aberration yield observed in control cells of the X-ray experiment. Although a direct and quantitative comparison between the efficiency of X-rays and ^{239}Pu-derived α-particles cannot be given here, the data show that the latter are very effective in causing chromosomal instability in haemopoietic cells, not only *in vitro* as shown by Kadhim *et al.* (1992), but also *in vivo* after incorporation of the nuclide.

Bone marrow cells from the offspring after *in utero* ^{239}Pu administration also had an increased yield of unstable aberrations when cultured as CFU-A colonies. The frequency of unstable aberrations detected between days 22 and 55 after birth in the offspring's bone marrow cells after *in utero* ^{239}Pu exposure was well within the range of that observed in bone marrow from the mother and in the foetal liver. The increase, when compared with unexposed cells, was smaller in bone marrow cells sampled from the offspring (relative excess = 1.23, confidence range = 0.7–2.1, deviation from 1 not statistically significant) than in those sampled from the mother. This, however, might in part be due to the higher number of unstable aberrations found in unexposed bone marrow cells from the offspring than from the mother.

The variations in the relative frequency of unstable aberrations not only in X-irradiated but also in control BM and FL cells makes it likely that the method itself might influence the outcome of the assay. Thus, as delayed chromosomal instability seems to be influenced by the number of cell divisions following the initiating event (Kennedy and Little 1984), this process might be influenced by the *in vitro* assay. One candidate is the addition of growth factors by the conditioned medium which leads to variable accelerated proliferation of the stem cells. Although this could make the assay more sensitive (latent damages in resting cells would be revealed after cells are forced to divide), it is also likely to make the

results less robust against variations in the culture conditions.

Taking all data together, it could be concluded that neither in terms of absolute aberration yields nor in terms of relative excess, does *in utero* exposure with ^{239}Pu have a higher efficiency in causing chromosomal instability in the offspring than in the mother.

Plutonium administered to pregnant mice rapidly accumulates in the foetal liver between day 15 p.c. and birth (Lord *et al.* 1992, Mountford-Lister and Lambert 1992, Lambert *et al.* 1993). Thus, it is unlikely that the observed low efficiency in terms of long-term chromosomal instability in the offspring is caused by reduced access of the isotope to the foetal liver cells.

A possible explanation for the low efficiency of ^{239}Pu in inducing instability in the bone marrow cells of the new born mice could be that haemopoietic stem cells from the foetal liver carrying chromosomal aberrations fail to seed the bone marrow and are eliminated. The rapid proliferation in the foetal haemopoietic system might lead to a faster manifestation of latent damage compared with adult bone marrow. According to the authors' own observations and studies published by other investigators (Lord *et al.* 1992, Mason *et al.* 1992), the reduction of CFU-As or CFU-S observed after X- or α-irradiation per unit dose is more pronounced in haemopoietic cells from the foetus than from an adult. This suggests that cells carrying chromosomal instabilities are eliminated more readily from the foetal than from the adult haemopoietic system. Furthermore, the haemopoietic microenvironment shows a long-term deficiency after foetal irradiation (Mason *et al.* 1992, Yang *et al.* 1995), which may increase the selection pressure on haemopoietic stem cells bearing unstable aberrations.

In terms of the potential leukaemogenic effect of *in utero* exposure, a long-term experiment in CBA/Ca mice irradiated at the foetal stage with a similar ^{239}Pu dose to the present study, also failed to show an effect in terms of AML induction in the offspring (Mountford-Lister 1997). Similar observations have been made by others (Schmal 1988, Antal *et al.* 1997) in NMRI or C57xDBA F1-mice receiving *in utero* γ- or X-irradiation. In these studies, a reduction of AML after *in utero* exposure (compared with the spontaneous incidence) was observed.

However, the unexpectedly low efficiency of *in utero* plutonium exposure in causing chromosomal instability in the mouse offspring suggests caution in extrapolation to the human situation. The pre- and post-natal development of the haemopoietic system is significantly different between mice and man. Whereas migration of the blood stem cells from the foetal liver to the bone marrow in mice takes place mainly post-natally, in man this process is almost complete at birth. Thus, radiation exposure of the mouse foetal liver in the later stages of pregnancy might only be comparable with foetal irradiation in the first or second trimester in humans.

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