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Sensitivity of murine haemopoietic stem cell populations to X-rays and 1 MeV fission neutrons *in vitro* and *in vivo* under hypoxic conditions

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Abstract. The radiosensitivity of primitive haemopoietic stem cells that repopulate the bone marrow with precursors of granulocytes and macrophages (MRA[CFU-C]), mature stem cells capable of forming spleen colonies in lethally irradiated recipients (CFU-S-7) and colony-forming units in culture (CFU-C) were determined *in vitro* and under hypoxic conditions *in vivo* for 1 MeV fission neutrons and 300 kV X-rays. The obtained D_0's were compared with previously observed D_0's after irradiation *in vivo* under normal oxic conditions. With 1 MeV fission neutron irradiation no significant difference in radiosensitivity of the cell populations was observed between normal *in vivo* irradiation and *in vitro* irradiation. With 300 kV X-rays a lower radiosensitivity for all three cell populations was observed after *in vitro* compared to *in vivo* irradiation. *In vivo* irradiation with fission neutrons under hypoxic conditions led to a small decrease in radiosensitivity. The obtained oxygen enhancement ratio (*OER*) for fission neutrons varied from 1·2 for MRA[CFU-C] to 1·5 for CFU-C. After *in vivo* irradiation with 300 kV X-rays under hypoxic conditions much higher *OER*s were observed. An *OER* = 1·8 was obtained for CFU-S and for MRA[CFU-C] and for CFU-C OER 3·0 and 2·9 were observed. These results indicate that the radioresistance of primitive haemopietic stem cells (MRA[CFU-C]) compared to mature stem cells (CFU-S-7) is mainly due to intrinsic factors and not to differences in localization or oxygenation between primitive and mature stem cells.

1. Introduction

In a previous study we determined the radiosensitivity of various haemopoietic stem cell and progenitor cell populations after *in vivo* irradiation with 300 kV X-rays (Meijne *et al.* 1991) and 1 MeV fission neutrons (Meijne *et al.*, submitted). Primitive haemopoietic stem cells, which repopulate the irradiated bone marrow with secondary CFU-S or *in vitro* clonable progenitors of granulocytes and macrophages (MRA[CFU-S-12], MRA[CFU-C]) (Hodgson and Bradley 1979, Ploemacher and Brons 1989), were found to be more resistant to ionizing irradiation than more mature stem cells (CFU-S). When mature stem cells differentiated into *in vitro* clonable progenitors of granulocytes and macrophages (CFU-C) their radiosensitivity decreased again. The radiosensitivity of the various cell populations may however be influenced by local conditions in the irradiated femora. Variations in the atomic composition of the various tissue in the mammalian body may lead to inhomogeneities in dose-distribution, especially at the bone-soft tissue interface (Broerse and Barendsen 1968a). Dependent on their location in the bone marrow, some cell populations may therefore absorb more or less dose than other cell populations. Another factor that may influence the observed radiosensitivity of the haemopoietic cell populations are local differences in oxygen tension (Broerse *et al.* 1968b, Allalunis *et al.* 1983, Allalunis-Turner and Chapman 1986). To investigate to what extent these two factors influence the *in vivo* radiosensitivity of haemopoietic stem cell and progenitor cell populations we irradiated bone marrow cells *in vitro* and *in vivo* under hypoxic conditions.

2. Materials and methods

2.1. *Mice*

Inbred CBA/H mice (H-2k) were bred at the Netherlands Energy Research Foundation (Petten, The Netherlands). The mice were maintained under clean conventional conditions. The animal care procedures have been described elsewhere (Davids 1970). Male donor mice were irradiated or sham-irradiated with graded doses of X-rays or fission neutrons at the age of 12–14 weeks. Male or female mice aged ≥14 weeks were used as recipients in the CFU-S and MRA assays.

The experiments were conducted with permission of the experimental animal welfare commission

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2.2. *Irradiation procedures*

Mice or cell suspensions were irradiated with fast fission neutrons from a ^{235}U-converter in the Low Flux Reactor at Petten. The design of the exposure facility, the tissue dosimetry, and the neutron spectrometry have been described elsewhere (Davids *et al.* 1969). The animals were exposed bilaterally to a fast neutron dose-rate of 0·1 Gy/min. The absorbed doses are given as neutron centre-line doses; they do not include the 9% γ-ray contribution. The neutron spectrum has a mean energy of 1·0 MeV and the mean track average LET of the recoil protons produced in tissue is equal to 57 keV/μm in water.

Total body X-irradiation (TBI) was performed with a Philips Müller X-ray tube, operating at 300 kV constant potential and 5 mA (HVL 2·1 mm Cu). The dose-rate was equal to 0·30 Gy/min in the centre-line of the animals. The distance from the focus to the centre-line was 69 cm. During irradiation the animals were confined in polycarbonate tubes, which were mounted in a rotating disk of Perspex.

For irradiation under hypoxic conditions (Millard and Blackett 1981) mice were killed by CO_2 gassing and subsequently kept at room temperature for 12 min before starting X-irradiations, or 30 min before starting the fission neutron irradiations. Due to the distance between the laboratory and the reactor and the start-up procedures it was not possible to shorten the time interval between killing of the mice and start of the neutron irradiations beyond 30 min. Immediately after irradiation both femurs were excised and placed in ice-cold Hanks (Gibco). *In vitro* irradiations were performed on marrow cell suspensions in 5-ml polystyrene tubes (Falcon) at 0°C. The tubes were agitated immediately before irradiation in order to keep the cells in suspension. During irradiation the cell suspensions were kept on ice.

2.3. *Haemopoietic cell suspensions*

Mice were killed by CO_2 gassing. Femurs were freed from muscles and tendons and ground in a mortar using 1 ml RPMI (Flow) containing 0·04% bovine serum albumin (BSA; Sigma), penicillin (100 IU/ml) and streptomycin (100 mg/ml) per femur. The cell suspension was put in a Falcon tube and the larger bone particles were allowed to settle for 45 s. The supernatant was sieved through a Nylon filter (pore size 100 μm) and the cellularity determined with a Coulter counter.

2.4. *CFU-S assay*

CFU-S were assayed according to Till and McCulloch (1961). Recipient mice received 9 Gy X-rays 1–4 h before injection of cell suspensions. This dose reduced endogenous CFU-S-7 to 0. Cell suspensions were always made from at least three donor mice. Ten mice were used as recipient for the CFU-S-7 assay. Each recipient received 2×10^4 normal bone marrow cells or equivalent cell doses irradiated bone marrow cells in 0·2 ml Hanks (Gibco) by lateral tail vein injection. Seven days later the mice were killed, their spleens were excised and fixed in Telleyesniczky's solution. The macroscopic surface colonies were counted with a stereo-microscope at 10× magnification and their diameters measured with an eyepiece micrometer.

2.5. *CFU-C assay*

CFU-C (including CFU-M, CFU-G and CFU-GM) were quantified in a semisolid (0·8% methylcellulose, Methocel, AP4 Premium; Dow Chemical) culture medium (Alpha medium) containing 1% BSA, 10% FCS and 10% Poke Weed Mitogen mouse spleen conditioned medium (PWM-MSCM). 2×10^4–$1{\cdot}5 \times 10^6$ cells were plated in 35–mm Costar dishes in 1 ml final culture medium. Duplicate cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air. Colonies (>50 cells) were counted after 7 days of culture using an inverted microscope.

2.6. *Marrow repopulating ability* (*MRA*)

For the repopulation assays five lethally irradiated recipients were injected with 1–5 times the number of donor bone marrow cells as used for the CFU-S assays. Twelve days after transplantation aliquots of their femoral marrow were assayed for the presence CFU-C. MRA was expressed as the number of haemopoietic precursor cells (MRA[CFU-C]) generated over a 12-day period in one femur of a lethally irradiated recipient per 10^5 cells injected (Hodgson *et al.* 1982). Control irradiated mice, which received no bone marrow transplantation, were included in each experiment,

and precursor cell contents were corrected for endogenous precursors if present.

2.7. *Experimental procedures and statistics*

Log-linear dose–effect curves were obtained by least squares regression analysis. D_0's were obtained from the slope of these curves. D_0's from three-to-four individual experiments were pooled. Each experiment in which mice or cells were irradiated with X-rays contained seven dose groups and experiments in which mice or cells were irradiated with fission neutrons contained five dose groups. Statistical comparison of D_0's was performed with a Chi-square test.

3. Results

3.1. In vitro *radiosensitivity of haemopoietic cell populations*

The survival curves for MRA[CFU-C], CFU-S-7 and CFU-C irradiated *in vitro* and *in vivo* with graded doses 1 MeV fission neutrons or X-rays are shown in figures 1–3 respectively. From these survival curves D_0's were calculated, summarized in Table 1. *In vitro* irradiation of MRA[CFU-C] with fission neutrons led to a small increase in radiosensitivity compared to the *in vivo* situation. The D_0 decreased from 0·46 ±0·01 Gy for *in vitro* irradiation to 0·40 ±0·04 Gy for *in vivo* irradiation. The decrease in D_0 was however not significant. The radiosensitivity of CFU-S-7 and CFU-C irradiated *in vitro* with fission neutrons was similar to the radiosensitivity observed after *in vivo* irradiation. The D_0's observed for MRA[CFU-C], CFU-S-7 and CFU-C after *in vitro* irradiation with 300 kV X-rays were higher for all three cell populations. The D_0 of 1·25 Gy observed after *in vitro* irradiation of MRA[CFU-C] was 13% higher than the D_0 of 1·11 Gy observed after *in vivo* irradiation. The increase was however not significant. The D_0's calculated for CFU-S-7 and CFU-C after *in vitro* irradiation with 300 kV X-rays were respectively 22 and 34% higher. Although the decrease in radiosensitivity observed after *in vitro* X-irradiation varied from 13% for MRA[CFU-C] to 34% for CFU-C, the radiosensitivity pattern within the stem cell hierarchy was identical after *in vitro* irradiation compared to *in vivo* radiation. Irrespective the mode of irradiation primitive MRA[CFU-C] stem cells were more resistant to ionizing radiation

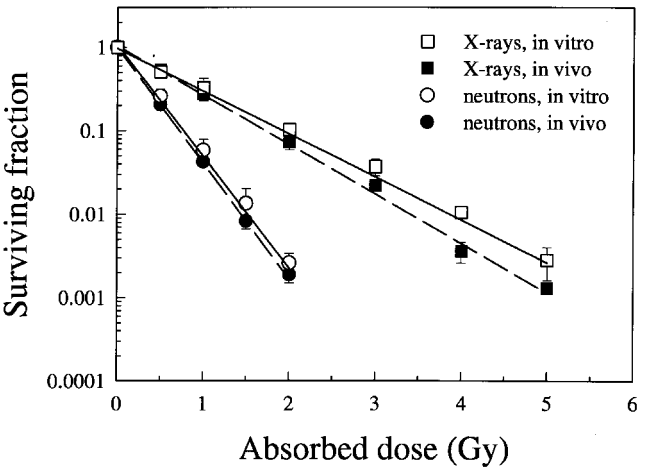

Figure 2. Dose–response curves for CFU-S-7 irradiated *in vitro* (solid lines) or *in vivo* (dashed lines) with X-rays or fission neutrons.

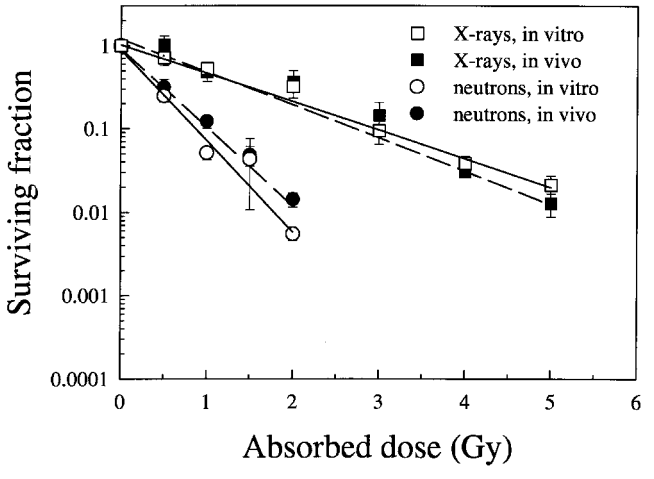

Figure 1. Dose–response curves for MRA[CFU-C] irradiated *in vitro* (solid lines) or *in vivo* (dashed lines) with X-rays or fission neutrons.

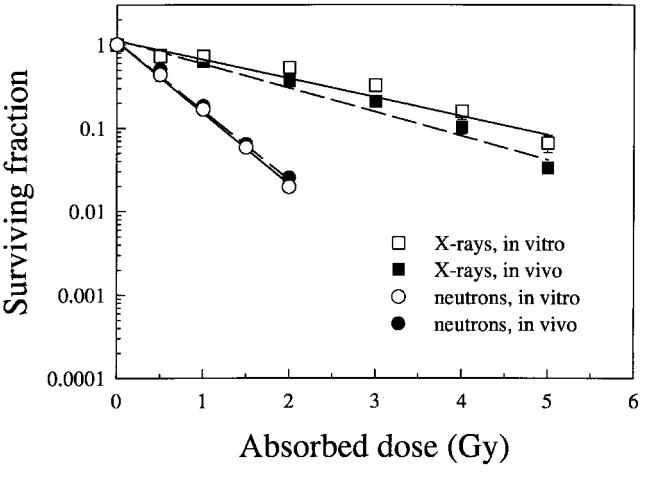

Figure 3. Dose–response curves for CFU-C irradiated *in vitro* (solid lines) or *in vivo* (dashed lines) with X-rays or fission neutrons.

Table 1. Radiobiological characteristics of haemopoietic stem and progenitor cell populations irradiated *in vivo* or *in vitro* with X-rays and 1 MeV fission neutrons.

	D_0 after *in vivo* irradiation (Gy)	D_0 after *in vitro* irradiation (Gy)
1 MeV fission neutrons		
MRA[CFU-C]	0·46 ±0·01	0·40 ±0·04
CFU-S-7	0·31 ±0·01	0·33 ±0·02
CFU-C	0·51 ±0·01	0·50 ±0·01
X-rays		
MRA[CFU-C]	1·11 ±0·03	1·25 ±0·08
CFU-S-7*	0·74 ±0·01	0·90 ±0·03
CFU-C*	1·50 ±0·05	2·01 ±0·13

* D_0 observed after *in vitro* irradiation significantly differs from D_0 observed after *in vivo* irradiation (CFU-S-7, $p < 0{\cdot}001$; CFU-C, $p < 0{\cdot}025$).

than more mature CFU-S-7. CFU-C which belong to the committed progenitor compartment, were most radioresistant to *in vivo* as well as *in vitro* irradiation.

3.2. *Radiosensitivity of haemopoietic cell populations under hypoxic conditions*

The survival curves for MRA[CFU-C], CFU-S-7 and CFU-C irradiated *in vivo* under hypoxic conditions are shown in figures 4–6. TBI with 1 MeV fission neutrons under hypoxic condition led to a significant decrease ($p < 0{\cdot}001$) in radiosensitivity for all three cell populations compared to normal *in vivo* irradiation. The D_0 of MRA[CFU-C] increased from 0·46 ±0·01 Gy for normal *in vivo* irradiation to 0·55 ±0·02 Gy for irradiation under hypoxic conditions and the D_0's for CFU-S-7 and CFU-C increased from 0·31 ±0·01 to 0·41 ±0·01 and from 0·51 ±0·01 to 0·74 ±0·03 Gy respectively. The calculated oxygen enhancement ratios (*OER*s) were small and ranged from 1·2 for MRA[CFU-C] to 1·5 for CFU-C.

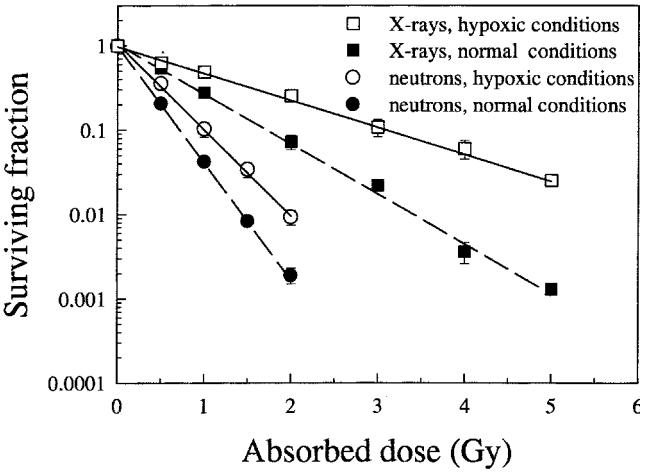

Figure 5. Dose–response curves for CFU-S-7 irradiated *in vivo* under hypoxic (solid lines) or normal oxic conditions (dashed lines) with X-rays or fission neutrons.

Irradiation of the three haemopoietic cell populations with 300 kV X-rays under hypoxic conditions led to a large decrease in radiosensitivity. This decrease in radiosensitivity was most prominent for MRA[CFU-C] and CFU-C. The D_0 for MRA[CFU-C] increased from 1·11 ±0·01 Gy for normal *in vivo* irradiation to 3·27 ±0·32 Gy for irradiation under hypoxic conditions and the D_0 for CFU-C increased from 1·50 ±0·01 to 4·33± 0·27 Gy. The calculated *OER*s were 3·0 for MRA[CFU-C] and 2·9 for CFU-C. The decrease in radiosensitivity of CFU-S-7 for X-irradiation under hypoxic conditions was much lower. The D_0 increased from 0·74 ±0·01 to 1·33 ±0·07 Gy for

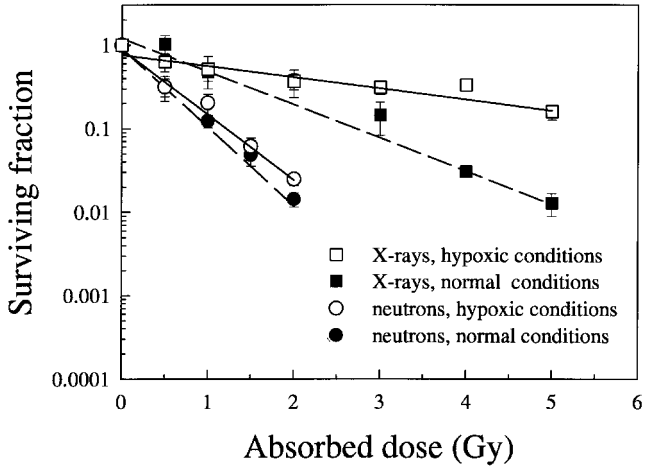

Figure 4. Dose–response curves for MRA[CFU-C] irradiated *in vivo* under hypoxic (solid lines) or normal oxic conditions (dashed lines) with X-rays or fission neutrons.

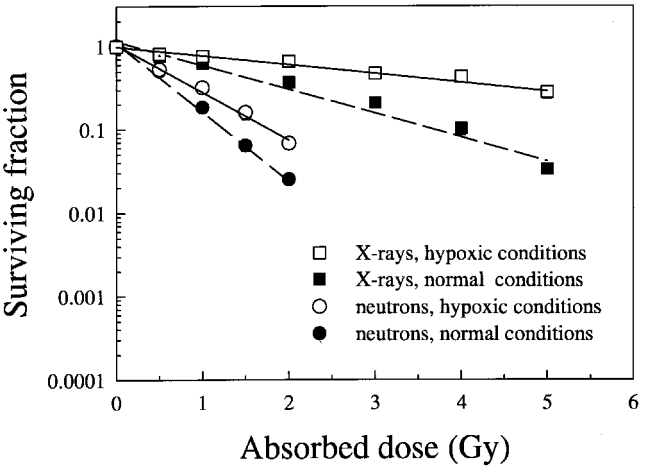

Figure 6. Dose–response curves for CFU-C irradiated *in vivo* under hypoxic (solid lines) or normal oxic conditions (dashed lines) with X-rays or fission neutrons.

irradiation *in vivo* under hypoxic conditions. The calculated *OER* was 1·8. The results are summarized in Table 2.

4. Discussion

In the present study we determined the radiosensitivity of haemopoietic stem cell and progenitor cell populations *in vitro* and *in vivo* under hypoxic conditions in order to determine if the previously observed differences in radiosensitivity between these cell populations in normal air breathing mice are caused by local differences in absorbed dose and/or oxygen tension. These two factors can influence the radiosensitivity *in vivo* because the spatial distribution of cells in the bone marrow is by no means random and probably each definable cell type has its own specific distribution. CFU-C have been reported to have a bimodal distribution in the mouse femur. The maximal concentration of CFU-C is found at approximately 120 μm from the bone surface (Lord *et al.* 1975). The concentration of CFU-S decreases from the marginal zone to the longitudinal axis of the femur. A 2–3 times higher concentration of CFU-S can be found close the femur shaft than in the centre of the marrow spaces (Lord *et al.* 1975, Gong *et al.* 1978). Not only the concentration of CFU-S varies across the femoral axis, but also the quality of the CFU-S. The primitive CFU-S, which are proliferatively quiescent reside close to the centre of the marrow spaces, while the mature CFU-S (CFU-S-7) can be found close to the bone surface (Lord 1986). The concentration of MRA[CFU-S-12] is highest close to the centre of the bone (Lord 1992). Since MRA[CFU-C] can be considered a reflection of MRA[CFU-S-12] (Hodgson *et al.* 1982) it is most likely that the MRA[CFU-C] are also located near the centre of the femur.

The above described variations in the spatial distribution of the various cell populations leads to small differences in absorbed dose due to perturbation of the secondary particle equilibrium at the bone bone-marrow interface caused by the difference in atomic composition of these two tissues. Neutrons mainly transfer energy through elastic collisions with hydrogen nuclei. Due to the lower hydrogen content of the bone, the absorbed dose in bone marrow cells in a 30 μm thick layer adjacent to the bone is 12% lower and in the bone marrow layer remote from the bone 6% lower than in soft tissue after irradiation with 15 MeV neutrons (Broerse and Barendsen 1968a). The range of recoil protons produced by 15 MeV neutrons is on average large compared to the diameter of a cell. A proton of 10 MeV energy has a range of about 1200 μm compared to a range of about 25 μm observed for 1 MeV protons (Broerse and Barendsen 1968a). For 1 MeV fission neutrons, used in this study, a lower absorbed dose will only be observed in a small layer of approximately 25 μm adjacent to the bone. At a larger distance from the bone a new secondary particle equilibrium will have been formed. Therefore, CFU-S located within the first 25 μm next to the bone will receive a lower dose compared to CFU-S irradiated *in vitro*. The D_0 observed after *in vitro* irradiation of CFU-S was however not significant different from the D_0 observed after *in vivo* irradiation. This means that the difference in absorbed dose due to the local perturbation of the secondary charged particle equilibrium is not large enough to exert a substantial effect. Alternatively, the proportion of the CFU-S population, which is effectively located within the first 25 μm from the bone, might be small in the mouse strain used in our studies. The large difference in marginal and axial CFU-S concentration have not been observed by all authors (Maloney *et al.* 1978).

Table 2. Radiobiological characteristics of haemopoietic stem and progenitor cell populations irradiated under normal or under hypoxic conditions with X-rays or 1 MeV fission neutrons

	D_0 (Gy) in normal (air-breathing) mice	D_0 (Gy) in hypoxic (asphyxiated) mice	Oxygen enhancement ratio
1 MeV fission neutrons			
MRA[CFU-C]*	0·46 ±0·01	0·55 ±0·02	1·2
CFU-S7*	0·31 ±0·01	0·43 ±0·01	1·4
CFU-C*	0·51 ±0·01	0·74 ±0·03	1·5
X-rays			
MRA[CFU-C]*	1·11 ±0·01	3·27 ±0·32	3·0
CFU-S-7*	0·74 ±0·01	1·33 ±0·07	1·8
CFU-C*	1·50 ±0·05	4·33 ±0·27	2·9

* D_0 observed after *in vivo* irradiation under hypoxic conditions significantly differs from D_0 observed after *in vivo* irradiation under normal conditions ($p < 0{\cdot}001$).

When an animal is exposed to whole-body X-irradiation, the energy absorbed by the bone marrow is greater than that absorbed by soft-tissue parts distant from bone. The bone marrow cells close to the femur shaft receive a relatively high dose owing to an excess of secondary electrons produced by photoelectric absorption in the minerals of the bone. The dose absorbed by cells in an adjacent layer of 30 μm next to the bone will receive a 17% higher dose and cells in a bone marrow layer remote from bone still receive a 3% higher dose compared to the mean absorbed dose in soft muscle tissue after irradiation with 250 kV X-rays (Epp *et al.* 1959, Broerse and Barendsen 1968a). For 300 kV X-rays identical percentages might be expected. The radiosensitivity of the haemopoietic cell populations irradiated *in vitro* in suspension was indeed lower (higher D_0's) compared to cells irradiated within the animal. Surprisingly the increase in D_0 for CFU-C irradiated *in vitro* compared to *in vivo* was higher than the increase observed for CFU-S-7. Because CFU-S-7 are located more closely to the bone a higher increase for CFU-S-7 would be expected. The *OER* for CFU-S-7 is however lower than the *OER* for CFU-C, indicating that the environmental conditions in which the CFU-S-7 reside in the air-breathing animal are more hypoxic than those of CFU-C. The higher dose the CFU-S-7 receive *in vivo* due to more short-range secondary electrons may therefore be partly compensated by a less oxygenated environment. The decrease in radiosensitivity observed for the three cell populations in however higher than expected based on microdosimetric factors. Possibly more cells survived irradiation *in vitro* because the cells in suspensions were not so well oxygenated as the cells in the animal.

Differences in oxygenation level between the various cell populations were determined by comparing the radiosensitivity of the cells under normal and under hypoxic conditions. In general the radiosensitivity of a tissue for neutron irradiation is not much dependent on the oxygenation level of the tissue. Indeed the *OER*s observed for MRA[CFU-C], CFU-S-7 and CFU-C after fission neutron irradiation were low and varied from 1·2 to 1·5. These values are comparable to the *OER* of 1·5 observed for human kidney cells after 1 MeV fission neutron irradiation (Broerse *et al.* 1968b). The $(OER)_{\mathrm{Xrays}}$ for MRA[CFU-C] and CFU-C were higher than the *OER* of 1·8 for CFU-S-7. This indicates that in normal air breathing mice, the oxygenation level at the location the CFU-S-7 reside is lower than the oxygenation level at the place of MRA[CFU-C] and CFU-C, since induction of hypoxia increased the survival of CFU-S less than the survival of MRA[CFU-C] and CFU-C. The $(OER)_{\mathrm{Xrays}}$ of 1·8 for CFU-S is lower than the $(OER)_{\gamma\mathrm{rays}}$ of 2·4 observed by Millard and Blackett (1981) for CFU-S irradiated with γ-rays. Also the $(OER)_{\mathrm{Xrays}}$ of 2·7 observed by Broerse and Barendsen (1973) after *in vitro* irradiation of CFU-S-9 was higher. The *OER* of 2 observed by Blackett (1974) and Hendry and Howard (1972) was however similar. On the other hand the $(OER)_{\mathrm{Xrays}}$ of 2·9 observed for CFU-C was higher than the $(OER)_{\gamma\mathrm{rays}}$ of 2·1 (Allalunis *et al.* 1983) and 1·3 (Millard and Blackett 1981) observed for agar colony-forming units. It is however difficult to compare D_0 and *OER* of various investigators. Differences in mouse strain, radiation source and conditions, days of colony culture, culture medium, and especially colony stimulating factor (Baird *et al.* 1990) used may cause large differences in the observed radiosensitivity. Therefore it is very important to compare the radiosensitivity of different cell population within one mouse strain and within one institute.

In summary our data show that differences in radiosensitivity between MRA[CFU-C], CFU-S and CFU-C are mainly caused by intrinsic differences between the cell populations and not by differences in oxygenation level of localization in the bone marrow.

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