RADIOSENSITIVITY OF THE MURINE HEMOPOIETIC STEM CELL COMPARTMENT

The effects of 1 MeV fission neutrons and 300 kV X-rays

DE STRALINGSGEVOELIGHEID VAN HET HEMOPOËTISCHE STAMCELCOMPARTIMENT VAN DE MUIS

De effecten van 1 MeV splijtingsneutronen en 300 kV röntgenstraling

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GENERAL INTRODUCTION

This thesis describes the effects of 300 kV X-rays and 1 MeV fission neutrons on hemopoietic stem cells and their microenvironment. In the first part of this chapter the characteristics of ionizing radiation in general and of X-rays and fission neutrons in particular are described; the second part describes the hemopoietic system.

1.1 IONIZING RADIATION

Ionizing radiation is a very potent cytotoxic agent, which exerts its effects by depositing energy in biological material. The amount of energy deposited in irradiated cells or tissues per unit mass is called absorbed dose. The international unit (S.I. unit) for absorbed dose is the gray (Gy), 1 Gy corresponds to 1 joule (J) of absorbed radiation energy per kg tissue. The gray has replaced the rad, which was the former unit used to quantify absorbed dose (1 Gy = 100 rads). The important characteristic of radiation, which is responsible for its high efficiency as a cytotoxic agent, is the localized release of energy. A total body dose of 4 Gy X-rays or gamma-rays, which leads to death of an individual in 50% of the cases, represents an absorption of energy of only 280 J (or 67 cal), assuming the irradiated individual being a "standard man" weighing 70 kg. The same amount of energy is absorbed by drinking a sip of warm coffee (Hall, 1988). The effectiveness of ionizing radiation is therefore not due to the total energy absorbed but to the unevenly deposition in tissues and cells of large individual packets of energy. In the case of ionizing radiation this energy is high enough to eject one or more orbital electrons from a molecule, which can lead to a break in a chemical bond and initiate a chain of effects that eventually culminates in a biological effect. Table 1.1 illustrates the large number of ionizations that occur in a mammalian cell after irradiation with 1 Gy X-rays. Most ionizations are not deleterious to the cell because of the redundancy and normal rate of replacement of most molecules. Molecule replacement is the common mechanism for the repair of molecules and organelles that are present in multiple copies in a cell. However, for DNA, which is a single copy molecule, enzyme repair mechanisms are responsible for the maintenance of the structural and functional integrity of the molecule. The DNA molecule, which contains the genetic information a cells needs to live and propagate itself, is the most important molecule in living cells and is generally considered to be the critical target for radiation-induced lethality in

rapidly proliferating cell systems. Ionizing radiations produce many different types of molecular damage to DNA. Table 1.2a gives an estimate of the physical and biochemical lesions that occur in a mammalian cell when irradiated with 1 Gy of low-LET radiation such as gamma-rays. Table 1.2b gives an estimate of some of these lesions after 1 MeV fission neutron irradiation. Together these lesions produce conditions of atomic and molecular disturbance which eventually can lead to cellular responses such as cell death, mutation, chromosome rearrangement and oncogenic transformation.

Component	Number of molecules/cell	Ionizations /cell
H_2O	$9.4\ 10^{13}$	580000
Inorganic ions	$6\ 10^{11}$	~ 9000
Miscellaneous small metabolites	$2.4\ 10^{11}$	25000
Proteins	$1\ 10^{10}$	150000
Phospholipids	$1.4\ 10^{11}$	25000
Other lipids	$1.2\ 10^{11}$	16500
Polysaccharides	$4.8\ 10^{10}$	16500
RNA	$3\ 10^{09}$	9000
DNA	40 - 48[a]	~ 2000

Table 1.1: Molecular composition of a typical mammalian cell[b] and the number of ionizations produced by 1 Gy of low-LET radiation (adapted from Chapman et al., 1991).
[a]The number of chromosomes per cell varies per species. Murine cells for example contain 40 (Lee et al., 1990), human cells 46 and ape cells 48 chromosomes (IJdo et al., 1991).
[b]Cell weight of $\pm 4\ 10^{-9}$ g; cell volume of $\pm 4\ 10^{-9}$ cm^3

1.1.2 CHARACTERISTICS OF DIFFERENT TYPES OF IONIZING RADIATION

1.1.2.1 Electromagnetic versus particulate radiation

There are two types of ionizing radiation, electromagnetic and particulate radiation. X- and γ-rays are both electromagnetic radiation and can be considered from two different standpoints: first, as a wave of electrical and

Effect	Number of events
Initial physical damage	
Ionizations in cell nucleus	~ 100000
Ionizations directly in DNA	~ 2000
Excitations directly in DNA	~ 2000
Selected biochemical damage	
DNA single-strand breaks	1000
8-Hydroxy adenine	700
T^ (thymine damage)	250
DNA double strand breaks	40
DNA-protein cross links	150
Selected cellular effects	
Lethal events	~ 0.2 - 0.8
Chromosome aberrations	~ 1
Hprt mutations	~ 10^{-5}

Table 1.2a: Some of the damage in a mammalian cell nucleus caused by 1 Gy of low-LET radiation (after Goodhead et al., 1994; Ward, 1988).

Selected biochemical damage	
DNA single-strand breaks	600
DNA double strand breaks	70

Table 1.2b: Some of the damage in a mammalian cell nucleus caused by 1 Gy 1 MeV fission neutrons (Goodhead, 1990).

magnetic energy or secondly, as a stream of photons. Compared to non-ionizing electromagnetic radiation (like for example radiowaves, microwaves and ultraviolet light) X- and γ-rays have a short wavelength (λ). Since all waves have the same velocity ($c = \lambda * \nu$), the frequency (ν) of X- and γ-rays is much larger than the frequency of non-ionizing electromagnetic radiation. Alternatively X- and γ-rays can be thought of as photons with an energy (E) and a frequency (ν) ($E = h * \nu$, in which h is a constant known as Planck's

constant). Electromagnetic radiation with a short wavelength and thus large frequency (X-rays and γ-rays) will have a large energy per photon. Electromagnetic radiations are usually considered to be ionizing if they have a photon energy in excess of 124 eV, which corresponds to a wave length shorter than 10^{-6} cm. As a result of their high photon energy, X- and γ-rays are able to break chemical bonds and produce biological effects. X-rays and γ-rays do not differ in nature or in properties although the photon energy of γ-rays is somewhat higher than that of X-rays. The distinction between X or γ reflects simply the way in which both types of radiation are produced. X-rays are produced extranuclearly (in an electrical device), while γ-rays are produced intranuclearly (decay products of radioactive isotopes). The most common forms of particulate radiation are α-particles, electrons or ß-particles, protons and neutrons. All these types of radiation interact with tissues by depositing energy in these tissues and thus causing excitations and ionizations of molecules.

1.1.2.2 Directly ionizing versus indirectly ionizing radiation

Charged particles (e.g. α-particles, protons and electrons) can disrupt the atomic structure of the material through which they pass directly as a consequence of the electrical forces induced on orbital electrons. Electromagnetic radiation (X- and γ-rays) and neutrons are both electrically neutral and thus indirectly ionizing. They do not produce chemical or biological damage themselves but transfer their energy to secondary charged particles. X-rays and γ-rays interact with electrons, negatively charged particles with a very small mass, which are subsequently ejected from their orbit. The ionizations formed along the track of these electrons are well separated in space and this type of radiation is therefore said to be sparsely ionizing. 1 MeV fast fission neutrons interact for 90% with hydrogen atoms. The so-formed recoil protons which are positively charged have a mass of about 2000 times that of an electron. As a result the ionization density along the track of a recoil proton is much higher. This type of radiation is therefore called densely ionizing radiation. The difference in ionization density between various types of radiation can be expressed by the mean track average Linear Energy Transfer (LET) values. LET is the energy transfer by a particle per unit of length in a medium. The unit of LET is keV per micron of path and does not specify how the energy is dissipated but refers strictly to energy absorption. The track-average LET value of the recoil protons

produced by 1 MeV fast fission neutrons is equal to 57 keV per micron in water, whereas the LET value of the fast electrons produced by 300 kV X-rays is only 3 keV per micron in water (Davids et al., 1969). LET values are only significant when used to describe monoenergetic particles. Because 300 kV X-rays and especially the 1 MeV fission neutrons used in this study are not monoenergenic energy sources but are composed of an energy spectrum, their average LET values can only be used as a crude way to indicate the quality of both types of radiation.

1.1.2.3 Direct versus indirect action of radiation

When any type of radiation (X- or γ-rays, charged or uncharged particles) is absorbed in biological material there is a possibility that it will interact directly with the critical target in the cell and ionize or excitate the atoms of the DNA molecule itself. This so called **direct action** of radiation is the predominant process with high-LET radiation. Alternatively, the radiation may interact with other molecules in the cell to produce free radicals that are able to diffuse far enough to reach and damage the DNA molecule. This is called the **indirect action** of radiation. A free radical is a free (not combined) atom or molecule carrying an unpaired orbital electron in the outer shell. The absence of a second electron with an opposing spin in these radicals causes chemical instability which results in a high level of chemical reactivity. For X-rays the indirect action is dominant. Since cells consist for 80-90% of water most of the deposited energy results in the ejection of electrons from water: $H_2O \rightarrow H_2O^+ + e^-$. Subsequent reactions with surrounding water result in the formation of e^-_{aq}, $HO^\cdot$, $H^\cdot$, H_2 and H_2O_2 (hydrogen peroxide). Of these products the $HO^\cdot$ radical is the most reactive and therefore most hazardous. It is estimated that about two third of the X-ray damage to DNA is caused by $OH^\cdot$ radicals. Hydrogen peroxide, which can produce $OH^\cdot$ radicals by interaction with transition metal ions, is also important because it is relatively stable and able to diffuse freely (Riley, 1994). In this way the potential distance between the initial ionization and damage caused by an $OH^\cdot$ radical is increased. $OH^\cdot$ can damage DNA by producing a radical on the deoxyribose chain by the following reaction: $RH + OH^\cdot \rightarrow R^\cdot + H_2O$. Radical transfer within the deoxyribose chain can subsequently lead to a single or double strand break. Radiation damage caused by radical attack can be repaired by radical scavengers (e.g. glutathione) $R^\cdot + GSH \rightarrow RH + GS^\cdot$ or it can be fixed in the presence of oxygen $R^\cdot + O_2 \rightarrow ROO^\cdot$. The significance of the oxygen addition is that it

prevents restoration of the original state even in the presence of radical scavengers.

1.1.3 BIOLOGICAL EFFECTIVENESS

In order to compare the biological effectiveness of two types of radiation the concept Relative Biological Effectiveness has been introduced. Due to the differences in LET value between 300 kV X-rays and 1 MeV fast fission neutrons an equal dose of both types of radiation produces a different biological effect (Davids, 1972, 1973). The biological effectiveness of two types of radiation can be compared by determining the Relative Biological Effectiveness (RBE) of a test radiation compared to a standard radiation. The RBE of some test radiation is the ratio D_R/D_T, where D_R and D_T are the doses of the reference radiation (in this thesis 300 kV X-rays) and the test radiation (in this thesis 1 MeV fission neutrons) which produces the same biological effect. Although RBE is conceptually very simple it is a very complex quantity which is dependent on a) radiation quality (LET) b) radiation dose c) number of dose fractions d) dose rate and e) biological system. In radiation protection RBE is therefore for practical reasons replaced by a radiation weighting factor (w_R), which has a fixed value for different types of radiation (w_R-neutrons, energy >100 keV to 2 MeV = 20, w_R-X-rays, γ-rays = 1, w_R-α-particles = 20) (ICRP, 1990).

1.1.4 RADIATION DAMAGE AND CELL SURVIVAL

It is well established that the damage caused by ionizing radiation depends on the initial physical properties of the type of radiation used. Which of the lesions mentioned in Table 1.2 are relevant for the changes induced by radiation at a cellular level is still a matter of research. Only a very small minority of the initially induced lesions results in permanent damage. Simple lesions, including single strand breaks (SSBs) tend to correlate poorly with biological effectiveness. An equitoxic dose of H_2O_2 produces about 400 times more SSBs than low LET ionizing radiation (Ward et al., 1985). Moreover, the yield of SSBs is about 10^3 cell^{-1} after 1 Gy low-LET radiation, a dose which leads to <1 lethal event per cell on average.

For many years the DNA double strand break (DSB) has been considered to be the most important type of lesion caused by ionizing radiation in cells, which, if left unrepaired, leads to cell death. This notion, which was supported by a mathematical model of cell killing based on DSB (Chadwick & Leenhouts, 1973), was based on several studies: a) studies with radiosensitive mutant cell lines partially defective in their ability to repair this lesion (Kemp et al., 1984; Wlodek & Hittelman, 1987) b) studies with radiomodifying agents such as aminothiols, in which a dose correlation was reported between the ability to protect against cell killing and the induction of DSBs in the DNA of irradiated mammalian cells (Radford, 1986; Murray et al., 1989, 1990, 1994) c) studies in which radiations of differing LET were used (Blöcher, 1988). Most DSBs, however, do not lead to an observable cellular effect. A dose of 1 Gy low-LET radiation for example, which induces <1 lethal event induces 40 DSBs (Goodhead, 1994). Moreover, the differences in DSB induction between different types of radiation are much less than the differences in cell survival. In recent literature the RBE for DSB induction, within the dose range used for cell survival experiments, is in general about unity for radiation types ranging from 2 (250 kV X-rays) to 120 keV/μm (^{238}Pu α-particles) (Jenner et al., 1993; Belli et al., 1994; Prise, 1994). The RBE for cell inactivation following irradiation with high LET radiation is much higher. Irradiation of V79 cells with ^{60}Co γ-rays and ^{238}Pu α-particles resulted in an RBE of 0.68 - 0.85 for DSB induction compared to an RBE for cell inactivation at the 1% survival level of 4.0 (Jenner et al.,1993) and irradiation with 0.88 MeV protons and 200 kV X-rays resulted in an RBE of 0.86 for DSB induction (Belli et al., 1994) and 7.3 for cell inactivation (Belli et al., 1989, 1992). Comparing radiation of differing qualities showed a clear difference in potential lethality of the DSBs induced (Weber et al., 1993). DSBs induced by high LET fission neutron radiation are about 3 times more likely to produce a lethal event than DSBs induced by 300 kV X-rays (Prise, 1994). These data suggest that DSBs, as a homogeneous class of DNA damage, are not the principle determinant of cell death. Initial clusters of ionizations in and/or very near to a few base pairs of DNA that lead to clustered damage in DNA (often more complex than a simple double strand break) are considered to be important for the induction of reproductive cell death. These severe types of DNA damage are least likely to be correctly repaired and therefore most likely lead to permanent effects (Goodhead, 1994).

1.1.5 CELL SURVIVAL MODELS

The survival of cell populations after irradiation can be described using several models. Two of these models have been used to analyze the experiments in this thesis.

The **single-hit model** assumes that one particle track or alternatively the hit of one critical target is sufficient to kill a cell. This model:

$$S(D)=e^{-\alpha D}=e^{-D/D_0} \quad \text{or} \quad \ln S(D)=-D/D_0$$

in which S represents cell survival, D the dose and D_0 the dose resulting in 1/e (37%) cell survival) describes most survival curves after high LET radiation. Also the survival curves of many hemopoietic cell types can be described using this model (Hendry, 1985; Huiskamp et al., 1986; Imai & Nakao, 1987). When plotted on a semi-logarithmic plot the single-hit model results in a straight line (Figure 1.1).

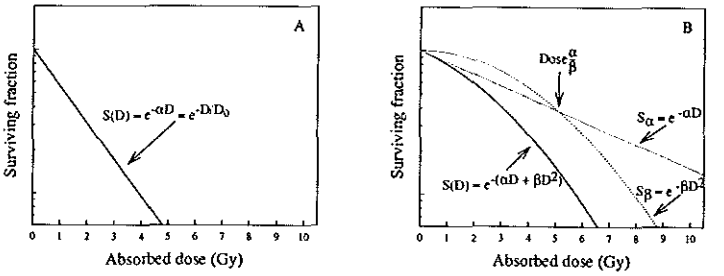

Figure 1.1: Cell survival models describing the survival of cell populations after irradiation. A. The single-hit model B. The linear-quadratic model.

The **linear-quadratic (LQ) model** developed by Kellerer and Rossi (1972) and Chadwick and Leenhouts (1973) describes cell survival as a resultant of two processes:

$$S(D)=e^{-(\alpha D+\beta D^2)} \quad \text{or} \quad \ln S(D)=-(\alpha D+\beta D^2)$$

(in which D is dose, α the rate constant for the single hit process and β the rate constant for the double hit process). Apart from damage caused by one

track or a single hit, which is described by a linear relationship, there is a second component, which increases quadratically with dose and describes the damage due to interaction of lesions from independent tracks or the interaction of two sublethal events which together produce one lethal event. In a semilogarithmic plot the LQ model results in a survival curve with a shoulder in the low dose region and which continuously keeps bending down at high doses (Figure 1.1). The linear component (α) of the LQ model has been associated with the intrinsic radiosensitivity of cells, the quadratic component (β) with the ability of cells to repair radiation induced damage. When dose survival data are fitted with the LQ model absolute values for the α and β parameter can be derived. Although the determination of the parameters is in principle very accurate, small changes in cell survival can have large consequences with respect to the absolute values of these parameters. Especially when cells have a high intrinsic radiosensitivity (high α), as is the case with hemopoietic cells, the determination of the β parameter becomes difficult (Peacock et al., 1988). The LQ model describes many cell survival curves obtained with low-LET radiation and is widely used (Van der Maazen et al., 1991; Prager et al., 1993; Petrovecki et al., 1994), particularly to describe survival curves obtained after fractionated irradiation.

1.1.6 IRRADIATIONS

1.1.6.1 Radiation facilities

The 1 MeV fission neutrons used in this study were generated in the Low Flux Reactor of the Netherlands Energy Research Foundation (ECN) at Petten (Figure 1.2). A uranium-235 (^{235}U) containing converter plate was exposed to thermal neutrons from the core of the reactor. The fission neutrons produced by fission of the ^{235}U have a mean energy of 1 MeV and a mean track average LET of 57 keV/μm. The design of the neutron exposure facility, the dosimetry and neutron spectrometry have been described elsewhere (Davids et al., 1969). Using this facility forty mice can be irradiated simultaneously (Figure 1.3). A homogeneous dose-distribution in small animals like mice was obtained by irradiating the animals bilaterally.

X-rays were produced by a Philips-Müller X-ray tube operating at 300 kV constant potential, at 5 mA with a measured HVL of 2.1 mm. Using this

facility 20 animals can be irradiated simultaneously. By rotating the mice during irradiations an uniform dose distribution was acquired.

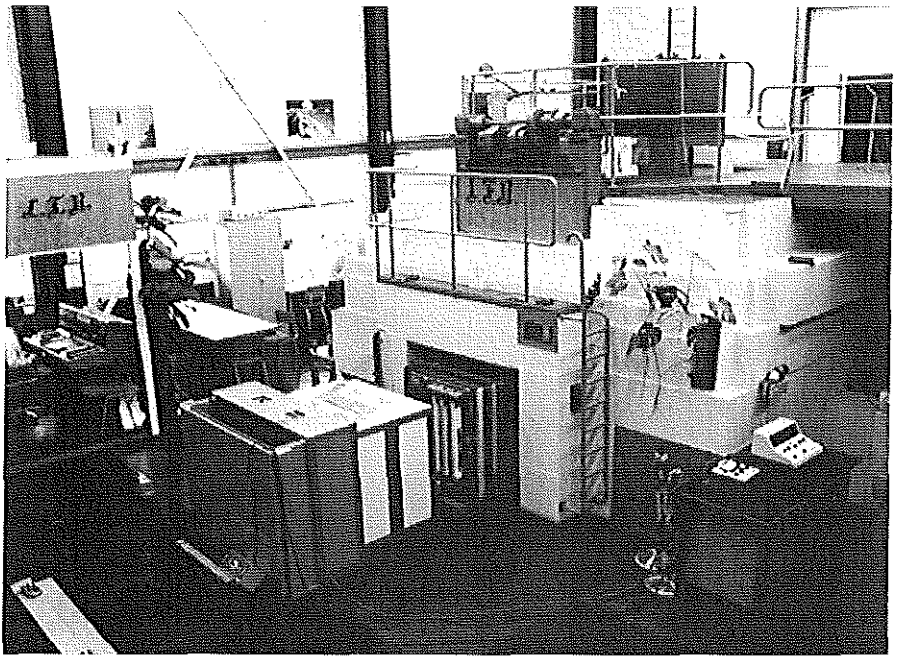

Figure 1.2: The Low Flux Reactor at Petten

1.1.6.2 Neutron RBE data

Neutron RBE data are relevant for radiation protection, fast neutron radiotherapy and protective treatment after accidental exposure to fast neutrons. Data about the effects of fission neutrons are limited. The reassessment of the atomic bomb dosimetry in Hiroshima and Nagasaki in 1986 showed that the neutron contribution to the total dose (especially in Hiroshima) had been overestimated in the past. New calculations revealed that neutrons made a minor contribution to the dose equivalent in both cities. Risks that were previously attributed to neutrons should have been attributed to γ-rays (Ellett, 1991). The elimination of human data from atomic bomb survivors as a source of data concerning the effects of high-LET neutron

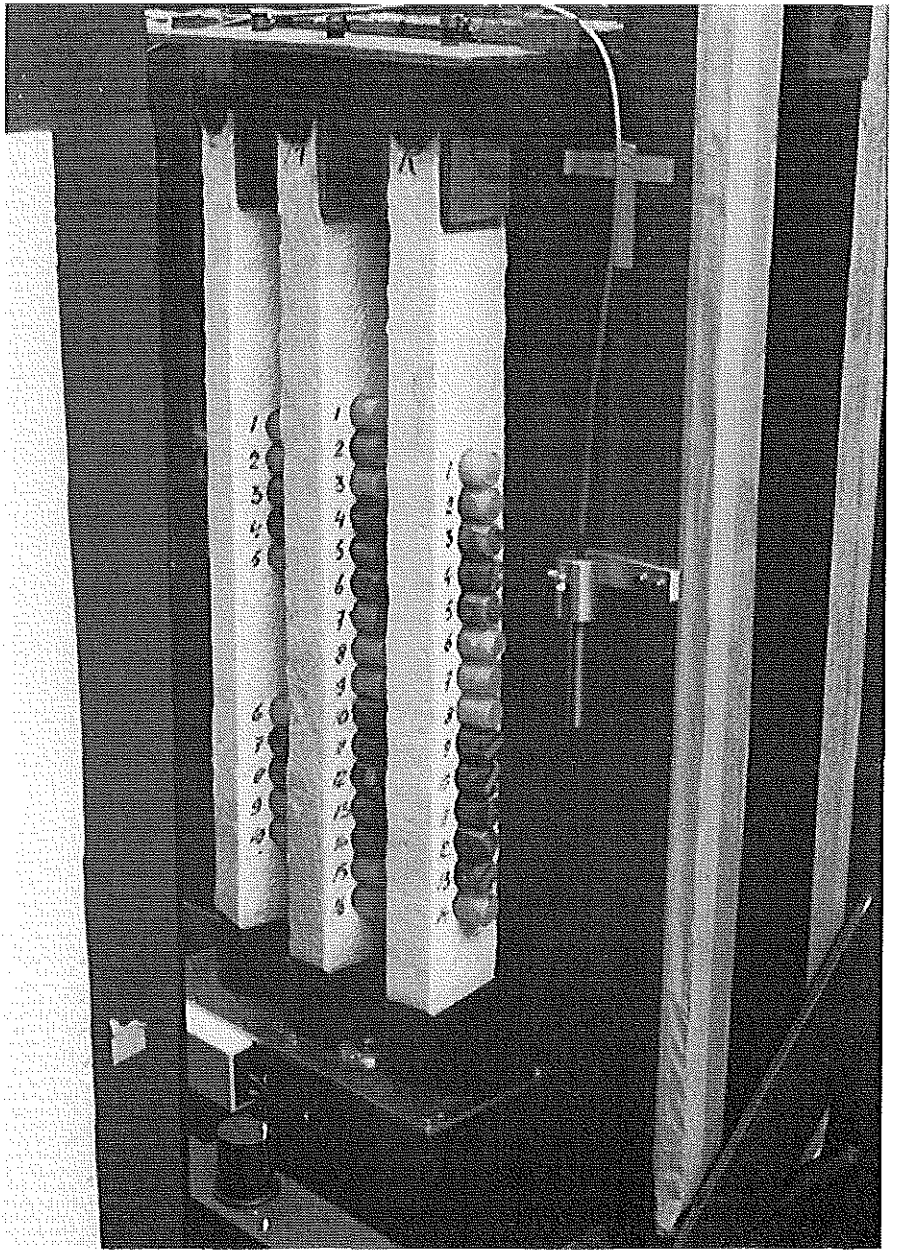

Figure 1.3: Fast neutron exposure facility for mice in the Low Flux Reactor (LFR) at ECN, Petten.

irradiation enhanced the importance of experimental studies in animals. Comparison of neutron RBE data obtained with fast neutrons of various mean energies showed that the RBE value for 1 MeV fission neutrons are among the highest for each effect category investigated (Broerse & Barendsen, 1973; Broerse, 1989; Hall et al., 1975). Therefore RBE data of 1 MeV fast fission neutrons can be regarded as the upper limit for neutron RBE data for each effect category.

Using the exposure facilities at Petten, RBE values for a number of tissues of an inbred CBA mouse strain were determined. For spermatogonial stem cells at the 1% survival level an RBE if 4.1 was found (De Ruiter-Bootsma et al., 1976), for gastric stem cells an RBE of 3.7 (Kingma-ter Haar, 1982) and for intestinal-crypt stem cells an RBE of 3.3 (Davids, 1973). Using the inbred CBA/H mouse strain the RBE for T cells present in the thymus 5 days after irradiation was determined. The fission neutron RBE for reduction of Thy-1$^+$ cells, T-200$^+$ cells, Lyt-1$^+$ cells and Lyt-2$^+$ cells to the 1% survival level varied from 2.6 - 2.8 (Huiskamp et al., 1986). In an earlier study a RBE value at the 1% survival level of 2.4 for hemopoietic stem cell killing in CBA/P mice was calculated (Davids, 1973). In this study the radiosensitivity of hemopoietic stem cells for X-rays and fission neutrons was measured by calculating for each X-ray or neutron dose the 50% effective bone marrow cell dose (ED_{50}) necessary for 50% 30-day survival of lethally irradiated recipients. The ED_{50} was assumed to be inversely proportional to the fraction of stem cells which is able to proliferate. The last decade it has become clear that this is an oversimplification of the hemopoietic system. Moreover, death within the first 30 days following exposure in the dose range used in these studies is due to the bone marrow syndrome. The causes of death from the bone marrow syndrome are granulocytopenia, thrombocytopenia and anaemia (Carsten, 1984) leading to haemorrhages and an inability to counteract infections. Rapid production of new granulocytes and thrombocytes from injected bone marrow cells originates from the precursor cells present in the cell suspension and not from stem cells. Stem cells are responsible for the long-term recovery after irradiation. An ED_{50} protocol using unsorted bone marrow cells can therefore not be regarded as being representative for the radiosensitivity of hemopoietic stem cells.

1.2 HEMOPOIESIS

1.2.1 HEMOPOIETIC STEM CELLS

The hemopoietic system is one of the most diverse and dynamic systems of the mammalian body. At least eleven different types of functional blood cells have been described: erythrocytes, thrombocytes, T-lymphocytes, B-lymphocytes, Killer cells (K-cells), Natural Killer cells (NK-cells), monocytes or macrophages, neutrophils, eosinophils, basophils and mast cells. They perform highly specialized functions which are vital for survival, like for example oxygen and carbon transport (erythrocytes), blood clotting (thrombocytes), antibody production (B-lymphocytes), cell mediated immune responses (K-cells, T-lymphocytes), elimination of tumour cells (NK-cells), elimination of invading organisms and their products (macrophages, monocytes, neutrophils, eosinophils and basophils), inflammatory reactions (mast cells) or cytokine production (lymphocytes, monocytes, macrophages and NK cells). Because most of these cells have a limited life span, varying from several hours (granulocytes) (Whetton & Dexter, 1986) to several weeks (erythrocytes), large amounts of new blood cells are produced continuously by a process called hemopoiesis. Each day an average human male produces 2×10^{11} red cells and 2×10^{9} granulocytes (Dexter et al., 1984).

All types of blood cells are derived from a small population of pluripotent hemopoietic stem cells (PHSC). Studies using genetically marked stem cells have shown that one PHSC in an initially engrafted population is sufficient for permanent blood formation during the entire life span of a mouse (Jordan & Lemischka, 1990; Ogata et al., 1995). PHSC are capable of self maintenance, i.e. the production of daughter cells with the same or almost the same characteristics as the parent cell, as well as generating progenitor cells, which are committed to a particular cell lineage.

Various models have been proposed to describe the organization of the hemopoietic system. Basically they can be divided into two categories: 1) stochastic models 2) deterministic models.

The first stochastic model was described by Till et al. (1964) to explain the growth of spleen colony forming cells. In this model each spleen colony forming cell may either produce two new spleen colony forming cells or develop into a differentiated cell, incapable of forming colonies.

Commitment to one pathway or the other was considered to be a random process. This model was extended by Nakahata et al. (1982) to explain the differentiation of in vitro colony forming cells. Also later studies using genetically marked stem cells, which showed clonal fluctuations in stem cell utilization in the first 4 - 6 months post-engraftment followed by stable engraftment dominated by a small number of totipotent clones, were argued to be indicative of a stochastic model of stem cell regulation (Snodgrass & Keller, 1987; Jordan & Lemischka, 1990). In these studies the longevity of individual stem cell clones could not be predicted. Stem cells can stay dormant for a prolonged period of time, increase or decrease their proliferative activity with time, extinguish completely within 6-8 weeks or display stable behaviour over a prolonged period of time (1.5 - 11.5 months after engraftment). From these studies it was concluded that hemopoietic stem cells form a homogeneous population that exhibits a random and heterogeneous kinetic behaviour (Lemischka, 1992).

Representatives of deterministic models are: a) clonal succession models (Kay, 1965; Rosendaal et al., 1979) b) the predetermined proliferation tree concept (Nicola & Johnson, 1982) c) extracellular control models (Dexter, 1982, 1990). Features of these models are a hierarchical organization of the hemopoietic system in which stem cells are organized according to their generation age (model a), a predetermined number of possible cell divisions and fixed differentiation options at any particular stage of development (model b) and external regulation of self-renewal and differentiation by the hemopoietic microenvironment and growth factors (model c). The basic principle of these three models is a hierarchical organized hemopoietic system in which cells loose proliferation potential with each cell division. According to the clonal succession model PHSC clones gradually loose self maintenance and proliferation capacities as they mature and are finally replaced by new clones from the original stem cell pool. Stem cells from a lineage of stem cells, which have generated many stem cells (older stem cells), are used in the animal before stem cells which have generated few stem cells (younger stem cells) (Rosendaal et al., 1979; Botnick et al., 1982).

The fact that distinct subclasses of hemopoietic stem cell subpopulations can be physically separated (Ploemacher & Brons, 1988b; 1989a; Jones et al., 1990; Ploemacher et al., 1993) favours a model of a

hierarchical organized stem cell compartment. Also some characteristic features attributed to stochastic models have been argued to be conjectures, not supported by the observations used to support their experimental basis. Due to the complexity of their control mechanism some processes just appear to be stochastic (Novak & Stewart, 1991).

The discovery that primary cells in culture loose telomere sequence with each cell division (Harley et al., 1990) has provided a mechanism to explain how cells remember their replicative history or age. Telomeres are the physical ends of eukaryotic chromosomes and contain both short tandem repetitive DNA sequences ($(TTAGGG)_n$) and proteins (Blackburn, 1991). Because conventional DNA polymerases are not able to replicate DNA ends completely (Levy et al., 1992) somatic human cells lose 50-100 bp of telomeric DNA with each successive cell division (Harley et al., 1990; Vaziri et al., 1994). This suggests that telomere length can be used as a measure of the replicative history of cells (Allsopp et al., 1992); short telomeres may trigger a checkpoint signalling entry into senescence and irreversible cell cycle arrest (Greider, 1994; Harley & Villeponteau, 1995). Telomere loss as a function of replicative aging in culture or as a function of donor age in vivo has been documented in a variety of human cells and tissues which undergo renewal, including candidate hemopoietic stem cells ($CD^{34+}CD^{38-}$cells) (Vaziri et al., 1994).

When DNAs from fetal liver cells, umbilical cord blood cells and adult bone marrow cells are compared a striking difference in mean length of the telomeric restriction fragments between fetal/neonatal and adult tissue is observed (Lansdorp, 1995). From these experiments it was concluded that hemopoietic stem cells have a very low turn-over rate in adults and decrease their proliferative potential with each division and, as a consequence, with age. Telomeres can be synthesized de novo onto chromosome ends by the enzyme telomerase. Telomerase, which is active in germ cells, is repressed in most somatic human cells including hemopoietic stem cells (Kim et al., 1994; Harley & Villeponteau, 1995; Lansdorp, 1995). The loss of telomeric sequences with each cell division and the absence of telomerase in human hemopoietic stem cells suggests that stem cells are not capable of true self-renewal, i.e. the production of daughter cells identical to the parent cell. However, telomere length measurements currently require at least 10^5 cells (Lansdorp, 1995). The possibility that a minor subpopulation of human

$CD^{34+}CD^{38-}$ stem cells expresses telomerase activity can therefore not be excluded. In contrast to the absence of telomerase activity in primary human somatic cells telomerase activity has been found in murine somatic tissue (Prowse & Greider, 1995). The question if true self-renewal exists can therefore only be answered when techniques become available to measure telomere length and telomerase activity in individual cells. Human fetal cells start their proliferative life with 5-10 kb $(TTAGGG)_n$ repeats, which is expected to be sufficient for in the order of 50-200 doublings. Even 50 doublings represents a tremendous proliferative capacity, which can in theory yield up to 10^{15} cells or approximately 1000 kg of cells. Since telomeres of murine cells are much longer (50 - 150 kb) (Kipling & Cooke, 1990; Starling et al., 1991) the proliferative capacity of murine stem cells will be even greater. Murine stem cells have been reported to be able to function normally through at least 15-50 life spans (Harrison & Astle, 1982). So even if PHSC are not capable of self-renewal their number and proliferative potential is large enough to ensure that sufficient stem cells persist throughout life, without loss of quality, despite physiological or accidental depopulation (Harrison, 1973, Harrison et al., 1989; Schofield et al., 1986; Keller & Snodgrass, 1990).

Stem cells are relatively few in number, representing approximately 0.001% (Dick et al., 1985; Lemischka et al., 1986; Harrison et al., 1988, 1989) to 0.4% (Lord, 1988) of the cells in the bone marrow. These differences in the number of stem cells quoted are due to the definition and the assay used to determine the number of stem cells. PHSC which are capable of multilineage hemopoietic reconstitution over an extended period of time are lower in number (0.001%) than the older more mature stem cells, which are capable of fast in vivo repopulation for a limited period of time (0.4%). To be able to produce the large amounts of functional blood cells necessary each day, the hemopoietic system is organized hierarchically in a three-tiered system which is composed of a stem cell compartment, a committed progenitor cell compartment and a mature cell compartment. PHSC give rise to more mature stem cells, which differentiate into committed progenitor cells. The committed progenitor cells, which form about 3% of the bone marrow cell population are restricted in their ability to undergo multilineage differentiation and have no ability to self-renew, i.e. they are committed to a particular lineage of cell development. They

gradually proliferate and differentiate into maturing cells and mature cells, which constitute about 95% of the total of hemopoietic cells in the bone marrow. This mechanism of differentiation and numerical amplification with each cell division enables the hemopoietic system to produce very large amounts of functional blood cells by only a limited number of stem cells. It also enables PHSC to remain most of the time in G_0 (Lajtha, 1979) to ensure adequate time for DNA repair (Cairns, 1975). The maintenance of steady-state hemopoiesis as well as the response to pathological and physiological events, which perturb normal hemopoiesis, is precisely regulated by a large family of cytokines. Cytokines can have overlapping, additive or synergistic actions and can be divided in lymphokines, interleukins, growth factors and growth inhibitors. Over twenty cytokines have been recognized, most of which are already molecularly cloned. Their hemopoietic activities, source and target cells and interaction with each other have been described in a number of reviews (Dorshkind, 1990; Moore, 1990; Bronchud, 1991; Haig, 1992, Rowe & Papoport, 1992; Quesenberry & Lowry, 1992; Metcalf, 1993). New cytokines are incessantly discovered and the list of activities for any of them is extended continuously.

1.2.1.1. Assays to study hemopoietic stem cells

The search for the hemopoietic stem cell began when it was first recognized that animals given lethal doses of irradiation died from bone marrow failure, and that this failure could be reversed by injection of unirradiated bone marrow or spleen cell suspensions (Jacobsen et al., 1949, 1951). Donor derived cells and not humoral factors were shown to provide protection against radiation inflicted death (Vos et al., 1956; Ford et al., 1956; Nowell et al., 1956). The major difficulty in defining stem cells is their rarity in hemopoietic tissues and the fact that they are not morphologically recognizable. It therefore lasted until 1961 before the first quantitative in vivo assay for mouse pluripotent stem cells was described by Till & McCulloch (1961). They observed that when bone marrow cells were transplanted into lethally irradiated syngeneic recipient mice, macroscopically visible colonies become visible on their spleens. These colonies are formed by spleen colony forming cells (CFC-S). Only a fraction of the potential CFC-S injected into an irradiated mouse actually settles in the spleen and results in a colony. Such cells are known as spleen colony-forming units (CFU-S) and are related to the number of CFC-S by the formula: CFU-S = f * CFC-S, where f is the seeding efficiency of colony

forming cells in the spleen. For normal bone marrow the f-factor is 6-10% (Lahiri & Van Putten, 1969; Lahiri et al., 1970; Lord, 1971; Lord & Hendry, 1973). Because of shrinkage of the spleen size shortly after irradiation and disappearance of transplanted CFC-S during the first day after transplantation only f-factors determined 24 hours after bone marrow transplantation are considered to be reliable estimates of the seeding efficiency of CFC-S (Lahiri et al., 1970; Testa et al., 1972; Lord & Hendry, 1973) and not the higher f-factors (17-33%) obtained 2 hours after bone marrow transplantation (Siminovitch et al., 1963; Lahiri & Van Putten, 1969; Lord, 1971). Spleen colonies were shown to be clonal (Wu et al., 1967) and may contain cells from erythrocytic, megakaryocytic, monocytic as well as granulocytic lineages, i.e. they are pluripotent. Some colonies have been shown to contain cells that, upon retransplantation are capable to give rise to secondary colonies. It was argued therefore that CFU-S can self-renew, a key function of the stem cell. Based on this evidence CFU-S seemed to possess the qualities required of PHSC, i.e. extensive proliferative capacity, pluripotentiality and self-renewal capacity and for many years spleen colony forming cells were referred to as stem cells (Van Bekkum et al., 1971; 1979).

An increasing number of observations were however incompatible with CFU-S being the ultimate stem cell. Characterization of spleen colonies showed that they were heterogeneous. The number of secondary CFU-S present in a spleen colony varied per colony (Siminovitch et al., 1963; Worton et al., 1969; Wolf & Priestley, 1986) and as early as 1969 it was shown that CFU-S with high self-renewal capacity could be physically separated on the basis of cell size by sedimentation (Worton et al., 1969). Later studies showed that the quality of CFU-S is dependent on the day their colonies become visible on a spleen. The majority of early CFU-S (CFU-S-day 7-9) disappear after a few days and at least half of the late colonies (CFU-S-day 11-13) arise from a separate group of hemopoietic cells (Priestley & Wolf, 1985). Colonies visible 7-9 days after bone marrow transplantation often contained only one myeloid cell type (Wu, 1969) and are derived from more mature cells than colonies derived from cells giving rise to spleen colonies 11-13 days after transplantation (Magli et al., 1982; Molineux et al., 1986). Altogether these data showed that at least early appearing CFU-S cannot be considered to be PHSC but are the more mature progeny of CFU-S-12.

Purification studies of mouse bone marrow on the basis of the surface antigens Thy-1.1, Lin and Stem cell antigen-1 (Sca-1) claimed that the Thy-1.1^{lo} Lin^- $Sca\text{-}1^+$ subpopulation, which was a 1000-fold enriched for CFU-S-12, contained a virtually pure population of PHSC. Only 30 cells were needed to protect 50% of lethally irradiated mice for at least 30 days and to establish multilineage repopulation (Spangrude et al., 1988; Uchida & Weissman, 1992). The Thy-1.1^{lo} Lin^- $Sca\text{-}1^-$ subpopulation, on the other hand, which was unable to save mice from lethal irradiation, contained almost all CFU-S-8 and more mature progenitors. These data implicated that a substantial fraction of CFU-S-12 represents the PHSC.

Although the correlation between radioprotective ability of a graft with the number of CFU-S-12 was in agreement with other data (Visser et al., 1984; Ploemacher & Brons, 1988c), the concept of CFU-S-12 being the ultimate stem cell was contradicted by the evidence obtained by a number of other investigators who showed the existence of more primitive stem cell populations within the stem cell compartment. Treatment of mice with 5-FU, which depletes bone marrow of most CFU-S-7/10 and many CFU-S-12, showed the existence of a 5-FU resistant cell population with marrow repopulating ability (MRA) (Hodgson & Bradley, 1979, Hodgson et al., 1982). 5-FU is a cycle-specific drug, targeting proliferating cells, because incorporation of the nucleotide analogue into DNA during the S phase of the cell cycle leads to cell death. The existence of a cell population resistant to the cytotoxic effects of 5-FU pointed to a more primitive cell population than CFU-S-12, also called pre-CFU-S, which cycles very slowly or not at all (Hodgson et al., 1982). These results were supported by cell separation studies on the basis of sedimentation velocity, light-scattering properties and rhodamine-123 (Rh123) retention which showed that MRA cells could almost completely be separated from CFU-S-12 and CFU-S-8 (Ploemacher & Brons, 1989a). This finding indicates that MRA cells and CFU-S-12 are two stem cell subpopulations with distinct properties, MRA cells being more primitive. MRA cells are assayed with a double transplantation technique which measures the regeneration of CFU-S or colony forming units in culture (CFU-C) in the bone marrow (MRA) or spleen (SRA) of a lethally irradiated host after reconstitution. The bone marrow and/or spleen cells from the repopulated mice are collected 12-13 days after transplantation and are transferred into a second lethally irradiated animal (MRA[CFU-S] or SRA[CFU-S]) or a semi-solid culture system (MRA[CFU-C] or

SRA[CFU-C]) at an appropriate concentration to form discrete spleen colonies or a CFU-C. MRA[CFU-S] lack CFU-S activity but repopulate the bone marrow with cells able to differentiate into cells that are CFU-S (Ploemacher et al., 1989a). Since the MRA assay is not a clonal assay no indication of the actual frequency of MRA cells can be obtained. Within strict limits the assay is however quantitative and thus allows comparison of the primitive hemopoietic stem cell activity in various cell populations (Van der Sluijs et al., 1992).

The notion of CFU-S-12 being the PHSC was further challenged by repopulation studies in vivo, which showed that only 1 out of every 39 Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells was able to induce more than 1% donor-type repopulation (Smith et al., 1991) and a study using counterflow elutriation, which showed that a subpopulation of low density bone marrow cells that completely lacked CFU-S-12 activity was capable of long-term repopulation of irradiated recipients. The intermediate and rapidly sedimenting fractions in this study containing almost all CFU-S-12 and cells capable of radiation protection and short-term reconstitution were not able to induce long-term repopulation (Jones et al., 1990). All these studies contrasted with the concept of CFU-S-12 being the PHSC and the Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ population being a pure population of PHSC.

Subsequent experiments showed that the Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ populations in the bone marrow are quite heterogeneous and contain in vitro CFU-C, CFU-S-12 and at least in Ly-6^b haplotype mice all cells capable of long-term repopulation (LTRA) (Uchida & Weissman, 1992; Li & Johnson, 1992). However, the expression of Sca-1, a member of the Ly-6 antigen family (Van de Rijn et al., 1989), shows a large variability and only in bone marrow cells of Ly-6^b haplotype mice the antigen is constitutively expressed. About 99% of all MRA in Ly-6^b haplotype mice (e.g. C57BL, DBA/J) can be recovered in the population Sca-1$^+$ bone marrow cells, whereas the same population in Ly-6^a haplotype mice (e.g. BALB/c, CBA/J) contained only 24% of the total MRA (Spangrude & Brooks, 1993). Dependent on the haplotype of the mouse strain used the Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ population therefore differs in long-term repopulating ability. Furthermore it was shown that protection against the lethal effect of irradiation in the $LD_{50/30}$ range (i.e. the bone syndrome) and long-term hemopoietic reconstitution were offered by two distinct subpopulation in the Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ fraction. Presently,

a PHSC is defined as a cell that is capable of extensive self-renewal and confers multilineage long-term repopulation in an irradiated host for at least four months, i.e. it has long-term repopulating ability. Short-term hemopoietic reconstitution is no longer considered to be a characteristic of the most primitive stem cells but reflects the proliferative activity of more mature stem cells (CFU-S-12) to produce new peripheral blood cells in the first weeks after bone marrow transplantation. These cells are also called short-term repopulating cells.

Although MRA cells are capable of repopulating a lethally irradiated recipient for more than four months, the MRA-assay has some disadvantages; the assay is not clonal and only takes a snapshot of the repopulation in the bone marrow at 13 days after transplantation. A recent study in which stem cell populations were sorted on the basis of WGA affinity showed that MRA cells differ from the most primitive LTRA cells and developmentally must be placed between CFU-S-12 and LTRA cells (Ploemacher et al., 1993).

To determine the LTRA of a graft several techniques and mouse models have become available that allow discrimination between donor- and recipient derived cells. These include the use of sex-mismatched transplantation (Visser et al., 1989; Jones et al., 1990), congenic mice strains (e.g. GPI-A vs. GPI-B) (Van Os et al., 1993; Down & Ploemacher, 1993), genetically anaemic mice (Van den Bos, 1992) and retrovirally tagged stem cells. Due to the random integration of retroviral vectors in DNA each transfected cell will be different and therefore the last method offers the possibility to identify the progeny of one transfected cell. Transplantation studies with individually tagged stem cells have shown that the first 4 to 6 months after bone marrow transplantation are characterized by frequent fluctuations of the clones contributing to hemopoiesis (Keller & Snodgrass, 1990; Jordan & Lemischka, 1990). Up to 4 to 5 months after transplantation part of these clones originate from CFU-S-12 (Van der Loo et al., 1994). Consequently, the LTRA of a graft can only be determined after a period of minimally 4 months. All the above mentioned evidence indicates that hemopoietic stem cells can be ordered on the basis of their primitiveness as is shown in Figure 1.4.

Characteristic	LTRA	MRA	CFU-S-12	CFU-S-7	Reference[a]
protection against radiation inflicted death	--	-	++	+	Ploemacher & Brons, 1988c, 1988d Sprangrude et al., 1988 Jones et al., 1990 Wagemaker, 1995
self-renewal[b] capacity	++++	+++	++	+	Magli et al., 1982 Wolf & Priestley, 1986
expression of					
Qa-m2			+	-	Harris et al., 1984
Qa-m7			+	-	Harris et al., 1985 Bertoncello et al., 1987
Qa-m8			+	-	Harris et al., 1985
Thy-1.1			+	+/-	Mulder, 1986
CD34	+	+	+	+	Lin et al., 1995
CD4	+/-		+/-		Wineman et al., 1992 Orlic et al., 1993
CD45 (T-200)			+ or -	-	Basch et al., 1992
H-2K density			++	+	Mulder, 1986
Sca-1 Ag (Ly6A/E)	+		+ or -	-	Spangrude et al., 1988 Uchida & Weissman, 1992
c-kit	+	+	+	+	De Vries et al., 1992 Orlic et al., 1993
ER-MP12 Ag[c]	+(++)		++(+)	++(+)	Van der Loo et al., 1995a
ER-MP20 Ag (Ly6C)	-	-	-	-	Van der Loo et al., 1995b
Fall 3 Ag	+		+ or -	+ or -	Müller-Sieburg, 1991
Hoechst-33342 uptake	-	-	+	++	Neben et al., 1991 Baines & Visser, 1983
Rh123 uptake	-	+/-	+/- or +	++	Harris et al., 1985 Mulder, 1987 Ploemacher et al., 1988a, 1988b, 1989a Visser & De Vries, 1988
WGA-affinity	+/-	+/-	+++	++	Ploemacher et al., 1988a, 1988b, 1989a, 1993

Table 1.3a: Some of the observed differences between LTRA cells, MRA cells , CFU-S-12 and CFU-S-8.

Characteristic	LTRA	MRA	CFU-S-12	CFU-S-7	Reference[a]
Sensitivity to 5-fluorouracil	-	+	++	+++	Hodgson & Bradley, 1979 Hodgson et al., 1982 Lerner & Harrison, 1990
busulphan	++++	+++	+	++	Down & Ploemacher, 1993 Down et al., 1994
dimethylbusulphan	++++	+++	+++	+++	Down et al., 1994
hydroxyurea		-	+	++	Rosendaal et al., 1979
melphalan	+	+	++	+++	Down et al., 1994
cyclophosphamide	+	+	+	+	Down et al., 1994
BCNU	+	+	+	+	Down et al, 1994
IMS	++	+	+	+	Down et al., 1994
CAFC formation	day 28/35	day 28	day 10	day 7	Ploemacher et al., 1989b, 1991, 1993

Table 1.3 continued: Some of the observed differences between LTRA cells, MRA cells , CFU-S-12 and CFU-S-8.

[a] References cited investigated at least one of the mentioned cell populations.

[b] Self-renewal in this table is considered to be self-renewal at cell population level and not self-renewal at the level of an individual stem cell .

[c] 95% of the LTRA cells are ER-MP12 medium and 5% are ER-MP12 high. 75% of the CFU-S-12 are ER-MP12 medium and 25% are ER-MP12 high

+/- indicates low (CD4), dim(WGA) or medium ER-MP12).

BCNU = bischloroethylnitrosourea

IMS = isopropylmethane sulphonate

CAFC = cobblestone area forming cell

Recently an assay has been developed to measure the frequency of HSC populations in vitro: the cobblestone area-forming cell assay (CAFC-assay) (Ploemacher et al., 1989b, 1991, 1993). This technique is based on the principle of long-term bone marrow cultures (LTBMC) (Dexter et al., 1977a, 1977b; Allen et al, 1984), which is performed in a miniaturized form. Stromal cells are grown in 96 well plates to establish a supporting microenvironment. By irradiating the stromal layers when they are confluent

with 20 Gy γ-rays the endogenous hemopoietic activity of the layers can be eradicated without affecting their capacity to support the clonal growth of newly overlaid HSC (Zuckerman et al., 1986). Primitive hemopoietic cells and groups of actively proliferating cells are located within the adherent stromal layer covered with adventitial reticulum cells, endothelial cells and macrophages (Hasthorpe et al., 1992) and appear as phase-contrast non-refractile cobblestone forming areas. Primitive cells able to form a cobblestone area are called cobblestone area forming cells (CAFC).

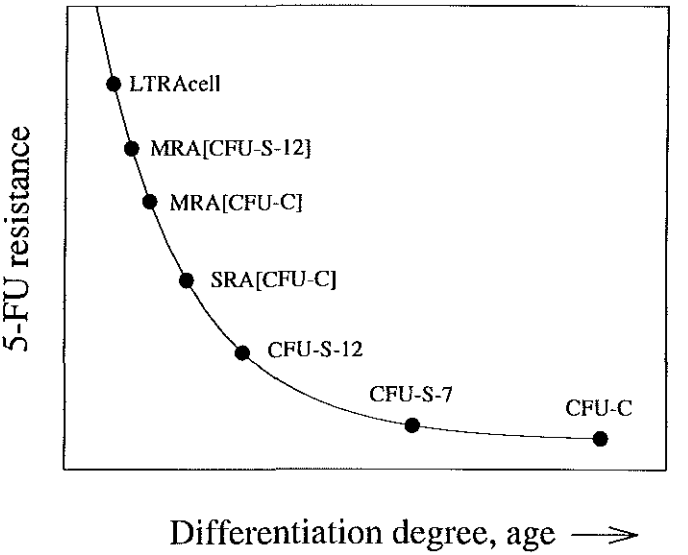

Figure 1.4: Variation in 5-FU sensitivity of hemopoietic stem cells in relation to their degree of differentiation (source: table 1.3)

It has been shown that the different cells in the stem cell hierarchy can be measured by scoring CAFC as a function of time after overlay on the stromal layers. Extensive correlation studies have shown that CAFC measured at day 7 after inoculation (CAFC-7) correlate highly with CFU-S-8; CAFC-10 with CFU-S-12; CAFC-28 with MRA; and CAFC-28 to CAFC-35 with the LTRA of a graft (Ploemacher et al., 1989b, 1991, 1993).

When bone marrow cell populations are inoculated at limiting dilution using 15-20 wells per dilution the frequency of the different HSC can be quantified by scoring CAFC frequencies as a function of time after overlay. The frequency of a CAFC is calculated from the proportion of negative wells, i.e the wells without CA, using Poisson statistics (Fazekas de St. Groth, 1982; Strijbosch et al., 1987). Some of the characteristics of LTRA, MRA, CFU-S-12 and CFU-S-7 reported in Table 1.3 have been assessed with this assay. Early CFU-S-7, late CFU-S-12, MRA cells and LTRA cells have been shown to differ in a number of characteristics. Without being comprehensive some of these differences are summarized in Table 1.3.

1.2.2 THE HEMOPOIETIC MICROENVIRONMENT (HM)

For the production of mature blood cells, hemopoietic stem cells require the presence of a supportive microenvironment which provides them with proper niches to proliferate and differentiate. In mammals, HSC can first be detected in the embryo yolk sac (Toles et al., 1989) then in the fetal liver. In adult animals, the major sites of hemopoiesis are the bone marrow and the spleen (Metcalf & Moore, 1971). In normal situations the bone marrow is the most important site of hemopoiesis. It is made up of vascular and non-vascular compartments. The bone marrow has a closed circulation consisting of arterioles, which empty into venous sinuses, which eventually emerge into a large central sinus connected with the efferent venous system (De Bruyn et al., 1970). The luminal surfaces of the sinuses are lined with endothelial cells. To enter the circulation, developing blood cells must pass through the sinus wall and also transplanted cells entering the bone marrow must pass the sinus wall. Between the sinuses lie the islets of hemopoietic tissue, which consist of a stromal network in which hemopoiesis takes place. The HM not only consists of a highly organized structure which supplies a supportive network, in which cells can lodge and respond to external influences, it also plays a conductive role in hemopoietic cell differentiation, probably through cytokine production (Roberts et al., 1988; Zipori et al., 1988, 1989; Dexter et al., 1990). Stromal cells form the cellular component of the HM and can be divided into endothelial cells, adventitial reticular cells, fibroblastic cells and macrophages and are responsible for the regulation of hemopoiesis. They exert profound effects on the differentiation and lodging of hemopoietic cells by the production of numerous cytokines.

The different cytokines produced by each stromal cell type have been summarized by Van der Sluijs (1993). Stromal derived cytokines are active either as membrane-anchored forms or bound to molecules of the extracellular matrix (Roberts et al., 1988). The extracellular matrix forms the noncellular component of the HM and is composed of collagen, fibronectin, laminin and proteoglycans. Macrophages are the only stromal cells which are of hemopoietic origin.

1.2.2.1 Assays to study the hemopoietic microenvironment

Several assays have been developed to assess the functional activity of the hemopoietic microenvironment: a) the fibroblast colony-forming unit assay (CFU-F) (Friedenstein et al., 1974), b) ectopic bone marrow or spleen implantations (Friedenstein et al., 1968; Tavassoli, 1984), and c) long-term bone marrow cultures (LTBMC) (Dexter et al., 1974, 1977, 1984). The CFU-F assay is an in vitro clonal assay for fibroblastoid precursor cells. Implantation of femurs or spleens, subcutaneously in the abdomen or under the kidney capsule, measures the capacity of the implanted organ stroma to support hemopoiesis. Endothelial cells have been suggested to represent a predominant component in the regeneration of these ectopic implants (Nikkels et al., 1987). Stromal layers in long-term bone marrow cultures form the in vitro representative of the hemopoietic stroma. They are capable to sustain hemopoiesis for several months up to one year. In this thesis the first two assays have been used to determine the radiosensitivity of the HM for X-rays and fission neutrons.

1.3 RADIOSENSITIVITY OF THE HEMOPOIETIC STEM CELLS

Exposure to high doses of ionizing radiation can occur following radiation accidents at nuclear power stations (Baverstock & Ash, 1983, Baverstock, 1984; Guskova et al., 1988). The best known radiation accidents are those that occurred at Oak Ridge (1958), Vinca (1969) and Chernobyl (1986). Large doses of external and internal radiation can also result from accidental contamination with radioactive material from radioactive sources as occurred in the city of Goiania in 1987 (Oliveira et al., 1991). The severity and the duration of the pancytopenia following these radiation accidents is dependent on the number of surviving HSC and the treatment given after

irradiation (BMT (Gale, 1987; Gale & Reissner, 1988; Champlin, 1988) and/or growth factor treatment (Butturini et al., 1988; Gale & Butturini, 1990a, 1990b; Brandao-Mello et al., 1991). Because of the difficulty of accurate dose-estimations, the relatively few radiation accidents which have occurred have not contributed much to our knowledge of stem cell radiosensitivity. Furthermore, accidental irradiation is in general distributed inhomogeneously and thus can result in a surviving HSC fraction, which may be higher than after a comparable dose of homogeneous TBI. Another group of people which can be exposed to ionizing radiation are cancer patients. Up till now total body irradiation (TBI) in combination with cyclophosphamide treatment is the most practical and effective conditioning regimen for the treatment of leukaemia patients (Vriesendorp, 1985). Precise knowledge of the radiosensitivity and frequency of HSCs is essential for the development of treatment regimens for victims of radiation accidents and for improvement of treatment modalities for cancer patients requiring TBI. These data are difficult to obtain in man. Using appropriate models, acute effects of TBI obtained in experimental animals can, however, be extrapolated to man if differences in dose limiting self-renewal systems between species, such as the proportion of HSCs and T-lymphocytes in the bone marrow, are taken into account (Vriesendorp, 1984, 1985).

The radiosensitivity of stem cells responsible for protection against the bone-marrow syndroom (CFU-S) can be estimated by determining the bone marrow cell doses required to rescue 50% of a group of lethally irradiated animals (Davids, 1973; Van Bekkum, 1991). As already stated in 1.2.1.1. cells responsible for survival after irradiation in the midlethal range (2-10 Gy depending on the species irradiated) (Carsten, 1984) are not the cells responsible for long-term engraftment (Ploemacher & Brons, 1988c, 1988d; Jones et al., 1990). Most studies determining the radiosensitivity of murine HSC have been carried out using the CFU-S assay. These data have been summarized by Hendry & Lord (1983). Table 1.4 summarizes the radiosensitivity of murine hemopoietic stem and progenitor cells obtained in representative studies. The differences in reported D_0 values could be explained by differences in radiation set-up, dosimetry, sex, age and mouse strain used. When the radiosensitivity of in vitro clonable progenitors is determined, differences in culture conditions and techniques may also influence the results. In the case of in vitro radiation differences in oxygenation (Broerse et al., 1968) and pH (Freeman et al., 1981) may also

influence the reported radiosensitivity. When comparing the radiosensitivities of different cell populations it is therefore better to compare data obtained within one laboratory. For CFU-C D_0 values varying from 0.42 - 1.29 Gy (Baird et al., 1990) have been obtained using different single recombinant growth factors and D_0 up to 1.75 Gy (Baird et al., 1991) for a combination a growth factors, indicating that the radiosensitivity of CFU-C depends on the type of colony stimulating factor used. The D_0 values quoted in Table 1.4 are obtained by using heterogeneous factors. The radiosensitivity of CFU-S is dependent on their source (spleen, bone marrow or fetal liver), fetal liver derived CFU-S being the most radioresistant (Siminovitch et al., 1965; Silini, 1967).

		In vitro irradiation		In vivo irradiation		
Cell population	**Radiation type**	**D_0 (Gy) mean ± s.e.**	**n**	**D_0 (Gy) mean ± s.e.**	**n**	**Reference**
MRA[CFU-C]	γ-rays			1.25 ± 0.12		Ploemacher et al. (1992)
CFU-S-12	γ-rays			0.91 ± 0.01		Ploemacher et al. (1992)
(femoral)	X-rays			0.70		Shen (1989)
CFU-S-7/10	γ-rays	0.84 ± 0.08	1.6 ± 0.5	0.99 ± 0.08	0.7 ± 0.2	Hendry (1972)
(splenic)	X-rays	0.64 ± 0.10	0.8 ± 0.4	0.66 ± 0.05	0.8 ± 0.3	Hendry (1972)
CFU-S-7/10	γ-rays	1.00 ± 0.03	1.4 ± 0.1	1.15 ± 0.08	1.6 ± 0.2	Hendry & Lord (1983)
(femoral)	γ-rays			0.79 ± 0.01		Ploemacher et al. (1992)
	X-rays	0.79 ± 0.07	2.1 ± 0.5	0.77 ± 0.03	1.7 ± 0.1	Hendry & Lord (1983)
	X-rays			0.57		Shen (1989)
CFU-S-7/10	γ-rays			1.46 ± 0.26	1.1 ± 0.5	Siminovitch et al. (1965
(fetal liver)	X-rays	1.26 ± 0.08	2.0	1.64 ± 0.08	1.5	Silini (1967)
CFU-C	γ-rays	1.60				Senn (1970)
	γ-rays			1.33 ± 0.10		Ploemacher et al. (1992)
	X-rays			1.57 ± 0.11	1.1	Imai (1987)
CFU-Mix	X-rays			1.44 ± 0.30		Imai & Nakao (1987)
BFU-E	X-rays			0.69 ± 0.09	0.9	Imai & Nakao (1987)
CFU-E	X-rays			0.53 ± 0.03	0.7	Imai & Nakao (1987)

Table 1.4: Radiosensitivity of murine hemopoietic stem and progenitor cells

REFERENCES

Allen, T.D. and Dexter, T.M. The essential cells of the hemopoietic microenvironment. Exp. Hematol. 12, 517-521 (1984).

Allsopp, R.C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E.V., Futcher, A.B., Greider, C.W. and Harley, C.B. Telomere length predicts replicative capacity of human fibroblasts. Proc. Natl. Acad. Sci. USA 89, 10114-10118 (1992).

Baines, P. and Visser, J.W.M. Analysis and separation of mouse bone marrow stem cells by Ho33342 fluorescence-activated cell sorting. Exp. Hematol. 11, 701-708 (1983).

Baird, M.C., Hendry, J.H. and Testa, N.G. Radiosensitivity increases with differentiation status of murine hemopoietic progenitor cells selected using enriched marrow subpopulations and recombinant growth factors. Radiat. Res. 123, 292-298 (1990).

Baird, M.C., Hendry, J.H., Dexter, T.M. and Testa, N.G. The radiosensitivity of populations of murine hemopoietic colony-forming cells that respond to combinations of growth factors. Exp. Hematol. 19, 282-287 (1991).

Basch, R.S., Oh, Y.D., Saha, C.S., Frederickson, G.G. and Hirst, J.A. Separation of self-renewing hematopoietic progenitors on the basis of CD45 (T-200) antigen expression. Exp. Hematol. 20, 11-16 (1992).

Baverstock, K.F. and Ash, P.J.N.D. A review of radiation accidents involving whole body exposure and the relevance to the $LD_{50/60}$ for man. Brit. J. Radiol. 56, 837- 845 (1983).

Baverstock, K.F. The response of man to accidental irradiation. In: Response of different species to total body irradiation. Eds. Broerse, J.J. and MacVittie, T.J. Martinus Nijhoff Publishers, Dordrecht, pp. 189-203 (1984).

Belli, M., Cherubini, R., Finotto, S., Moschini, G., Sapora, O., Simone, G. and Tabocchini, M.A. RBE-LET relationship for the survival of V79 cells irradiated with low energy protons. Int. J. Radiat. Biol. 55, 93-104 (1989).

Belli, M., Cera, F., Cherubini, R., Ianzini, F., Moschini, G., Sapora, O., Simone, G., Tabocchini, M.A. and Tiveron, P. RBE-LET relationship for survival and mutation induction of V79 cells irradiated with low-energy protons: re-evaluation of the LET values at the LNL facility. Int. J. Radiat. Biol. 61, 145-146 (1992).

Belli, M., Cera, F., Cherubini, R., Ianzini, F., Moschini, G., Sapora, O., Simone, G., Tabocchini, M.A. and Tiveron, P. DNA double-strand breaks induced by low energy protons in V79 cells. Int. J. Radiat. Biol. 65, 529-536 (1994).

Bertoncello, I., Hodgson, G.S., Bradley, T.R. Multiparameter analysis of transplantable hemopoietic stem cells. I. The separation and enrichment of stem cells homing to marrow and spleen on the basis of rhodamine-123 fluorescence. Exp. Hematol. 13, 999-1006 (1985).

Bertoncello, I., Bartelmez, S.H., Bradley, T.R. and Hodgson, G.S. Increased Qa-M7 antigen expression in characteristic of primitive hemopoietic progenitors in regenerating bone marrow. J. Immunol. 139, 1096-1103 (1987).

Blackburn, E.H. Structure and function of telomeres. Nature 350, 569-572 (1991).

Blöcher, D., DNA double-strand break repair determines the RBE of alpha-particles. Int. J. Radiat. Biol. 54, 761-771 (1988).

Botnick, L.E., Hannon, E.C., Obbagy, J. and Hellman, S. The variation of hematopoietic stem cell self-renewal capacity as a function of age: Further evidence for heterogenicity of the stem cell compartment. Blood 60, 268-271 (1982).

Brandao-Mello, C.E., Oliveira, A.R., Valverde, N.J., Farina, R. and Cordeiro, J.M. Clinical and hematological aspects of ^{137}Cs: The Goiania accident. Health Physics 60, 31-39 (1991).

Broerse, J.J., Barendsen, G.W. and Kersen, G.R. Survival of cultured human cells after irradiation with fast neutrons of different energies in hypoxic and oxygenated conditions. Int. J. Radiat. Biol. 6, 559-572 (1968).

Broerse, J.J. and Barendsen, G.W. Relative biological effectiveness of fast neutrons for effects on normal tissues. Curr. Top. Radiat. Q. 8, 305-350 (1973).

Broerse, J.J. Influence of physical factors on radiation carcinogenesis in experimental animals. In: Low dose radiation. Biological bases of risk assessment. Eds.: Baverstock, K.F. and Stather, J.W. Taylor & Francis, London, pp.: 181- 194 (1989).

Bronchud, M.H. Haemopoietic growth factors - Unravelling the secrets of blood formation. Eur. J. Cancer. 27, 369-372 (1991).

Butturini, A., Gale, R.P., Lopes, D.M., Cunha, C.B., Ho, W.G., Sanpai, J.M., De Souza, P.C., Cordiero, J.M., Neto, C., De Souza, C.E.P., Tabak, D.G., Burla, A. and the Navy Hospital Radiation Team. Use of recombinant granulocyte-macrophage colony stimulating factor in the Brazil radiation accident. The Lancet, 471-474 (1988).

Cairns, J. Mutation selection and the natural history of cancer. Nature 225, 197- (1975).

Carsten, A.L. Acute lethality. The hemopoietic syndrome in different species. In: Response of different species to total body irradiation. Eds. Broerse, J.J. and MacVittie, T.J. Martinus Nijhoff Publishers, Dordrecht, pp. 59-86 (1984).

Chadwick, K.H. and Leenhouts, H.P. A molecular theory of cell survival. Phys. Med. Biol. 18, 78-87 (1973).

Champlin, R. Treatment for victims of nuclear accidents: the role of bone marrow transplantation. Radiat. Res. 113, 205-210 (1988).

Chapman, J.D. and Allalunis-Turner, M.J. Cellular and molecular targets in normal tissue radiation injury. In: Radiation injury to the nervous system. Eds. Gutin, P.H., Leibel, S.A. and Sheline, G.E. Raven Press Ltd., New York, pp. 3-15 (1991).

Davids, J.A.G., Mos, A.P.J. and Oude, A. de. Fast-neutron facility for biological exposures in an Argonaut reactor: design, tissue dosimetry and neutron spectrometry. Phys. Med. Biol. 14, 573-584 (1969).

Davids, J.A.G. Application of a fast-nautron facility in an Argonaut reactor for radiobiological research. In: Radiobiological applications of neutron irradiation, International Atomic Energy Agency, Vienna, pp. 27-40 (1972).

Davids, J.A.G. Acute effects of 1 MeV fast neutron s on the haematopoietic tissues, intestinal epithelium and gastric epithelium in mice, In: Advances in radiation research, Eds. Duplan, J.F. and Chapiro, A., Gordon & Breach, New York, vol 2, pp. 565-576 (1973).

De Bruyn, P.P., Breen, P.C. and Thomas, T.B. The microcirculation of the bone marrow. Anat. Rec. 168, 55-68 (1970).

De Ruiter-Bootsma, A.L., Kramer, M.F., Rooij, D.G. de and Davids, J.A.G. Response of stem cells in the mouse testis to fission neutrons of 1 MeV mean energy and 300 kV X-rays. Methodology, dose-response studies, relative biological effectiveness. Radiat. Res. 67, 56-68 (1976).

De Vries, P., Brasel, K.A., McKenna, H.J., Williams, D.E. and Watson, J.D. Thymus reconstitution by c-kit-expressing hematopoietic stem cells purified from adult mouse bone marrow. J. Exp. Med. 176, 1503-1509 (1992).

Dexter, T.M. and Lajtha, L.G. Proliferation of haemopoietic stem cells in vitro. Brit. J. Haematol. 28, 525-530 (1974).

Dexter, T.M., Allen, T.D. and Lajhta, L.G. Conditions controlling the proliferation of haemopoietic stem cells in vitro. J. Cell Physiol. 91, 335-344 (1977a).

Dexter, T.M., Wrigth, E.G., Krisza, F. and Lajtha, L.G. Regulation of haemopoietic stem cell proliferation in long-term bone marrow cultures. Biomedicine 27, 344-349 (1977b).

Dexter, T.M. Stromal cell associated haemopoiesis. J. Cell Phys. Suppl. 1, 87-94 (1982).

Dexter, T.M., Spooncer, E., Simmons, P. and Allen, T.D. Long-term marrow culture: An overview of techniques and experience. In: Long-term bone marrow culture. Eds Wright, D.G. and Greenberger, J.S. Alan R. Liss, Inc., New York, pp. 57-96 (1984).

Dexter, T.M., Heyworth, C.M., Spooncer, E. and Pointing, L.O. The role of growth factors in self-renewal and differentiation of haemopoietic stem cells. Phil. Trans. Royal Soc. Lond. B 327, 85-98 (1990).

Dick, J.E., Magli, M.C., Huszar, D., Phillips, R.A. and Bernstein, A. Introduction of a selectable gene into primitive stem cells capable of long-term reconstitution of the hemopoietic system of W/W` mice. Cell 42, 71-79 (1985).

Dorshkind, K. Regulation of hemopoiesis by bone marrow stromal cells and their products. Ann. Rev. Immunol. 8, 111-137 (1990).

Down, J.D. and Ploemacher, R.E. Transient and premanent engraftment potential of murine hematopoietic stem cell subsets: differential effects of host conditioning with gamma radiation and cytotoxic drugs. Exp. Hematol. 21, 913-921 (1993).

Down, J.D., Boudewijn, A., Dillingh, J.H., Fox, B.W. and Ploemacher, R.E. Relationships between ablation of distinct haematopoietic cell subsets and the development of donor bone marrow engraftment following recipient pretreatment with different alkylating drugs. Br. J. Cancer 70, 611-616 (1994).

Ellett, W.H. Neutrons at Hiroshima: How their disapppearance affected risk estimates. Radiat. Res. 128, S147-S152 (1991).

Fazekas de St. Groth, S. The evaluation of limiting dilution assays. J. Immunol. Methods 49, R11-R23 (1982).

Ford, C.E., Hamerton, D.W.H., Barnes, J.F. and Loutit, J.F. Cytological identification of radiation chimeras. Nature 177, 452-454 (1956).

Freeman, M.L., Boone, M.L.M., Ensley, B.A. and Gilette, E.L. The influence of environmental pH on the interaction and repair of heat and radiation damage. Int. J. Radiat. Biol. Phys. 7, 761-764 (1981).

Friedenstein, A.J. Chailakhyan, R.K., Latsinik, N.V., Panasuyk, A.F. and Keiliss-Borok, I.V. Stromal cells responsible for transfering the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. Transplantation 17, 331-340 (1974).

Friedenstein, A.J., Petrakova, K.V., Kurolesova, A.I. and Prolova, G.P. Heterotopic transplants of bone marrow. Transplantation 6, 230-247 (1968).

Gale, R.P. The role of bone marrow transplantation following nuclear accidents. Bone Marrow Transplantation 2, 1-6 (1987).

Gale, R.P. Perspective: The medical response to radiation and nuclear accidents: lessons for the future. J. Natl. Cancer Inst. 80, 995-998 (1988).

Gale, R.P. and Reissner, Y. The role of bone marrow transplantation after nuclear accidents. The Lancet, 923-925 (1988).

Gale, R.P. and Butturini, A. Use of hematopoietic growth factors in radiation accidents. Int. J. Radiat. Oncol. Biol. Phys. 19, 1291-1295 (1990a).

Gale, R.P. and Butturini, A. The role of hematopoietic growth factors in nuclear and radiation accidents. Exp. Hematol. 18, 958-964 (1990b).

Goodhead, D.T. Personnel communication (1990).

Goodhead, D.T. Initial events in cellular effects of ionizing radiations: clustered damage in DNA. Int. J. Radiat. Biol. 65, 7-17 (1994).

Greider, C.W. Mammalian telomere dynamics: healing, fragmentation shortening and stabilization. Curr. Opin. Genet. Dev. 4, 203-211 (1995).

Guskova, A.K., Barobanova, A.V., Baranov, A.E., Gruszdev, G.P., Pyatkin, Y.K., Nadezhina, N.M., Metlyaeva, M., Selidovkin, G.D., Moiseev, A.A., Gusev, I.A., Dorefeeva, E.M., Zykova, I.E. Acute radiation effects in victims of the Chernobyl nuclear power plant accident. In: United nations scientific committee on the effects of atomic radiation. Sources, effects and risks of ionizing radiation. Report to the general assembly, with annexes. New York, United nations, pp. 613-647 (1988).

Haig, D.M. Haemopoietic stem cells and the development of the blood cell repertoire. J. Comp. Path. 106, 121-136 (1992).

Hall, E.J., Novak, J.K., Kellerer, A.M., Rossi, H.H., Marino, S. and Goodman, J. RBE as a function of neutron energy. I. Experimental observations. Radiat. Res. 64, 245-255 (1975).

Hall, E.J. Radiobiology for the radiologist, 3rd edition, J.B. Lippincott Company, Philadelphia (1988).

Harley, C.B., Futcher, A.B. and Greider, C.W. Telomeres shorten during ageing of human fibroblasts. Nature 345, 458-460 (1990).

Harley, C.B. and Villeponteau, B. Telomeres and telomerase in aging and cancer. Curr. Opin. Genet. Dev. 5, 249-255 (1995).

Harris, R.A., Hogarth, P.M., Wadeson, R.J., Collins, P., McKenzie, I.F.C. and Pennington, D.G. An antigenic difference between cells forming early and late haemopoietic spleen cell colonies (CFU-S). Nature 307, 638-641 (1984).

Harris, R.A., Sandrin, M.S., Sutton, U.R., Hogarth, P.M., McKenzie, I.F.C. and Pennington, D.G. Variable expression of Qa-M7, Qa-M8 and Qa-M9 antigenic determinants on primitive hemopoietic precursor cells. J. Cell. Physiol. 123, 451-458 (1985).

Harrison, D.E. Normal production of erythrocytes by mouse bone marrow continuous for 73 months. Proc. Natl. Acad. Sci. USA 70, 3184- 3190 (1973).

Harrison, D.E. and Astle, C.M. Loss of stem cell repopulating ability upon transplantation. J. Exp. Med. 156, 1767-1779 (1982).

Harrison, D.E., Astle, C.M. and Lerner, C. Number and continuous proliferation pattern of transplanted primitive immunohemopoietic stem cells. Proc. Natl. Acad. Sc. USA 85, 822-826 (1988).

Harrison, D.E., Astle, C.M. and Stone, M. Numbers and functions of transplantable primitive immunohematopoietic stem cells. Effect of age. J. Immunol. 142, 3833-3840 (1989).

Harrison, D.E. and Lerner, C.P. Most primitive hematopoietic stem cells are stimulated to cycle rapidly after treatment with 5-fluorouracil. Blood 78, 1237-1240 (1991).

Hasthorpe, S., Bogdanovski, M., Rogerson, J. and Radley, J.M. Characterization of endothelial cells in murine long-term bone marrow culture. Implication for hemopoietic regulation. Exp. Hematol. 20, 476-481 (1992).

Hendry, J.H. The response of haemopoietic colony-forming units and lymphoma cells irradiated in soft tissue (spleen) or a bone cavity (femur) with single doses of X-rays , γ-rays or D-T neutrons. Brit. J. Radiol. 45, 923-932 (1972).

Hendry, J.H. and Lord, B.I. The analysis of the early and late response to cytotoxic insults in the haemopoietic cell hierarchy. In: Cytotoxic insult to tissue. Effect on cell lineages. Eds: Potten, C.S. and Hendry, J.H., Churchill Livingstone, New York, pp. 1-66 (1983).

Hendry, J. Review: Survival curves for normal-tissue clonogens: a comparison of assessments using in vitro, transplantation, or in situ techniques. Int. J. Radiat. Biol. 47, 3-16 (1985).

Hodgson, G.S. and Bradley, T.R. Properties of haemopoietic stem cells surviving 5-fluorouracil treatment: Evidence for a pre-CFU-S cell. Nature 281, 381-382 (1979).

Hodgson, G.S., Bradley, T.R. and Radley, J.M. The organization of hemopoietic tissue as inferred from the effects of 5-fluorouracil. Exp. Hematol. 10, 26-35 (1982).

Huiskamp, R., Davids, J.A.G. and Ewijk, W. van. The effect of graded doses of fission neutrons or X-rays on the lymphoid compartment of the thymus in mice. Radiat. Res. 105, 247-258 (1986).

ICRP publication 60. 1990 recommendations of the international commission on radiological protection. Pergamon Press, Oxford, p 6 (1990).

IJdo, J.W., Baldini, A., Ward, DE.C., Reeders, S.T. and Wells, R.A. Origin of human chromosome 2: An ancestral telomere-telomere fusion. Proc. Natl. Acad. Sci. USA 88, 9051-9055 (1991).

Imai, Y. and Nakao, I. In vivo radiosensitivity and recovery pattern of the hematopoietic precursor cells and stem cells in mouse bone marrow. Exp. Hematol. 15, 890-895 (1987).

Jacobsen, L.O., Marks, E.K., Robson, M.J., Gaston, E.O. and Zirkle, R.E. The role of the spleen in radiation injury. Proc. Soc. Exp. Biol. Med. 70, 740-742 (1949).

Jacobsen, L.O., Simons, E.L., Marks, E.K. and Eldredge, J.H. Recovery from radiation injury. Science 113, 510 (1951).

Jenner, T.J., Lara, C.M. de, O'Neill, P.O. and Stevens, D.L. Induction and rejoining of DNA double-strand breaks in V79-4 mammalian cells following γ- and α-irradiation. Int. J. Radiat. Biol. 64, 265-273 (1993).

Jordan, C.T. and Lemischka, I.R. Clonal and systemic analysis of long-term hematopoiesis in the mouse. Genes & Devel. 4, 220-232 (1990).

Jones, R.J., Wagner, J.E., Celano, P., Zicha, M.S. and Sharkis, S.J. Separation of pluripotent haemopoietic stem cells from spleen colony forming cells. Nature 347, 188-189 (1990).

Kay, H.E.M. How many cell generations? Lancet 2, 418-419 (1965).

Keller, G. and Snodgrass, R. Life span of multipotential hematopoietic stem cells in vivo. J. Exp. Med. 171, 1407-1418 (1990).

Kellerer, A.M. and Rossi, H.H. The theory of dual radiation action. Curr. Top. Radiat. Res. Quart. 8, 85-158 (1972).

Kemp, L., Sedgwick, S. and Jeggo, P. X-ray sensitive mutants of Chinese hamster ovary cells defective in double-strand break rejoining. Mutat. Res. 132, 189-196 (1984).

Kim, N.W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West. M.D., Ho, P.L.C., Coviello, G.M., Wright, W.E., Weinrich, S.L. and Shay, J.W. Specific association of human telomerase activity with immortal cells and cancer. Science 266, 2011-2014 (1994).

Kingma-ter Haar, J.M. Effects of fission neutrons and X-rays on the epithelium of the mouse stomach. Thesis, State University, Utrecht, 1982.

Kipling, D. and Cooke, H.J. Hypervariable ultra-long telomeres in mice. Nature 347, 400-402 (1990).

Lahiri, S.K. and Van Putten, L.M. Distribution and multiplication of colony forming units from bone marrow and spleen after injection in irradiated mice. Cell Tissue Kinet. 2, 21-28 (1969).

Lahiri, S.K., Keizer, H.J. and Van Putten, L.M. The efficiency of the assay for haemopoietic colony forming cells. Cell Tissue Kinet. 3, 355-362 (1970).

Lajtha, L.G. Stem cell concepts. Nouv. Rev. Fr. Hematol. 21, 59-65 (1979).

Lansdorp, P.M. Telomere length and proliferation potential in hematopoietic stem cells. Commentary. J. Cell Sci. 108, 1-6 (1995).

Lee, J.J., Warburton, D. and Robertson, E.J. Cytogenetic methods for the mouse: Preparation of chromosomes, karyotyping, and in situ hybridization. Anal. Biochem. 189, 1-17 (1990).

Lemischka, I.R., Raulet, D.H. and Mulligan, R.C. Developmental potential and dynamic behavior of hematopoietic stem cells. Cell 45, 917-927 (1986).

Lemischka, I.R. What have we lerned from retroviral marking of hematopoietic stem cells. Curr. Top. Microbiol. Immunol. 177, 59-71 (1992).

Lerner, C. and Harrison, D.E. 5-fluorouracil spares hemopoietic stem cells responsible for long-term repopulation. Exp. Hematol. 18, 114-118 (1990).

Levy, M.Z., Allsopp, R.C., Futcher, A.B., Greider, C.W. and Harley, C.B. Telomere end-replication problem and cell aging. J. Mol. Biol. 225, 951-960 (1992).

Li, C.L. and Johnson, G.R. Long-term hemopoietic repopulation by Thy-1^{lo}, Lin^{-}, Ly6A/E^{+} cells. Exp. Hematol. 20, 1309-1315 (1992).

Lin, G., Finger, E. and Gutierrez-Ramos, J.C. Expression of CD34 in endothelial cells, hematopoietic progenitors and nervous cells in fetal and adult mouse tissue. Eur. J. Immunol 25, 1508-1516 (1995).

Lord, B.I. The relationship between spleen colony production and spleen cellularity. Cell Tissue Kinet. 4, 211-216 (1971).

Lord, B.I. and Hendry, J.H. Observations on the settling and recoverability of transplanted hemopoietic colony-forming units in the mouse spleen. (1973).

Lord, B.I., The hemopoietic system. In: Hematopoiesis. Long-term effects of chemotherapy and radiation. Testa, N.G. and Gale, R.P. Eds., Marcel Dekker, Inc., New York, pp. 1-26 (1988).

Maazen, R.W.M. van der, Kleiboer, B.J., Verhagen, I. and Kogel, A.J. van der. Irradiation in vitro discriminates between different O-2A progenitor cell subpopulations in the perinatal central nervous system of rats. Radiat. Res. 128, 64-72 (1991).

Magli, M.C., Iscove, N.N. and Odartchenko, N. Transient nature of early haemopoietic spleen colonies. Nature 295, 527-529 (1982).

Metcalf, D. and Moore, M. Haemopoietic cells. North Holland, Amsterdam (1971).

Metcalf, D. Hematopoietic regulators: Redundancy of subtlety? Blood 82, 3515-3523 (1993).

Molineux, G., Schofield, R. and Testa, N.G. Development of spleen CFU-S colonies from day 8 to day 11: relationship to self-renewal capacity. Exp. Hematol. 14, 710-713 (1986).

Moore, M. Haemopoietic growth factor interactions: in vitro an in vivo preclinical evaluation. Cancer Surv. 9, 7-80 (1990).

Mulder, A.H. Differentiation of hemopoietic stem cells in the thymus. Thesis, Erasmus University, Rotterdam (1986).

Mulder, A.H. and Visser, J.W.M. Separation and functional analysis of bone marrow cells separated by rhodamine-123 fluorescence. Exp. Hematol. 15, 99-104 (1987).

Müller-Sieburg, C.E. Separation of pluripotent stem cells and early B lymphocyte precursors with antibody Fall-3. J. Exp. Med. 174, 161-168 (1991).

Murray, D., Prager, A. and Millas, L. Radioprotection of cultured mammalian cells by the aminothiols WR-1065 and WR-255591: Correlation between protection against DNA double-strand breaks and cell killing after γ-radiation. Radiat. Res. 120, 154-163 (1989).

Murray, D.. Prager. A., VanAnkeren, S.C., Altschuler, E.M., Kerr, M.S., Terry, N.H.A. and Milas, L. Comparative effect of the thiols dithiothreitol, cysteamine and WR-151326 on survival and on the induction of DNA damage in cultured chinese hamster ovary cells exposed to γ-radiation. Int. J. Radiat. Biol. 58, 71-91 (1990).

Murray, D., Simpson, R., Rosenberg, E., Carraway, A. and Britten, R. Correlation between γ-ray-induced DNA double-strand breakage and cell killing after biologically relevant doses: analysis by pulsed-field gel electrophoresis. Int. J. Radiat. Biol. 65, 419-426 (1994).

Nakahata, T., Gross, A.J. and Ogawa, M. A stochastic model of self-renewal and commitment to differentiation of the primitive hemopoietic stem cells in culture. J. Cell Phys. 113, 455-458 (1982).

Neben, S. Redfearn, W.J., Parra, M., Brecher, G. and Pallavicini, M.G. Short- and long-term repopulation of lethally irradiated mice by bone marrow stem cells enriched on the basis of light scatter and Hoechst 33342 fluorescence. Exp. Hematol. 19, 958-967 (1991).

Nicola, N.A. and Johnson, G.R. The production of committed hemopoietic colony-forming cells from multipotential precursor cells in vitro. Blood 60, 1019-1029 (1982).

Nikkels, P.G.J., Jong, H. de, Ploemacher, R.E. Long-term effects of cytostatic agents on the hemopoietic stroma: A comparison of four different assays. Leuk. Res. 11, 817-825 (1987).

Novak, J.P. and Stewart, C.C. Stochastic versus deterministic in haemopoiesis: what is what? Brit. J. Haematol. 78, 149-154 (1991).

Nowell, P.C., Cole, L.J., Habermeyer, J.G. and Roan, P.L. Growth and continued function of rat marrow cells in X-radiated mice. Cancer Res. 16, 258-261 (1956).

Ogata, H., Bradley, W.G., Inaba, M., Ogata, N. and Ikehara, S. Long-term repopulation of hematolymphoid cells with only a few hemopoietic stem cells in mice. Proc. Natl. Acad. Sci. USA 92, 5945-5949 (1995).

Oliveira, A.R., Hunt, J.G., Valverde, N.J.L., Brandao-Mello, C.E. and Farina, R. Medical and related aspects of the Goiania accident: an overview. Health Physics 60, 17-24 (1991).

Orlic, D., Fischer, R., Nishikawa, S.I., Nienhuis, A.W. and Bodine, D.M. Purification and characterization of heterogeneous pluripotent hematopoietic stem cell populations expressing high levels of c-kit receptor. Blood 82, 762-770 (1993).

Peacock, J.H., Cassoni, A.M., McMillan, T.J. and Steel, G.G. Radiosensitive tumour cell lines may not be recovery deficient. Int. J. Radiat. Biol. 54. 945-953 (1988).

Petrovecki, M., Prager, A., Terry, N.H.A. and Murray, D. Relationship between DNA damage and the survival of murine bone marrow cells irradiated in situ. Radiat. Res. 138, 443-450 (1994).

Ploemacher, R.E. and Brons, N.H.C. Cells with marrow and spleen repopulating ability and forming spleen colonies on day 16, 12, and 8 are sequentially ordered on the basis of increasing rhodamine 123 retention. J. Cel Physiol. 136, 531-536 (1988a).

Ploemacher, R.E. and Brons, N.H.C. In vivo proliferative and differential properties of murine bone marrow cells separated on the basis of rhodamine-123 retention. Exp. Hematol. 16, 903-907 (1988b).

Ploemacher, R.E. and Brons, N.H.C. Isolation of hemopoietic stem cell subsets from murine bone marrow: I. Radioprotective ability of purified cell suspensions differing in the proportion of day-7 and day-12 CFU-S. Exp. Hematol. 16, 21-26 (1988c).

Ploemacher, R.E. and Brons, N.H.C. Isolation of hemopoietic stem cell subsets from murine bone marrow: II. Evidence for an early precursor fo day-12 CFU-S and cells associated with radioprotective ability. Exp. Hematol. 169, 27-32 (1988d).

Ploemacher, R.E. and Brons, N.H.C. Separation of CFU-S from primitive cells responsible for reconstitution of the bone marrow hemopoietic stem cell compartment following irradiation: Evidence for a pre-CFU-S cell. Exp. Hematol. 17, 263-266 (1989a).

Ploemacher, R.E., Sluijs, J.P. van der, Voerman, J.S. and Brons, N.H.C. An in vitro limiting-dilution assay of long-term repopulating hematopoietic stem cells in the mouse. Blood 74, 2755-2763 (1989b).

Ploemacher, R.E. and Van der Sluijs, J.P. In vitro frequency analysis of spleen colony-forming and marrow-repopulating hemopoietic cells in the mouse. J. Tiss. Cult. Methods 13, 63-68 (1991).

Ploemacher, R.E., Os, R. van, Beurden, C.A.J. van, and Down, J.D. Murine hemopoietic stem cells with long-term engraftment and marrow repopulating ability are less radiosensitive to gamma radiation than spleen colony forming cells. Int. J. Radiat. Biol. 61, 489-499 (1992).

Ploemacher, R.E., Loo, J.C.M. van der, Beurden, C.A.J. van and Baert, M.R.M. Wheat germ agglutinin of murine hemopoietic stem cell subpopulations is an inverse function of their long-term repopulating ability in vitro and in vivo. Leukemia 7, 120-130 (1993).

Prager, A., Terry, N.H.A. and Murray, D. Influence of intracellular thiol and polyamine levels on radioprotection by aminothiols. Int. J. Radiat. Biol. 64, 71-81 (1993).

Priestley, G.V. and Wolf, N.S. A technique for the daily examination of spleen colonies in mice. Exp. Hematol. 137, 733-735 (1985).

Prise, K.M. Use of radiation quality as a probe for DNA lesion complexity. Int. J. Radiat. Biol. 65, 43-48 (1994).

Prowse, K.R. and Greider, C.W. Developmental and tissue-specific regulation of mouse telomerase and telomere length. Proc. Natl. Acad. Sci. USA 92, 4818-4822 (1995).

Quesenberry, P.J. and Lowry, P.A. The colony-stimulating factors - an overview. Cancer 70, 909-912 (1992).

Radford, R. Effect of radiomodifying agents on the ratios of X-ray-induced lesions in cellular DNA: Use in lethal lesion determination. Int. J. Radiat. Biol. 49, 621-637 (1986).

Riley, P.A. Free radicals in biology: oxidative stress and the effects of ionizing radiation. Int. J. Radiat. Biol. 65, 27-33 (1994).

Roberts, R., Gallagher, J., Spooncer, E., Allen, E.D., Bloomfield, F. and Dexter, T.M. Heparan sulphate bound growth factors: A mechanism for stromal cell mediated haemopoiesis. Nature 332, 376-378 (1988).

Rosendaal, M., Hodgson, G.S. and Bradley, T.R. Organization of haemopoietic stem cells: The generation-age hypothesis. Cell Tissue Kinet. 12, 17-29 (1979).

Rosendaal, M., Dixon, R. and Panayi, M. Haemopoietic stem cells: Possibility of toxic effects of 5-fluorouracil on spleen colony formation. Blood cells 7, 561-569 (1981).

Rosendaal, M., Hodgson, G.S. and Bradley, T.R. Organization of haemopoietic stem cells: The generation age-hypothesis. Cell Tissue Kinet. 12, 17-29 (1979).

Rowe, J.M. and Papoport, A.P. Hemopoietic growth factors - A review. J. Clin. Pharmacol. 32, 486-501 (1992).

Schofield, R., Dexter, T.M., Lord, B.I. and Testa, N.G. Comparison of haemopoiesis in young and old mice. Mech. Aging Devel. 34, 1-12 (1986).

Senn, J.S. and McCulloch, E.A. Radiation sensitivity of human bone marrow cells measured by a cell culture method. Blood 35, 56-60 (1970).

Shen, Y. Comparison of the radiosensitivity and 5-fluorouracil susceptability of two stem cell subpopulations in mouse bone marrow. Thesis, University of Washington, Seattle, 1989.

Silini, G. Control of haemopoiesis and the action of radiation on the blood forming organs. In: Effects of ionising radiation on haemopoietic tissue, IAEA, Vienna, p52 (1967).

Siminovitch, L., McCulloch. E.A. and Till, J.E. The distribution of colony-forming cells among spleen colonies. J. Cell Comp. Physiol. 62, 327-336 (1963).

Siminovitch, L., Till, J.E. and McCulloch. E.A. Radiation responses of hemopoietic colony-forming cells derived from different sources. Radiat. Res. 24, 482-493 (1965).

Smith, L.G., Weissman, I.L. and Heimfeld, S. Clonal analysis of hematopoietic stem-cell differentiation in vivo. Proc. Natl. Acad. Sci. USA 88, 2788-2792 (1991).

Snodgrass, R. and Keller, G. Clonal fluctuation within the haematopoietic system of mice reconstituted with retrovirus-infected stem cells. Embo J. 6, 3955-3960 (1987).

Spangrude, G.J., Heimfeld, S. and Weissman, I.L. Purification and characterization of mouse hematopoietic stem cells. Science 241, 58-62 (1988).

Spangrude, G.J. and Brooks, D.M. Mouse strain variability in the expression of the hematopoietic stem cell antigen Ly-6A/E by bone marrow cells. Blood 82, 3327-3332 (1993).

Starling, J.A., Maule, J., Hasite, N.D. and Allshire, R.C. Extensive telomere repeat arrays in mouse are hypervariable. Nucleic Acid. Res. 18, 6881-6888 (1991).

Strijbosch, L.W.G., Buurman, W.A., Does, R.J.M.M., Zinken, P.H. and Groenewegen, G. Limiting dilution assays: Experimental design and statistical analysis. J. Immunol. Methods 97, 133-140 (1987).

Tavassoli, M. Hemopoiesis in ectopically implanted bone marrow. In: Long-term bone marrow culture. Eds Wright, D.G. and Greenberger, J.S. Alan R. Liss, Inc., New York, pp. 31-54 (1984).

Testa, N.G., Lord, B.I. and Shore, N.A. The in vivo seeding of hemopoietic colony-forming cells in irradiated mice. Blood 40, 654-661 (1972).

Till, J.E. and McCulloch, E.A. A direct measurement of the radiation sensitivity of normal bone marrow cells. Radiat. res. 14, 213-222 (1961).

Till, J.E., McCulloch, E.A. and Siminovitch, L. A stochastic model of stem cell proliferation, based on the growth of spleen colony-forming cells. Proc. Nat. Acad. Sci. USA 51, 29-36 (1964).

Toles, J.F., Chui, D.H., Belbeck, L.W., Starr, E. and Barker, J.E. Haemopoietic stem cells in murine yolk sac and peripheral blood. Proc. Nat. Acad. Sc. USA 86, 7456-7459.

Uchida, N. and Weissman. I.L. Searching for hematopoietic stem cells: evidence that Thy-1.1^{lo}Lin^{-}Sca-1^{+} cells are the only stem cells in C57Bl/Ka-Thy-1.1 bone marrow. J. Exp. Med. 175, 175-184 (1992).

Van Bekkum, D.W., Van Noord, M.J., Maat, B. and Dicke, K.A. Attempts at identification of hemopoietic stem cell in mouse. Blood 5, 547-559 (1971).

Van Bekkum, D.W., Van den Engh, G.J., Wagemaker, G., Bol, S.J.L. and Visser, J.W.M. Structural identity of the pluripotential hemopoietic stem cell. Blood cells 5, 143-159 (1979).

Van Bekkum, D.W. Radiation sensitivity of the hemopoietic stem cell. Radiat. Res. 128, S4-S8 (1991).

Van den Bos, C., Van Gils, F.C.J.M., Bartstra, R.W. and Wagemaker, G. Flow cytometric analysis of peripheral blood erythrocyte chimerism in α-thalassemic mice. Cytometry 13, 659-662 (1992).

Van der Loo, J.C.M., Van den Bos, C., Baert, M.R.M., Wagemaker, G. and Ploemacher, R.E. Stable multilineage hematopoietic chimerism in a-thalassemic mice induced by a bone marrow subpopulation that excludes the majority of day-12 spleen colony forming units. Blood 83, 1769-1777 (1994).

Van der Loo, J.C.M., Slieker, W.A.T., Kieboom, D. and Ploemacher, R.E. Identification of hematopoietic stem cell subsets on the basis of their primitiveness using antibody ER-MP12. Blood, in press (1995a).

Van der Loo, J.C.M., Slieker, W.A.T. and Ploemacher, R.E. Use of ER-MP12 as a positive marker for the isolation of murine long-term repopulating stem cells. Exp. Hematol., in press (1995b).

Van de Rijn, M., Heimfeld, S., Spangrude, S.J. and Weissman, I.L. Mouse hematopoietic stem-cell antigen Sca-1 is a member of the Ly-6 antigen family. Proc. Natl. Acad. Sci USA 86, 4634-4638 (1989).

Van Os, R., Thames, H.D., Konings, A.W.T. and Down, J.D. Radiation dose-fractionation and dose-rate relationships for long-term repopulating hemopoietic stem cells in a murine bone marrow transplantation model. Radiat. Res. 136, 118-125 (1993).

Van der Sluijs, J.P., Van Beurden, C.A.J., Baert, M.R.M. and Ploemacher, R.E. Evaluation of in vivo and in vitro repopulation assays in the mouse. Comp. Haematol. Int. 2, 117-124 (1992).

Van der Sluijs, J.P., Van den Bos, C., Baert, M.R.M., Van Beurden, C.A.J. and Ploemacher, R.E. Loss of long-term repopulating ability in long-term bone marrow culture. Leukemia 7, 725-732 (1993).

Van der Sluijs, J.P. The hematopoietic micro-environment of the bone marrow. In: The distinct nature of hematopoietic stem cell subpopulations studied in long-term stroma-associated culture in the mouse. Thesis Erasmus University, pp. 35-67 (1993).

Vaziri, H., Dragowska, W., Allsopp, R.C., Thomas. T.E., Harley, C.B. and Lansdorp, P.M. Evidence for a mitotic clock in human hematopoietic stem cells: loss of telomeric DNA with age. Proc. Natl. Acad. Sci. USA 91, 9857-9860 (1994).

Visser, J.W.M., Bauman, J.G.J., Mulder, A.H., Eliason, J.F. and Leeuw, A.M. de. Isolation of murine pluripotent hemopoietic stem cells. J. Exp. Med. 59, 1576-1590 (1984).

Visser, J.W.M. and De Vries, P. Isolation of spleen-colony forming cells (CFU-S) using Wheat Germ Agglutinin and Rhodamine 123 labelling. Blood Cells 14, 369-384 (1988).

Visser, J.W.M., Hogeweg-Platenburg, M.G.C., Vries, P. de, Bayer, J. and Ploemacher, R.E. Culture of purified pluripotent haemopoietic stem cells. In: The biology of hematopoiesis. Eds: Daniak, N., Alan R. Liss, New York, pp. 1-16 (1989).

Vos, O., Davids, J.A.G., Weyzen, W.W.H. and Van Bekkum, D.W. Evidence for the cellular hypothesis in radiation protection by bone marrow cells. Acta Physiol. Pharmacol. Neerl. 4, 482-486 (1956).

Vriesendorp, H. Susceptibility to total body irradiation. In: Response of different species to total body irradiation. Eds. Broerse, J.J. and MacVittie, T.J. Martinus Nijhoff Publishers, Dordrecht, pp. 43-57 (1984).

Vriesendorp, H. Engraftment of hemopoietic cells. In: Bone marrow transplantation. Biological mechanisms and clinical practice. Eds: Van Bekkum, D.W. and Löwenberg, B., Dekker, New York, pp 73-145 (1985).

Ward, J.F., Blakely, W.F. and Joner, E.I. Mammalian cells are not killed by DNA strand breaks caused by hydroxyl radicals from hydrogen peroxide. Radiat. Res. 103, 383-392 (1985).

Ward, J.F., Evans, J.W., Limoli, C.L. and Calabro-Jones, P.M. Radiation and hydrogen peroxide induced free radical damage to DNA. Br. J. Cancer 55 (Suppl. VIII), 105-112 (1987).

Ward, J.F. DNA damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation and reparability. Prog. Nucl. Acid Res. Mol. Biol. 35, 95-125 (1988).

Weber, K.J. and Flentje, M. Lethality of heavy ion-induced DNA double-strand breaks in mammalian cells. Int. J. Radiat. Res. 64, 169-178 (1993).

Whetton, A.D. and Dexter, T.M. Haemopoietic growth factors. T.I.B.S. 11, 207-211 (1986).

Wineman, J.P., Gilmore, G.L., Gritzmacher, C., Torbett, B.E. and Müller-Sieburg, C.E. CD4 is expressed on murine pluripotent hematopoietic stem cells. Blood 80, 1717-1724 (1992).

Wlodek, D. and Hittelman, W.N. Repair of double-strand breaks correlates with radiosensitivity of L5178Y-S and L5178Y-R cells. Radiat. Res. 112, 1-10 (1987).

Wolf, N.S. and Priestly, G.V. Kinetics of early and late spleen colony development. Exp. Hematol. 14, 676-682 (1986).

Worton, R.G., McCulloch, E.A. and Till, J.E. Physical separation of hemopoietic stem cells differing in their capacity for self-renewal. J. Exp. Med. 130, 91-103 (1969).

Wu, A.M., Till, J.E., Siminovitch, L. and McCulloch, E.A. A cytological study of the capacity for differentiation of normal hemopoietic colony-forming cells. J. Cell Physiol. 69, 177-184 (1967).

Zipori, D., Kalai, M. and Tamir, M. Restrictins: Stromal cell associated factors that control cell organization in hemopoietic tissues. Nat. Immun. Cell Growth Regul. 7, 185-192 (1988).

Zipori, D. Stromal cells from the bone marrow: Evidence for a restrictive role in regulation of hemopoiesis. Eur. J. Haematol. 42, 225-232 (1989).

Zuckerman, K.S., Prince, C.W., Rhodes, R.K. and Rhibadeneira, M. Resistance of the stromal cells in murine long-term bone marrow cultures to damage by ionizing radiation. Exp. Hematol. 14, 1056-1062 (1986).

SCOPE OF THE PRESENT STUDY

Since the discovery that BM cells transplanted into lethally irradiated recipients could produce spleen colonies, the CFU-S assay has been used for more than 25 years as the assay to measure the radiosensitivity of hemopoietic stem cells. CFU-S were shown to be clonal, multipotential and were able to produce secondary CFU-S (at that time assumed to be the confirmation of self-renewal capacity). In addition the radiosensitivity of CFU-S highly correlated with the radiosensitivity of cells capable to rescue lethally irradiated recipients of radiation inflicted death. In summary, CFU-S possessed all the features at that time attributed to PHSC. However, the concept of CFU-S being the PHSC has been re-evaluated as well as the functional definitions of PHSC that had been used for many years. The stem cell compartment has been shown to contain various functionally distinct cell populations, which can be globally ordered on the basis of their primitiveness. CFU-S are considered to be the most mature cells within the stem cell compartment, which are capable of fast but transient repopulation of an irradiated host. Sustained multilineage long-term repopulation over extended periods of time is provided for by more primitive stem cells (MRA and LTRA). As a consequence CFU-S are no longer considered to be the most primitive HSC and short-term hemopoietic reconstitution (30-day radioprotective ability) is no longer considered to be a characteristic of these cells, but merely reflects the capacity of more mature stem cells to rapidly repopulate a radiation depleted host. At this moment PHSC are defined as cells capable of extensive self-renewal and able to confer multilineage long-term repopulation in an irradiated host for at least 4 months.

In this study we investigated if the different hemopoietic stem cell populations, which are heterogeneous with respect to repopulating capacity, surface marker phenotypes and sensitivity to cytotoxic drugs also differed in their sensitivity to 300 kV X-rays and 1 MeV fission neutrons. In addition we investigated the effect of these two types of radiation on the hemopoietic stroma. As described in chapter 1 ionizing radiation can be divided in electromagnetic radiation (such as X- and γ-rays), which is sparsely ionizing and particulate radiation (such as neutrons and α-particles), which has a much denser ionization pattern. This difference in ionization pattern is responsible for the higher relative biological effectiveness of 1 MeV fission neutrons compared to X-rays for a number of effects. If differences in RBE

values also exist between different hemopoietic stem cell populations, this might have implications for the treatment of victims of radiation accidents.

In chapter 3 the effects of total body irradiation with 300 kV X-rays on hemopoietic stem cells and progenitor cells of CBA/H mice is described. The radiosensitivity of stem cells was assayed using the classical CFU-S assay and a marrow repopulation assay (MRA[CFU-S-12] assay). The experimental procedures used to measure the different cell populations are shown in Figure 2.1.

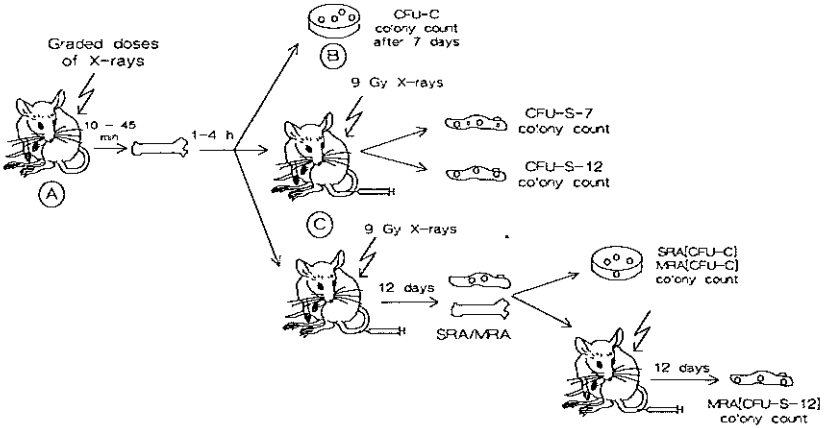

Figure 2.1: Mice were irradiated with graded doses of X-rays (A). Immediately after irradiation the femurs were excised and bone marrow cell suspensions were made. A part of the cell suspension (B) was assayed for the number of surviving colonies in vitro (CFU-C); the other part (C) was injected into three groups of lethally irradiated mice. The first two groups were used to determine the number of macroscopic spleen colonies 7 or 12 days after bone marrow transplantation. The third group of mice was used to determine the ability of injected bone marrow cells to generate either nucleated cells or CFU-C and CFU-S-12 in the spleen and bone marrow. To this purpose the splenic and femoral cellularity were determined 12 days after injection of donor cells, and aliquots of the spleens and bone marrow were assayed for the presence of cells capable of forming macroscopic spleen colonies and colonies in vitro.

Finally, the results of this thesis are discussed in a wider context in chapter 9 along with their implications for the treatment of victims of radiation accidents.

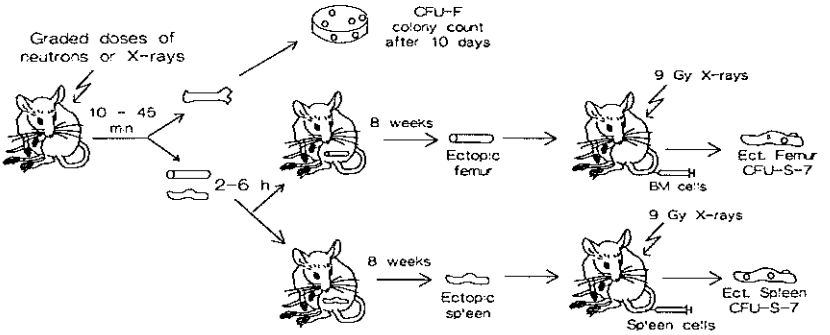

Figure 2.2: Mice were irradiated with graded doses of X-rays or fission neutrons. Immediately after radiation femurs and the spleens were excised. The right femurs were used to determine the radiosensitivity of fibroblastoid colony forming units (CFU-F). The left femurs and spleens were used to determine the regenerative capacity of the stroma after subcutaneous implantation into recipient mice. Because splenic regeneration is dependent on the total spleen volume, splenic recipients were irradiated with 5 Gy of X-rays one day before implantation. Eight weeks after implantation the ectopic bone marrow and spleen were assayed for the number of CFU-S-7 or CFU-C present, which is a measure of the functional capacity of the stroma.

THE EFFECTS OF X-IRRADIATION ON HEMOPOIETIC STEM CELL COMPARTMENTS IN THE MOUSE

Adapted from:
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ABSTRACT

The sensitivity for X-irradiation of a series of hemopoietic stem cell populations has been determined. The most primitive cells identified, cells with marrow-repopulating ability (MRA), showed the highest degree of radioresistance. These MRA cells which generate many secondary day-twelve spleen colony-forming units (MRA[CFU-S-12]) or colony-forming units in culture (MRA[CFU-C]) in the marrow of primary recipients had D_0 values equal to 1.18 Gy and 1.13 Gy, respectively. The more mature CFU-S-12 had intermediate radiosensitivity (D_0 = 0.94 Gy), while the less primitive CFU-S-7 were the most radiosensitive (D_0 = 0.71 Gy). The in vitro colony-forming precursor cells (CFU-C) showed low radiosensitivity. These data clearly show that the most primitive hemopoietic stem cell measured is less sensitive to ionizing radiation than generally has been assumed on the basis of measurements on CFU-S-7 or CFU-S-12.

INTRODUCTION

The spleen colony forming unit (CFU-S) assay of Till and McCulloch (1961) has for a long time been used as an assay of hemopoietic stem cells. However, the CFU-S appear to be a heterogeneous cell population with respect to the time required for colony-formation (Magli et al., 1982; Wolf & Priestley, 1986) their capacity to generate secondary CFU-S (Magli et al., 1982; Worton et al., 1969; Hellman et al., 1978; Bertoncello et al., 1988; Ploemacher et al., 1987), their capacity to 'home' to either the bone marrow or spleen (Hodgson & Bradley, 1979) and their ability to rescue recipients from radiation inflicted death. It has been proposed that the spleen colony method is not relevant to the study of hemopoietic stem cells capable of restoring hemopoiesis on a long-term basis when macroscopic colonies are scored before 11 days (Magli et al., 1982; Hodgson & Bradley, 1979). However cells able to generate colonies on day 12 in the spleen (CFU-S-12) have recently been reported to be defective in generating new CFU-S and cells that can rescue recipients from radiation inflicted death (Ploemacher et al., 1987; 1988a). These functions are performed by a separate class of primitive hemopoietic stem cells (pre-CFU-S) (Hodgson & Bradley, 1979; Ploemacher et al., 1988a, 1988b, 1989), that repopulate the bone marrow (marrow repopulating ability, MRA) of irradiated mice with cells capable of

forming spleen colonies in secondary recipients (MRA[CFU-S-12]). Thus, the hemopoietic system consists of a series of subpopulations that can be physically separated on the basis of their rhodamine 123 retention (Mulder & Visser, 1987; Ploemacher & Brons, 1988b) and wheat germ agglutinin (WGA) affinity (Ploemacher & Brons, 1988a). These subpopulations also differ in their sensitivity to 5-FU, hydroxyurea, and bromodeoxyuridine (Hogdson & Bradley, 1979, 1984). Because most studies on the effects of ionizing radiation have been carried out using the less relevant CFU-S-7/10 we investigated the effect of graded doses of X-rays on MRA[CFU-S-12] in comparison with CFU-S-12, CFU-S-7, and colony forming units in culture (CFU-C).

MATERIALS AND METHODS

Animals

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 mice were irradiated or sham-irradiated with graded doses of X-rays at the age of 12-14 weeks. Male or female mice at the age of >20 weeks or more were used as recipients in the CFU-S assays and MRA/spleen repopulating (SRA) assays. In each experiment, all the recipients used were of the same sex.

Irradiation procedure

Total body irradiations were 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. Twenty animals could be irradiated simultaneously. During irradiation the animals were confined in polycarbonate tubes, which were mounted in a rotating disk of perspex. Donor mice were irradiated with graded doses of X-rays. Recipient mice, which were used in the CFU-S and MRA/SRA assays, received a dose of 9.0 Gy, which is lethal for this mouse strain. Transplantation of bone marrow cells into lethally irradiated hosts was always performed within 1- 4 hours after irradiation.

Hemopoietic cell suspensions

Immediately after irradiation both femurs were excised from the donor mice and put on ice. The femurs were freed from muscles and tendons and ground in a mortar using 1 ml RPMI containing 0.04% bovine serum albumin (BSA), 100 IU/ml penicillin and 100 mg/ml streptomycin per femur. The cell suspension was put in a Falcon tube and the larger bone particles were allowed to settle for 45 sec. The supernatant was sieved through a nylon filter (pore size 100 μm) and the cellularity determined with a Coulter counter. Viability was determined by the trypan blue dye exclusion test.

CFU-S assay

CFU-S were assayed according to Till and McCulloch (1961). Recipient mice received 9 Gy 1- 4 hours before injection of cell suspensions. This dose reduced endogenous CFU-S-7 to 0 and CFU-S-12 to < 0.04 colony per spleen. Cell suspensions were always made from at least three donor mice. Five mice were used as recipient for the CFU-S-7 assay and 8-10 mice were used as recipient for the CFU-S-12 assay. Each recipient received 2 x 10^4 normal bone marrow cells or equivalent cell doses irradiated bone marrow cells in 0.2 ml Hanks' balanced salt solution (Gibco) by lateral tail vein injection. Seven or twelve days later the mice were killed, their spleens were excised and fixed in Telleyesniczky's solution. The macroscopic surface colonies were counted using a stereo-microscope at 10x magnification, and their diameters measured using an eye piece micrometer.

CFU-C assay

CFU-C were quantified in a semisolid (0.8% methylcellulose, Methocel, AP4 Premium; Dow Chemical) culture medium (alpha medium) containing 1% BSA, 10% fetal calf serum (FCS) and 10% concanavaline A - stimulated mouse spleen conditioned medium (Con-A-MSCM) or Poke Weed Mitogen mouse spleen conditioned medium (PWM-MSCM). Cells (2 x 10^4 to 1.5 x 10^6) 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.

MRA and SRA

For the repopulation assays five lethally irradiated recipients were injected with one to five times the number of donor bone marrow cells used

for the CFU-S assays. Twelve days after transplantation, aliquots of their femoral marrow were assayed for both the presence of CFU-S-12 and CFU-C and aliquots of their spleen cells for the presence of CFU-C. MRA and SRA were expressed as the number of either nucleated cells (MRA[cell], SRA[cell]) or hemopoietic precursor cells (MRA[CFU-S-12 or CFU-C], SRA[CFU-C]) generated over a 12-day period in one femur or spleen 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.

Statistical analysis

Log-linear dose-effect curves were obtained by least squares regression analysis. D_0 values were obtained from the slope of these curves. Statistical comparison of D_0 values was performed with a Chi square test. Values for α/β were obtained using the linear-quadratic model (release 2.05; Stata).

RESULTS

Premature deaths

Thirty-four percent of the lethally irradiated recipients that received no bone marrow cells died before the 12th day after irradiation. No deaths were seen before day 7 after irradiation. When normal or irradiated bone marrow cells were injected (2×10^4 cells or equivalent doses) the average number of premature deaths decreased to 10% for CFU-S-12 assays. Only 2% of the mice that received bone marrow cells in the repopulation assays died prematurely.

In vivo radiosensitivity of CFU-S and CFU-C

Animals were irradiated and killed within 1 hour after irradiation. No differences in the total number of nucleated cells between control and irradiated mice were found at this time. The mean viability of the cell suspensions was 91%; this did not differ between control and irradiated groups. The survival curves after graded doses of X-rays appeared to be exponential, with hardly a sign of a shoulder (Figure 3.1). CFU-C were the most radioresistant with a D_0 equal to 1.45 Gy if Con-A-MSCM was used as a source of colony stimulating factor and a D_0 equal to 1.47 Gy if

PWM-MSCM was used. A significant difference ($p < 0.005$) in radiosensitivity was found between CFU-S-7 (D_0 = 0.71 Gy) and CFU-S-12 (D_0 = 0.94 Gy). The survival curve of CFU-S-12 showed a little shoulder (n = 1.25), indicating that CFU-S-12 are capable of limited repair of sublethal damage.

Cell type	D_0 values[a] (Gy)	n	Correlation coefficient
MRA[CFU-S-12]	1.18 ± 0.01	1.39	- 0.9267
MRA[CFU-C]	1.13 ± 0.08	1.17	- 0.9147
SRA[CFU-C]	0.88 ± 0.03	1.31	- 0.9479
MRA[cell]	1.12 ± 0.04	1.48	- 0.9618
SRA[cell]	0.91 ± 0.03	1.25	- 0.9773
CFU-S-12	0.94 ± 0.03	1.25	- 0.9903
CFU-S-7	0.71 ± 0.01	1.04	- 0.9851
CFU-C[b]	1.45 ± 0.04	1.14	- 0.9737
CFU-C[c]	1.47 ± 0.15	1.28	- 0.9762

Table 3.1: D_0 values and extrapolation numbers (n) for different hemopoietic cell population exposed to graded doses of X-irradiation.
[a]Mean ± standard error of the mean (SEM) from three to six experiments.
[b]Con-A-MSCM stimulated CFU-C, mean of five experiments.
[c]PWM-MSCM stimulated CFU-C, one experiment.

In vivo radiosensitivity of marrow and spleen repopulating cells

Figure 3.2 shows the dose-response curve for the capability of injected donor cells to repopulate the bone marrow (MRA[cell]) and the spleen (SRA[cell]) with nucleated cells. MRA[cell] was less sensitive to X-irradiation than SRA[cell]. Figure 3.3 shows the survival curves for the primitive stem cells that repopulate the bone marrow or the spleen of lethally irradiated mice with in vitro clonable progenitors of granulocytes and macrophages. MRA[CFU-C] (D_0 = 1.13 Gy) were found to be more radioresistant than SRA[CFU-C] (D_0 = 0.88 Gy). The survival curve for stem cells that generate new CFU-S-12 in irradiated marrow (MRA[CFU-S-12]) is shown in Figure 3.4. The curve shows a little shoulder (n = 1.39), indicating that the MRA[CFU-S-12] have a higher ability to repair sublethal damage than either CFU-S-7 or CFU-S-12. The D_0 values for the different

cell populations are shown in Table 3.1. The radiosensitivity of cells capable of repopulating the bone marrow with either nucleated cells, CFU-C, or CFU-S-12 is lower than that of CFU-S-12, CFU-S-7, SRA[cell] and SRA[CFU-C]. CFU-C showed a remarkable low sensitivity to X-irradiation.

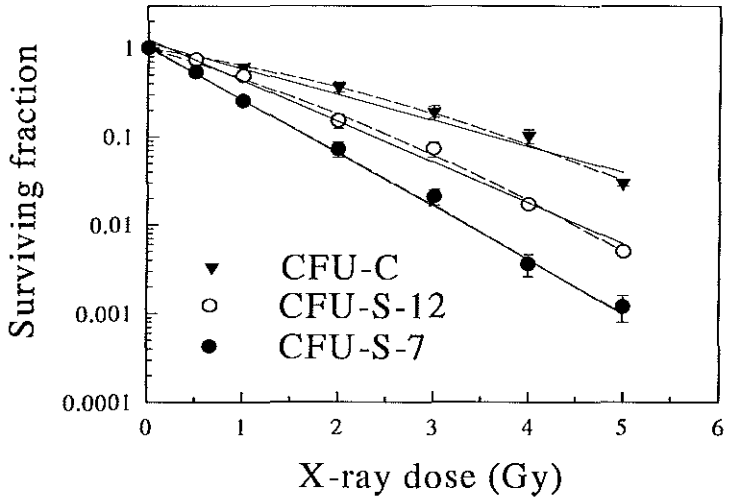

Figure 3.1: Survival curves of CFU-S-7, CFU-S-12 and CFU-C. Each solid line represents the regression line computed from three to six experiments using the linear model. The dotted line represents the fit of the LQ model. Mean absolute figures per 10^5 unirradiated cells were CFU-S-7: 39, CFU-S-12: 22, CFU-C: 173.

Radiosensitivity as a function of the degree of differentiation

When the D_0 values for the various stem cell subsets are expressed as a function of their respective position in the differentiation hierarchy of stem cells (Figure 3.5) it can be seen that the most primitive cells identified within this study, i.e. MRA[CFU-S-12], are most radioresistant, whereas cells differentiating into CFU-S-7 become progressively more sensitive. CFU-S-7 are the most sensitive. In contrast, the radiosensitivity of cells leaving the stem cell compartment and differentiating into CFU-C decreases again. These differences in radiosensitivity may be partly caused by differences in

the capacity to repair sublethal damage because the extrapolation number of the most primitive cell populations, with the highest self-renewal capacity (MRA) is higher than that of the more mature stem cell populations.

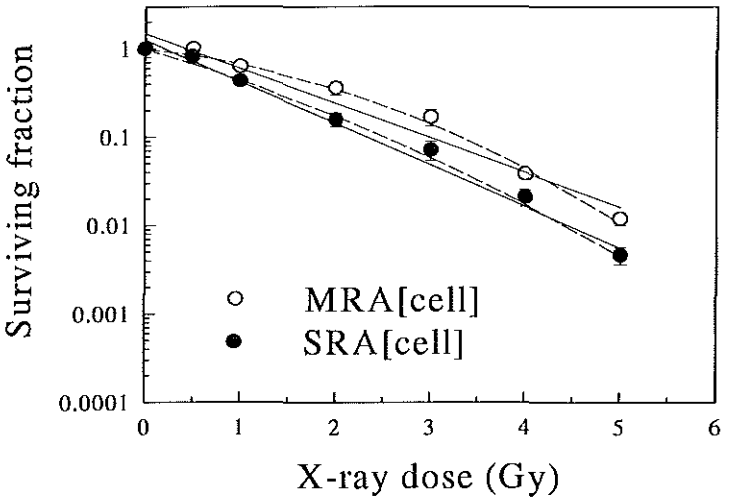

Figure 3.2: Dose response curve for the capability of injected donor cells to repopulate the bone marrow and the spleen with nucleated cells. Each solid line represents the regression line computed using the linear model. The dotted line represents the fit of the LQ model. Mean absolute cell values per organ per 10^5 unirradiated injected cells were: MRA[cell]: 4.3 x 10^6, SRA[cell]: 94.8 x 10^6. The background values found in 9 Gy irradiated control mice, which were not injected with bone marrow cells were: MRA[cell]: 1.9 x 10^6, SRA[cell]: 10.1 x 10^6.

α/β *values*

The linear-quadratic model was used to calculate the $\alpha\beta$ values of the various cell populations (Table 3.2). The α/β value of the most primitive cell identified, MRA[CFU-S-12], was 3.2 Gy. The more mature CFU-S-12 had an α/β value 11.0 Gy, and CFU-S-7 had an α/β value of 141 Gy. The very high α/β value obtained for CFU-S-7 was due to a very low β value.

Effect of irradiation on CFU-S colony size

The effect of X-irradiation on the proliferative ability of CFU-S was estimated by determining the size of the spleen colonies (Figure 3.6). Irradiated bone marrow cells formed slightly smaller colonies than unirradiated bone marrow cells. Irradiation with 5 Gy X-rays resulted in a 20% decrease in CFU-S-7 colony size, whereas CFU-S-12 spleen colonies were 9% smaller. In contrast, irradiation did not influence the colony size of secondary CFU-S-12-derived spleen colonies in the MRA[CFU-S-12] assay.

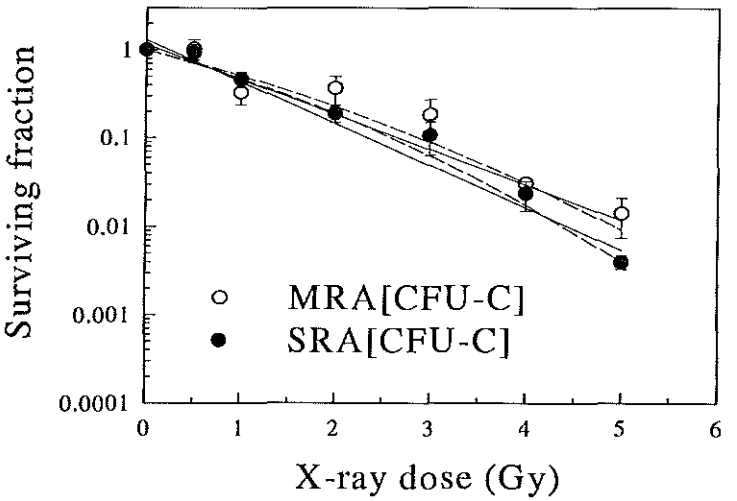

Figure 3.3: Dose response curves for stem cells that repopulate the irradiated bone marrow (MRA[CFU-C]) or spleen (SRA[CFU-C]) with in vitro clonable progenitors of granulocytes and macrophages. Each solid line represents the regression line computed using the linear model. The dotted lines represent the fit of the LQ model. Mean absolute values of organ CFU-C content per 10^5 injected unirradiated bone marrow cells were: MRA[CFU-C]: 3800, SRA[CFU-C]: 25.8 x 10^3. The background values found in 9 Gy irradiated control mice, which were not injected with bone marrow cells, were: MRA[CFU-C]: 415, SRA[CFU-C]: 110.

	α (Gy^{-1})	β (Gy^{-2})	α/β (Gy)	Correlation coefficient
MRA[CFU-S-12]	0.38 ± 0.09	0.117 ± 0.021	3.2 ± 0.9	-0.9724
MRA[CFU-C]	0.61 ± 0.08	0.065 ± 0.019[a]	9.5 ± 2.9	-0.9747
SRA[CFU-C]	0.66 ± 0.07	0.089 ± 0.016	7.4 ± 1.5	-0.9778
MRA[cell]	0.28 ± 0.03	0.126 ± 0.007	2.2 ± 0.3	-0.9888
SRA[cell]	0.75 ± 0.04	0.066 ± 0.010	11.3 ± 1.8	-0.9909
CFU-S-12	0.73 ± 0.04	0.066 ± 0.009	11.0 ± 1.7	-0.9970
CFU-S-7	1.34 ± 0.04	0.009 ± 0.009[a]	141 ± 140	-0.9937
CFU-C	0.39 ± 0.03	0.060 ± 0.006	6.4 ± 0.8	-0.9916

Table 3.2: Values of α and β from the linear-quadratic model for different hemopoietic cell population exposed to graded doses of X-irradiation.
Mean coefficient ± SEM of three to six experiments.
[a]β not significantly different from zero.

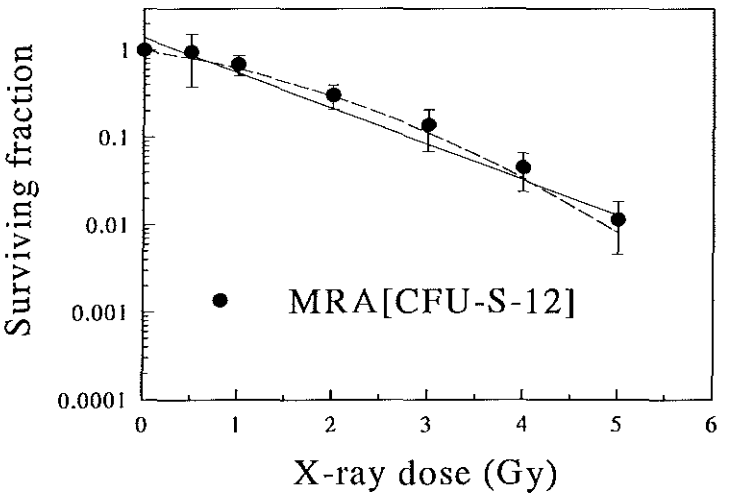

Figure 3.4: Dose response curve for MRA[CFU-S-12]). The solid line represents the regression line computed using the linear model. The dotted line represents the fit of the LQ model. Mean absolute organ CFU-S-12 content per 10^5 injected unirradiated bone marrow cells was: MRA[CFU-S-12]: 223. The background value found in 9 Gy irradiated control mice, which were not injected with bone marrow cells was: MRA[CFU-S-12]: 60

DISCUSSION

In order to guarantee a continuous production of functional blood cells, the bone marrow is composed of a hierarchically organized proliferative system with relatively few ultimate stem cells producing proliferating transit cells, which eventually differentiate and mature into the functional cells of the tissue. The CFU-S assay of Till and McCulloch (1961) has for a long time been used as a quantitative assay to determine the number of hemopoietic stem cells. Over the last few years, a considerable heterogeneity within the CFU-S population has been revealed (Magli et al., 1982; Ploemacher et al., 1987; Harris et al., 1984), indicating that the hemopoietic stem cell compartment can be divided into early appearing CFU-S (CFU-S-7/10), late appearing CFU-S (CFU-S-12/14) and pre-CFU-S. Of these cell populations, which can be separated on the basis of the Rh123 retention, WGA affinity and light scatter (Ploemacher & Brons, 1988a, 1988b), neither early appearing CFU-S (Magli et al., 1982; Hodgson & Bradley, 1984) nor the majority of late appearing CFU-S (Ploemacher et al., 1989; Visser et al., 1989) are capable of extensive proliferation and long-term reconstitution of the hemopoietic system. Long-term hemopoietic reconstitution after irradiation is dependent on cells that can generate many new stem cells and induce long-term hemopoietic chimerism (Bertoncello et al., 1988; Hodgson & Bradley, 1979; Ploemacher et al., 1989; Visser et al., 1989).

A means of measuring the radiosensitivity of the pre-CFU-S cell is the MRA technique of Hodgson and Bradley (1984). Although the MRA assay is a nonclonal assay, it is adequate to measure the radiosensitivity of the average pre-CFU-S cells. In the present investigation we compared the radiosensitivity of these primitive MRA cells with the more mature CFU-S-12 and CFU-S-7. MRA cells were found to be less radiosensitive than CFU-S-12 and CFU-S-7. These data support and extend the observations, reported by Hendry & Lord (1983) that the mean number of secondary CFU-S per CFU-S-11 has not decreased following irradiation of the graft but may have increased by a factor of up to 2.5. This increase indicates that the radiosensitivity of CFU-S with high self-renewal capacity is probably lower than that of the average CFU-S. On the other hand the distribution of CFU-S across the marrow has been reported to be non-uniform in some mice strains. According to these publications the CFU-S concentration increases from the longitudinal axis of the femur towards the internal surface of the bone (Lord & Hendry, 1972). CFU-S close to the axis of the long bones (axial CFU-S)

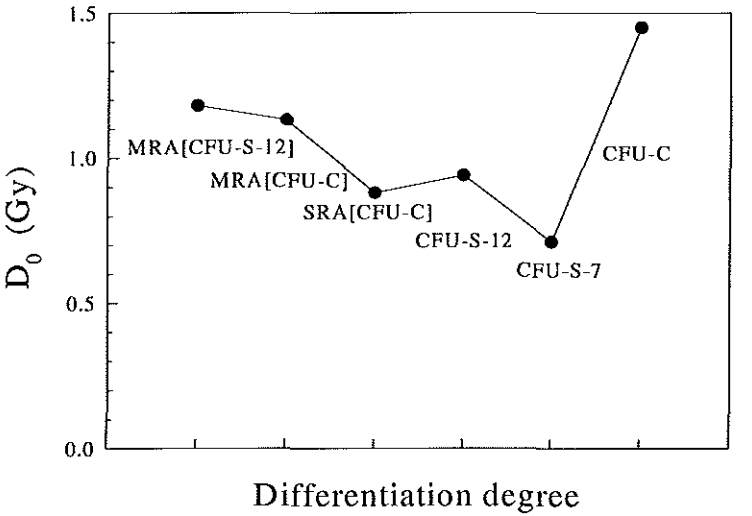

Figure 3.5: Radiosensitivity of the different hemopoietic cell populations as a function of differentiation degree. The most primitive cell identified within the stem cell compartment, the MRA[CFU-S-12], is the most radioresistant, whereas cells differentiating into CFU-S-7 become progressively more radiosensitive. When cells leave the stem cell compartment to become CFU-C their radiosensitivity decreases again

have a higher probability of self-renewal and exist in deeper levels of the quiescent G_0-state (Lord, 1986). With 250 kV X-rays, cells growing close to the bone in the mouse femur will receive up to 17% more dose due to secondary short-range electron emission from elements with a high atomic number in the bone than cells in the centre of the femur (Broerse & Barendsen, 1968). Because the most mature CFU-S (CFU-S-7/10) are supposed to be located close to the bone surface, their apparent greater radiosensitivity could be explained by the fact that they received a higher dose of X-rays. An increase in radiation dose of 17% can explain in part the observed differences in radiosensitivity between CFU-S-7 and CFU-S-12, but not between CFU-S-12 and MRA[CFU-S-12], because these are both located in the axial region of the bone. In addition, when in vivo irradiation is compared to in vitro irradiation a lower survival level for CFU-S-7/10 should

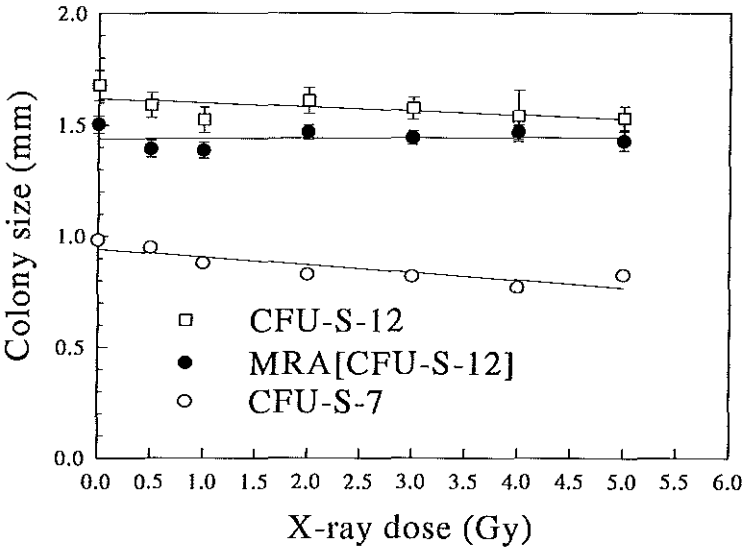

Figure 3.6: Size of spleen colonies produced after infusion of normal or X-irradiated bone marrow cells. Each point represents the arithmetic mean (±1 SEM) of 31-290 colony diameters determined in at least three independent experiments.

be observed after in vivo irradiation due to emission of secondary electrons, leading to a lower D_0 value. However, such differences have not been observed. For example, Hendry and Lord (1972) and Hendry (1972) found a lowered radiosensitivity when cells were irradiated in vivo (D_0 = 0.70 Gy) than in vitro (D_0 = 0.67 Gy), and a comparison of various experiments carried out in vivo and in vitro gave mean D_0 values of 0.77 Gy and 0.79 Gy for in vivo and in vitro irradiation, respectively (Hendry, 1983). Measurements of the proliferation rate of CFU-S in different regions show that the CFU-S close to the bone are rapidly proliferating (up to 70% are killed by a large dose of tritium-labelled thymidine ([^{3}H]TdR)). The CFU-S located at a distance of > 80 μm or more from the bone surface are proliferative quiescent (Hendry, 1983; Lord et al., 1975). Because cells in S-phase are more sensitive to X-rays than resting cells (Lahiri & Van Putten, 1972), the higher percentage of CFU-S-7 in S-phase compared to the more primitive cell

populations may also contribute to the observed differences in radiosensitivity. However, CFU-S-12 and MRA[CFU-S-12] are both in G_0, and the more radioresistant CFU-C are also rapidly proliferating (40%-50% killed by [^{3}H]TdR), indicating that other intrinsic factors must also contribute to the observed differences in radiosensitivity.

The D_0 value obtained for CFU-S-7, which can be considered to belong to the same cell population as CFU-S-8/10 (Magli et al., 1982; Hodgson & Bradley, 1984; Imai & Nakao, 1987), is comparable to the D_0 values of 0.72 Gy reported by Broerse et al. (Imai & Nakao, 1987) and 0.77 Gy reported by Hendry and Lord (1983) for CFU-S-9. However, the extrapolation number we found (n = 1.04) is lower than that reported by Broerse et al. (1971) (n = 2.47) and Hendry and Lord (1983) (n = 1.7), but comparable to Carstens et al. (1976) (n = 1.0) and Imai and Nakao (1987) (n = 1.04). It has been reported that a lower extrapolation number can be obtained when the CFU-S assay is not performed directly after irradiation of the donors (Van Putten, 1970). However, this was not the case in our experiments. SRA[CFU-C] can be considered to be less primitive than MRA[CFU-S-12] and MRA[CFU-C] because they have a higher mitochondrial activity (Ploemacher & Brons, 1988b). The D_0 value found for this cell population was 0.88 Gy, which is close to the D_0 value found for CFU-S-12. When cells leave the stem cell compartment and differentiate into committed progenitors of granulocytes and macrophages (CFU-C), their radiosensitivity decreases again (D_0 = 1.45 Gy - 1.47 Gy). This is in agreement with the D_0 value of 1.57 Gy found by Imai and Nakao (1987) and the results reported by Baird et al. (1988) who found that the radiosensitivity of CFU-C depended on the type of colony stimulating factor (CSF) used. It is possible that heterogeneous stimuli can stimulate a resistant subpopulation; this resulted in an increase in D_0 from 0.4 Gy when G-CSF was used to 1.7 Gy when a combination of IL-3, IL-1 and M-CSF was used (Baird et al., 1988). Both Con-A-MSCM and PWM-MSCM are heterogeneous stimuli that probably stimulate the same cell population. The high radioresistance of CFU-C compared to CFU-S may be due to a higher ability to repair potential lethal damage (Hendry & Lord, 1983).

The radiosensitivity of a tissue can be described using several models. Although most studies determining the radiosensitivity of hemopoietic stem cells used the single-hit model, much recent radiobiological work has focused on the linear-quadratic model. The linear-quadratic model assumes that the mean number of lethal events is $\alpha D + \beta D^2$ and comprises a linear

term, where the number of events is proportional to dose, and a quadratic term, where two sublesions interact to produce a lethal event (Thames & Hendry, 1987). Using this model the α and β values of cell populations can be calculated. The linear component (α) has been associated with the intrinsic radiosensitivity of cells, the quadratic component (β) with the ability of cells to repair sublethal damage. The ratio α/β is a measure of the relative importance of the α and β terms, that is, the curvature in the survival curve. In general the α/β value is high for early responding, fast proliferating tissues (> 6 Gy) and low for late responding, slowly proliferating tissues. The α/β value found for the most primitive stem cell identified (MRA[CFU-S-12]) was 3.2 Gy, and the α/β value of the MRA[cell] was surprisingly low (2.2 Gy). The α/β value of 11.0 Gy, obtained for CFU-S-12, was somewhat lower than the α/β value of 23 Gy for CFU-S-9 calculated for CFU-S-9 by Hendry and Moore (1985) and Hendry and Potten (1988). The more mature CFU-S-7 had a very high α/β value (141 Gy) due to a very low β value. However, the SE for this value was very high. These data are consistent with the thought that the α/β values of slowly cycling colony-forming cells in early responding hierarchical tissues are lower than for rapidly cycling cells [Hendry & Potten, 1988]. The strong correlation between the D_0 values and the α/β for MRA[cell] and MRA[CFU-S-12] indicates that the repopulation of the bone marrow over a 12-day period after lethal irradiation is dependent on more primitive cells than the CFU-S-12.

In summary, our data indicate that the most primitive stem cells identified in our studies, MRA[CFU-S-12], are much less radiosensitive than the stem cells belonging to the CFU-S compartment. Using a dose of 9 Gy X-rays, 150 times more MRA[CFU-S-12] survive in comparison to CFU-S-7. These results have important implications for radiotherapy and the treatment of victims of radiation accidents. Small increments (0.5 Gy) in total dose will not easily lead to complete ablation of hemopoietic stem cells required for allogeneic bone marrow transplantation in cancer treatment. On the other hand our data indicate, that optimisation of supportive care in the treatment of victims of radiation accidents can increase survival, since prolonged radiation-induced pancytopenia is probably not due to a lack of surviving primitive stem cells, but to a lack of time to reconstitute the hemopoietic system with sufficient numbers of functional blood cells.

Acknowledgements
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REFERENCES

Baird, M.C., Hendry, J.H. and Testa, N.G. The radiosensitivity of different sub-populations of colony forming cells in the haemopoietic hierarchy [abstr]. Int. J. Radiat. Biol. 53:1004 (1988).

Bertoncello, I., Hodgson, G.S. and Bradley, T.R. Multiparameter analysis of transplantable hemopoietic stem cells. I. The separation and enrichment of stem cells homing to marrow and spleen on the basis of rhodamine-123 fluorescence. Exp. Hematol. 13:999 (1988)

Broerse, J.J. and Barendsen, G.W. The importance of secondary particle equilibrium for neutron irradiations of cultured cells and intact organisms. In: Ebert HG (ed) Proc symposium on microdosimetry. Ispra, 1967, euratom, Brussels, EUR 3747, p353 (1968).

Broerse, J.J., Engels, A.C., Lelieveld, P., Van Putten, L.M., Duncan, W., Greene, D., Massay, J.B., Gilbert, C.W., Hendry, J.H. and Howard, A. The survival of colony-forming units in mouse bone-marrow after in vivo irradiation with D-T neutrons, X- and γ-irradiation. Int. J. Radiat. Biol. 19:101 (1971).

Carsten, A.L., Bond, V.P. and Thompson, K. The R.B.E. of different energy neutrons as measured by the hemotopoietic spleen-colony technique. Int. J. Radiat. Biol. 29:65 (1976).

Davids, J.A.G. Bone marrow syndrome in CBA mice exposed to fast neutrons of 1.0 MeV mean energy. Effect of syngeneic bone marrow transplantation. Int. J. Radiat. Biol. 17:173 (1970).

Harris, R.A., Hogarth, P.M., Wadeson, L.J., Collins, P., McKenzie, I.F.C. and Penington, D.G. An antigenic difference between cells forming early and late haematopoietic spleen colonies (CFU-S). Nature 307:638 (1984).

Hellman, S., Botnick, E.C., Hannon, E.C. and Vigneulle, R.M. Proliferative capacity of murine hematopoietic stem cells. Proc. Natl. Acad. Sci. USA 75:490 (1978).

Hendry, J.H. The response of haemopoietic colony-forming units and lymphoma cells irradiated in soft tissue (spleen) or a bone cavity (femur) with single doses of X rays, gamma rays or D-T neutrons. Brit. J. Radiol. 45:923 (1972).

Hendry, J.H., Lord, B.I. The analysis of the early and late response to cytotoxic insults in the hematopoietic cell hierarchy. In: Potten CS, Hendry JH (eds) Cytotoxic insult to tissue, Edinburgh, Churchill Livingstone, p 1 (1983).

Hendry, J.H. and Moore, J.V. Deriving absolute values of a and b for dose fractionation, using dose-incidence data. Br. J. Radiol. 58:885 (1985).

Hendry, J.H. and Potten, C.S. α/β Ratios and the cycling status of tissue target cells. Radiother Oncol. 12:79 (1988).

Hodgson, G.S. and Bradley, T.R. Properties of haematopoietic stem cells surviving 5-fluorouracil treatment: evidence for a pre-CFU-S cell? Nature 281:381 (1979).

Hodgson, G.S., Bradley T.R. and Radley J.M. The organization of hemopoietic tissues as inferred from the effects of 5-fluorouracil. Exp. Hematol. 10:26 (1982).

Hodgson, G.S. and Bradley, T.R. In vivo kinetic status of hematopoietic stem and progenitor cells as inferred from labelling with Bromodeoxyuridine. Exp. Hematol. 12:683 (1984).

Imai, Y. and Nakao, I. In vivo radiosensitivity and recovery pattern of the hematopoietic precursor cells and stem cells in mouse bone marrow. Exp. Hematol. 15:890 (1987).

Lahiri, S.K. and Van Putten, L.M. Location of the G_0-phase in the cell cycle of mouse haemopoietic spleen colony forming cells. Cell Tissue Kinet. 5:365 (1972).

Lord, B.I. and Hendry, J.H. The distribution of haemopoietic colony-forming units in the mouse femur, and its modification by X-rays. Brit. J. Radiol. 45:110 (1972).

Lord, B.I., Testa, N.G. and Hendry, J.H. The relative spatial distribution of CFU-S and CFU-C in the normal mouse femur. Blood 46:65 (1975).

Lord, B.I. The sensitivity of Go-state haemopoietic spleen colony-forming cells to a stimulus for proliferation. Cell Tissue Kinet. 19:305 (1986).

Magli, M.C., Iscove, N.N. and Odartchenko, N. Transient nature of early haemopoietic spleen colonies. Nature 295:527 (1982).

Mulder, A.H. and Visser, J.W.M. Separation and functional analysis of bone marrow cells separated by rhodaminefluorescence. Exp. Hematol. 15:99 (1987).

Ploemacher, R.E., Brons N.H.C. and Leenen, P.J.M. Bulk enrichment of transplantable hemopoietic stem cell subsets from lipopolysaccharide stimulated murine spleen. Exp. Hematol. 15:154 (1987).

Ploemacher, R.E. and Brons, N.H.C. Isolation of hemopoietic stem cell subsets from murine bone marrow: II Evidence for an early precursor of day-12 CFU-S and cells associated with radioprotective ability. Exp. Hematol. 16:27 (1988a).

Ploemacher, R.E. and Brons, N.H.C. Cells with marrow and spleen repopulating ability, and cells forming spleen colonies on day 16, 12, and 8, are sequentially ordered on the basis of increasing rhodamine 123 retention. J. Cell Phys. 136:531 (1988b).

Ploemacher, R.E.and Brons, N.H.C. Separation of CFU-S from primitive cells responsible for reconstitution of the bone marrow hemopoietic stem cell compartment following irradiation: evidence for a pre-CFU-S cell. Exp Hematol 17:263 (1989).

Thames, H.D. and Hendry, J.H. Fractionation in radiotherapy. London: Taylor & Francis, p 187 (1987).

Till, J.E. and McCulloch, E.A. A direct measurement of the radiation sensitivity of normal bone marrow cells. Radiat. Res. 14:213 (1961).

Van Putten, L.M. In:Bond VP, Suit HD, Marcial V (eds) Time and dose relationships in radiation biology as applied to radiotherapy. BNL report no. 50203 (C-57), Biology and Medicine-TID-4500, p 162 (1970).

Visser, J.W.M., Hogeweg-Platenburg, M.G.C., Vries, P. de, Bayer, J. and Ploemacher, R.E. Culture of purified pluripotent haemopoietic stem cells. In: Daniak N, Liss, AR (eds) The biology of hematopoiesis, New York, p 1 (1989).

Wolf, N.S. and Priestley, G.V. Kinetics of early and late spleen colony development. Exp. Hematol. 14:676 (1986).

Worton, R.G., McCulloch, E.A. and Till, J.E. Physical separation of hemopoietic stem cells differing in their capacity for self-renewal. J. Exp. Med. 130:91 (1969)

THE SENSITIVITY OF MURINE HEMOPOIETIC STEM CELL POPULATIONS FOR 1 MEV FISSION NEUTRONS

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Submitted to: Radiat. Res.

ABSTRACT

The sensitivity of hemopoietic stem cell populations for 1 MeV fission neutron irradiation was determined. The most primitive stem cells measured, cells with Marrow Repopulating Ability (MRA), were found to be the most radioresistant. MRA cells, which generate many secondary day-twelve spleen colony-forming units (MRA[CFU-S-12]) or colony forming units in culture (MRA[CFU-C]) in the marrow of lethally irradiated primary recipients, had D_0 values of 0.48 and 0.46 Gy, respectively. When MRA cells differentiate into more mature stem cells the radiosensitivity of the stem cells progressively increases. Cells repopulating the spleen and CFU-S-12 were characterized by a D_0 value of 0.37 Gy and 0.36 Gy and the more mature CFU-S-7 were even more radiosensitive (D_0, 0.31 Gy). When stem cells leave the stem cell compartment and differentiate into precursors of the granulocyte-macrophage lineage their radiosensitivity decreases, as is evidenced by a D_0 value of 0.51 Gy. These data clearly show that primitive hemopoietic stem cells (MRA[CFU-S-12] and MRA[CFU-C]) are less sensitive to ionizing radiation than CFU-S-7. The observed differences in radiosensitivity are due to intrinsic characteristics of the different cell populations and not to their location in the bone as has been suggested for X-irradiation by some investigators. By comparing D_0 values observed for the different stem cell populations after 1 MeV fission neutron irradiation with the D_0 values observed earlier after 300 kV X-irradiation RBE values were determined. The fission neutron RBE for all the stem cell subsets did not differ much and ranged from 2.2 to 2.6. The RBE for CFU-C was 2.8. The RBE value for the various stem cell populations was comparable to the RBE of 2.3 obtained for the $LD_{50/30}$ using the same CBA/H mouse strain.

INTRODUCTION

Exposure to ionizing total body radiation (TBI) causes a dose-dependent suppression of hemopoiesis. The severity and the duration of the resulting pancytopenia has been attributed to the number of surviving pluripotent hemopoietic stem cells (PHSC), which through self-renewal and differentiation regenerate the damaged bone marrow and induce the resumption of normal blood production. The spleen colony forming unit (CFU-S) assay has for a long time been used as an assay to measure the

radiosensitivity of PHSC (Till & McCulloch, 1961). CFU-S forming colonies 7-10 days after injection into a lethally irradiated syngeneic recipient (CFU-S-7/10) appeared to be very sensitive to X-rays and γ-rays (average value of D_0 = 0.77 ± 0.03 Gy for X-rays and 1.00 ± 0.03 Gy for γ-rays) (Hendry & Lord, 1983). Death due to TBI doses up to 10 Gy has therefore been attributed to a lack of surviving PHSC. During the last years, however, it has become clear that the stem cell compartment is heterogeneous. Using counterflow centrifugal elutriation and or flow cytometric sorting on the basis of Rh123 and/or wheat germ agglutinin (WGA) affinity different stem cell populations have been identified (Bertoncello et al., 1985; Mulder & Visser, 1987; Ploemacher & Brons, 1988a, Visser et al., 1990). These data indicate that the hemopoietic stem cell compartment consists of a series of cell populations organized for use according to their generation age (the number of cell generations in their history) (Rosendaal et al., 1979; Hodgson et al., 1982).

At least five subpopulations can be identified using in vivo assays that may or may not have overlapping functional characteristics: 1) cells responsible for long-term donor-type engraftment following their transplantation into hemopoietically suppressed recipients (LTRA cells) (Jordan & Lemischka, 1990; Ploemacher et al., 1992), 2) cells repopulating the bone marrow (MRA cells), 3) cells repopulating the spleen (SRA cells), 4) CFU-S-12 (enumerated by counting colonies at day 12) and 5) CFU-S-7/10 (Ploemacher & Brons, 1988a, Hodgson & Bradley, 1979; Ploemacher & Brons, 1989). Within this hierarchy LTRA cells are considered to be most primitive followed by MRA cells. MRA cells are able to generate many new CFU-S and cells that rescue recipients from radiation inflicted death (Ploemacher & Brons, 1989; Ploemacher & Brons, 1988b). MRA cells can be measured with a double transplantation assay. Twelve days after transplantation into a primary lethally irradiated recipient the marrow of the recipient is assayed for the number of CFU-S-12 generated by the donor cells by injecting it into a secondary lethally irradiated recipient (MRA[CFU-S-12]). MRA cells lack CFU-S or CFU-C activity but have a very potent repopulating capacity and have the potential to differentiate into cells that are CFU-S or CFU-C. CFU-S-7 are considered to be the most mature cells within the stem cell compartment. In an earlier study we determined the radiosensitivity of four of these different stem cell subpopulations for 300 kV X-rays (Meijne et al., 1991). The primitive MRA[CFU-S-12] appeared to be the most radioresistant, whereas stem cells differentiating towards CFU-S-7

became progressively more radiosensitive. In the present investigation we determined the radiosensitivity of the various stem cell populations for 1 MeV fission neutrons. By comparing the radiosensitivity of the hemopoietic subpopulations for 1 MeV fission neutrons with their radiosensitivity for 300 kV X-rays we have determined the RBE values. The RBE is defined as the ratio of the absorbed dose of 300 kV X-rays to the absorbed dose of 1 MeV fission neutrons which produces the same degree of effect. Comparison of neutron RBE data obtained with fast neutrons of various mean energies shows that those for fast fission neutrons of 1 MeV are among the highest for each effect category investigated (Broerse & Barendsen, 1973; Hall et al., 1975; Broerse, 1989). Therefore RBE data of fission neutrons can be regarded as the upper limit for neutron RBE data for a specific biological effect.

The difference in radiation quality between high LET fission neutron radiation and low LET X-radiation makes it possible to analyze the influence of various factors on the observed differences in radiosensitivity of the various stem cell populations for 300 kV X-rays. These factors include repair processes, and differences of various stem cell subsets with regard to their location in relation to the bone. In contrast to the occurrence of sublethal and potential lethal damage repair (SLDR, PLDR) processes following X-irradiation, virtually no intracellular repair occurs after irradiation with fission neutrons (Broerse & Barendsen, 1973 ; Hall, 1988). Also no secondary electron emission occurs on the transition of bone to marrow tissue as is the case with X-rays.

It has been reported that the distribution of stem cells across the marrow is non-uniform. The more primitive CFU-S are located close to the axis of the femora and the more mature CFU-S are located close to the bone (Lord, 1986). The higher cell killing of the more mature CFU-S-7 by X-rays in vivo could therefore partly be explained by assuming a locally higher dose due to the emission of secondary short-range electrons from the bone (Broerse & Barendsen, 1968). Data about the radiosensitivity of hemopoietic stem cell subpopulations for 1 MeV fission neutrons may elucidate whether the observed differences are primarily due to differences in SLDR or to the location of the cell population in the bone marrow. Alternatively, the observed differences in radiosensitivity to X-rays might be caused by intrinsic differences between stem cell populations.

MATERIALS AND METHODS

Mice

Inbred CBA/H mice (H-2^k) 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 mice were irradiated or sham-irradiated with graded doses of neutrons at the age of 12-14 weeks. Male or female mice at the age ≥ 20 weeks were used as recipients in the CFU-S assays and marrow repopulating ability (MRA)/spleen repopulating ability (SRA) assays. In each experiment all the recipients used were of the same sex.

The experiments were conducted with permission of the experimental animal welfare commission (DEC) of the Netherlands Energy Research Foundation, as required by Dutch law.

Irradiation procedure

The donor mice 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 mice 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% gamma-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.

X-irradiation 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 0.3 Gy/min at 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.

Donor mice were irradiated with graded doses of fast fission neutrons. Recipient mice, which were used in the CFU-S, MRA and SRA assays, received a dose of 9 Gy X-rays, which is lethal for this mouse strain (LD_{99} = 8.8 Gy, unpublished results). Transplantation of bone marrow cells into lethally irradiated hosts was always performed within 1-4 hours after irradiation.

Hemopoietic cell suspensions

Immediately after irradiation both femurs were excised from the donor mice and put on ice. The femurs were freed from muscles and tendons and ground in a mortar using 1 ml RPMI-1640 medium (Flow) per femur containing 0.04% bovine serum albumin (BSA; Sigma), 100 IU/ml penicillin, and 100 mg/ml streptomycin. The cell suspension was put in a Falcon tube and the larger particles were allowed to settle for 45 sec. The supernatant was sieved through a nylon filter (pore size 100 μm) and the cellularity was determined using a Coulter Counter. Viability was determined by trypan blue dye exclusion test.

CFU-S assay

CFU-S were assayed according to Till and McCulloch (1961). Recipient mice received 9 Gy X-rays 1- 4 hours before injection of cell suspensions. This dose reduced endogenous CFU-S-7 to 0 and CFU-S-12 to < 0.04 colony per spleen. Cell suspensions were always made from at least three donor mice. Five mice were used as recipients for the CFU-S-7 assay and eight to ten mice were used as recipients for the CFU-S-12 assay. Each recipient received 2 x 10^4 normal bone marrow cells or equivalent cell doses of irradiated bone marrow cells in 0.2 ml Hanks' balanced salt solution (Gibco) by lateral tail vein injection. Seven or 12 days later the mice were killed, and their spleens were excised and fixed in Telleyesniczky's solution (Culling, 1974). The macroscopic surface colonies were counted using a stereomicroscope at 10x magnification, and their diameters were measured using an eyepiece micrometer.

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% fetal calf serum (FCS), and 10% concanavalin A-stimulated mouse spleen-conditioned medium (Con-A-MSCM). Cells (2 x 10^4 to 1.5 x 10^6) were plated in 35-mm Costar dishes in 1 ml final culture medium. Duplicate cultures were incubated at 37 ^{0}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.

MRA and SRA

For the repopulation assays five lethally irradiated recipients were injected with one to five times the number of donor bone marrow cells used for the CFU-S assays. Twelve days after bone marrow transplantation, aliquots of their femoral marrow were assayed for both the presence of CFU-S-12 and CFU-C, and aliquots of their spleen cells were assayed for the presence of CFU-C. MRA and SRA were expressed as the number of either nucleated cells (MRA[cell], SRA[cell]) or hemopoietic precursor cells (MRA[CFU-C], SRA[CFU-C] or MRA[CFU-S-12]) generated over a 12-day period in one femur or spleen 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.

Experimental procedures

The experimental procedures were similar to those used for X-irradiation (Meijne et al., 1991). From each experiment the individual mean colony counts per dose point were used as input for statistical analysis to obtain dose effect curves. Log-linear dose-effect curves were obtained by least squares regression analysis. D_0 values were obtained from the slopes of these curves. The reported D_0 values were derived from the individual D_0 's per experiment (n = 4 - 14) using a weighted averaging procedure resulting in an average D_0 for the cell population studied. Statistical comparison of the resulting D_0 values was performed with a Chi-square test. The relative biological effectiveness (RBE) values of fission neutrons for the various cell populations were determined by calculating the ratio of the D_0 values obtained after fission neutron and X-irradiation. The SE of the RBE was obtained by multiplying the obtained ratios with the square root of the sum of the squares of the fractional errors of the terms devided.

RESULTS

In vivo radiosensitivity of CFU-S and CFU-C

Animals were irradiated and killed immediately after irradiation. No differences in the number of nucleated cells between control and irradiated mice were found at this time. The mean viability of the cell suspension was 90% and did not differ between irradiated and control groups. The survival

curves for CFU-S-7, CFU-S-12 and CFU-C are shown in Figure 4.1. The survival curves after 1 MeV fission neutron irradiation were exponential without any shoulder. CFU-S-7 were most radiosensitive and their radiosensitivity was characterized by a D_0 value of 0.31 ± 0.01 Gy. CFU-S-12 had an intermediate radiosensitivity with a D_0 value of 0.36 ± 0.01 Gy. CFU-C, which belong to the committed progenitor compartment, were the most radioresistant. Their survival curve was characterized by a D_0 value of 0.51 ± 0.01 Gy.

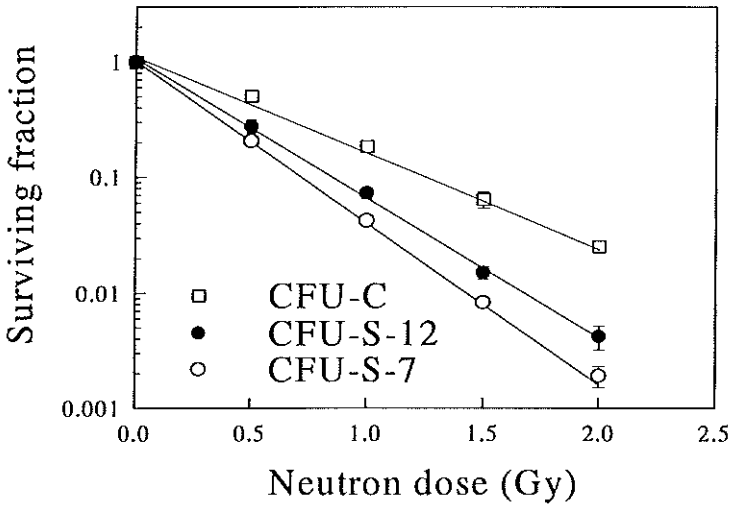

Figure 4.1: Survival curves of CFU-S-7, CFU-S-12 and CFU-C after whole body irradiation. Each curve represents the regression line computed from 4 to 14 experiments. The data points show the mean survival level per dose point.

In vivo radiosensitivity of marrow and spleen repopulating cells

Figure 4.2 shows the dose-response curve for the capability of injected donor cells to repopulate the bone marrow (MRA[cell]) and the spleen (SRA[cell]) with nucleated cells within a 12-day period. MRA[cell] was less sensitive to fission neutron irradiation than SRA[cell]. The survival curves for primitive stem cells that repopulate the bone marrow (MRA[CFU-C]) or spleen

(SRA[CFU-C]) of lethally irradiated mice with in vitro clonable progenitors of granulocytes and monocytes/macrophages are shown in Figure 4.3. MRA[CFU-C] were somewhat more radioresistant than SRA[CFU-C] and were characterized by a D_0 value of 0.46 ± 0.01 Gy. The more mature SRA[CFU-C] had a D_0 value of 0.39 ± 0.01 Gy. The survival curve for stem cells that generate many new CFU-S-12 in irradiated bone marrow (MRA[CFU-S-12]) is shown in Figure 4.4. MRA[CFU-S-12] were the most radioresistant cells within the stem cells compartment and were characterized by a D_0 value of 0.48 ± 0.05 Gy.

The survival parameters obtained for the different hemopoietic stem cell populations are summarized in Table 4.1. In an earlier study we determined the radiosensitivity of these different cell populations for 300 kV X-rays (Meijne et al., 1991). By dividing the D_0 value obtained after X-irradiation by the D_0 value obtained after neutron irradiation the RBE value

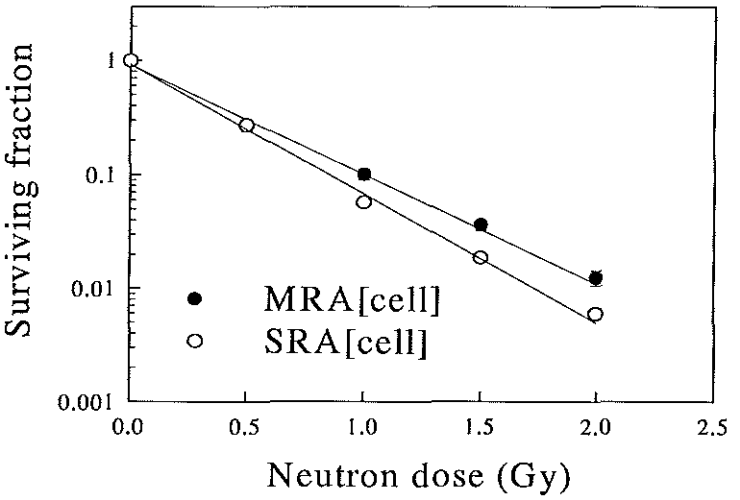

Figure 4.2: Dose-response curve for the capability of injected donor cells to repopulate the bone marrow (MRA[cell]) and spleen (SRA[cell]) with nucleated cells.

for the different cell populations was determined. The RBE value for the different cell population ranged from 2.2 for MRA[cell] to 2.8 for CFU-C.

Spleen colony size

The size of the spleen colonies as a function of neutron dose is shown in Figure 4.6. The mean diameter of colonies formed by the CFU-S-7 control group was 0.98 ± 0.01 mm. Irradiation with 1 MeV fission neutrons resulted in a dose-dependent reduction of the size of colonies generated by CFU-S-7. The mean diameter of spleen colonies formed by CFU-S-7 which had been

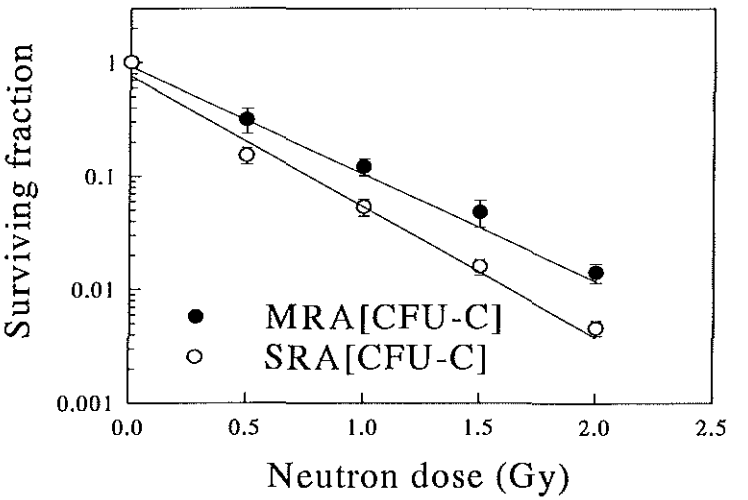

Figure 4.3: Dose response curve for stem cells that repopulate the irradiated bone marrow (MRA[CFU-C]) or spleen (SRA[CFU-C]) with in CFU-C

irradiated with 2 Gy fission neutrons was 0.78 ± 0.02 mm, a reduction of 20% compared to unirradiated CFU-S-7. The effect of fission neutron irradiation on CFU-S-12 colony size was much smaller. Irradiation with 2 Gy fission neutrons decreased the colony size with 5% from 1.56 ± 0.04 mm to 1.48 ± 0.01 mm. In contrast MRA[CFU-S-12] derived colonies were not decreased in size after irradiation of MRA[CFU-S-12] with fission neutrons.

LD_{50}

The radiosensitivity of the hemopoietic system of CBA/H mice as a whole is described by the $LD_{50/30}$ (the dose, which produces 50 percent lethality within 30 days due to anaemia, thrombocytopenia and granulocytopenia). The $LD_{50/30}$ for 11-17 weeks old male CBA mice was 3.41 Gy for fission neutron irradiation and 7.66 Gy for 300 kV X-irradiation. Based on the $LD_{50/30}$ values for the two types of radiation an RBE of 2.3 can be derived for the bone marrow syndrome.

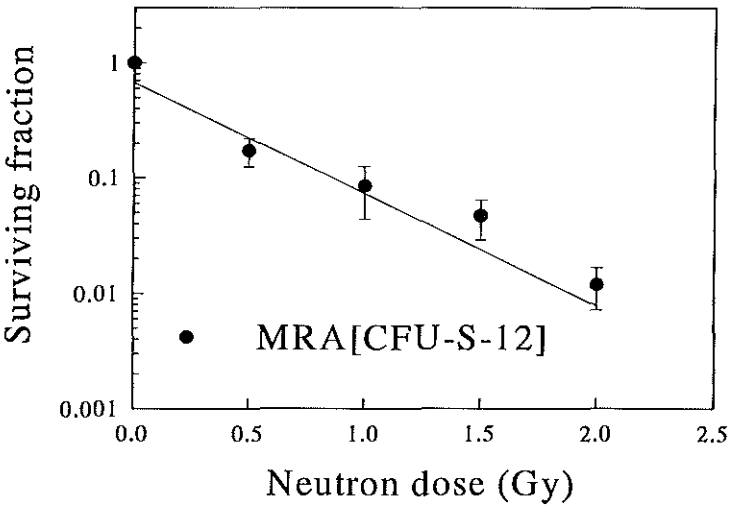

Figure 4.4: Survival curves of stem cells that repopulate the bone marrow of lethally irradiated mice with secondary CFU-S-12 (MRA[CFU-S-12]).

DISCUSSION

Application of bone marrow transplantation requires information on the number and recovery kinetics of hemopoietic stem cells surviving radiation conditioning of the recipient. The increasing insight in the

extensive heterogeneity of the hemopoietic stem cell compartment necessitates data acquisition on the radiosensitivity of the various stem cell subpopulations in order to allow critical evaluation of these parameters. Previous studies using CBA mice (Davids, 1970; Davids, 1972) showed that lethality after whole body irradiation with X-rays below 10 Gy, or 1 MeV fission neutrons below 4 Gy, occurred between 8 and 15 days due to hemopoietic damage. Although the processes leading to death of an animal involve many complex eactions, the susceptibility to hemopoietic death, which is expressed by the $LD_{50/30}$, has been attributed to the number of surviving pluripotent hemopoietic stem cells. The RBE value for the bone marrow syndrome was also found to correspond to the RBE for initial stem cell damage as measured by testing suspensions of irradiated bone marrow cells for their capacity to protect lethally X-irradiated recipients (Davids, 1972). CFU-S have for a long time been considered to be pluripotent stem cells (Till & McCulloch, 1961). Several investigators observed a correlation between CFU-S kinetics and survival (McCulloch & Till, 1964; Yuhas &

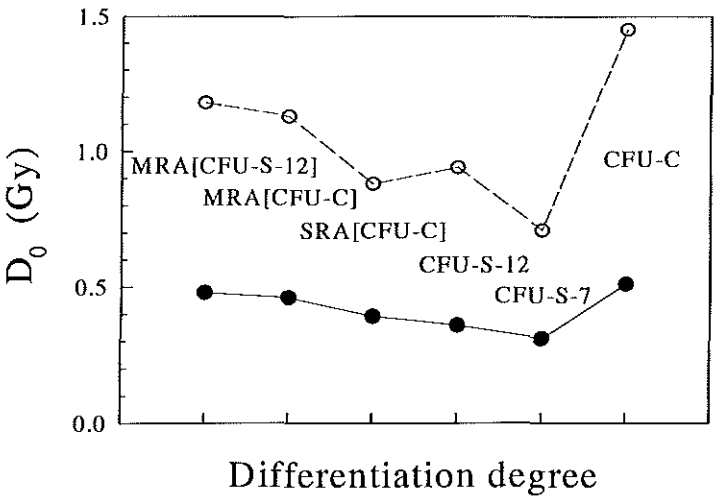

Figure 4.5: Radiosensitivity of different hemopoietic cell populations as a function of differentiation degree. Dotted line shows radiosensitivity for 300 kV X-rays (Meijne et al., 1991).

Storer, 1967; Van Bekkum, 1991), however, other studies did not show such a correlation (Ainsworth et al., 1969; Krebs & Jones, 1972; Yuhas & Storer, 1969; Schofield, 1978). Despite the fact that some experimental findings were inconsistent with the view that spleen colony forming cells (CFU-S) represented the most primitive hemopoietic stem cells, the CFU-S assay remained the most acceptable assay for measuring the pluripotent stem cell until the eighties (Carsten, 1984).

In the last decade it has become clear that the stem cell compartment is heterogeneous and can be divided in at least five subpopulations: LTRA cells, MRA cells, SRA cells, CFU-S-12 and CFU-S-7. These stem cell subpopulations differ with respect to self-renewal capacity, the time required for colony formation in lethally irradiated spleens (Magli et al., 1982) and long-term bone marrow cultures (Ploemacher et al., 1989, 1991, 1993), their

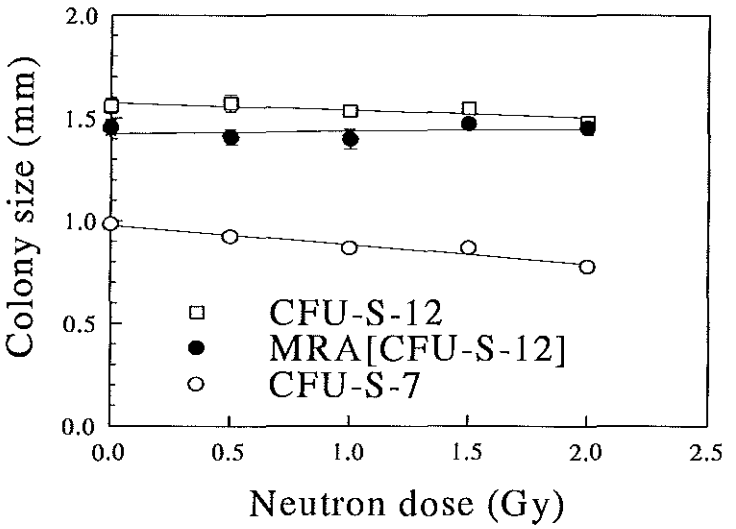

Figure 4.6: Size of spleen colonies produced after injection of normal or fission neutron irradiated bone marrow cells. Each point represents the arithmetic mean (1 SEM) of 39 to 469 colony diameters determined in at least four independent experiments.

capacity to home to either the bone marrow or the spleen (Hodgson & Bradley, 1979), their capacity to generate secondary CFU-S (Bertoncello et al., 1985; Magli et al., 1982; Ploemacher et al., 1993), the ability to rescue recipients from radiation inflicted death and their radiosensitivity for 300 kV X-rays (Meijne et al., 1991) and γ-rays (Ploemacher et al., 1992). Primitive MRA[CFU-S-12] have low, or negligible cycling activity and consequently their capacity to generate new stem cells is greater than that of more mature stem cells (Hodgson & Bradley, 1984, Pietrzyk et al., 1989). As they divide they differentiate into more mature stem cells, that give rise to committed progenitors of one of the hemopoietic differentiation lineages. CFU-S-7, which were the subject of most studies about the radiosensitivity of hemopoietic stem cells, are considered to be the most mature stem cells within the stem cell compartment and are not truely pluripotent and long-term repopulating stem cells (Jones et al., 1990).

In the present investigation we determined the radiosensitivity of four of these stem cell subpopulations for 1 MeV fission neutrons. The results show that the most primitive stem cells identified in this study, the MRA[CFU-S-12], are significantly ($p < 0.001$) more radioresistant to fission neutron irradiation ($D_0 = 0.48$ Gy) than are CFU-S-7 ($D_0 = 0.31$ Gy). When the primitive MRA[CFU-S-12] differentiate into the more mature MRA[CFU-C] their radiosensitivity does not change ($D_0 = 0.46$ Gy), but further differentiation into subsequently SRA[CFU-C], CFU-S-12 and eventually CFU-S-7 does lead to an increase in the radiosensitivity of the stem cells. SRA[CFU-C] are characterized by a D_0 value of 0.37 Gy, CFU-S-12 by a D_0 value of 0.36 Gy whereas CFU-S-7 were determined to have a D_0 value of 0.31 Gy. The D_0 of 0.31 Gy obtained for CFU-S-7 is in agreement with the value of 0.28 Gy reported for 1 MeV neutrons (Carsten et al., 1976). When CFU-S-7 differentiate into committed progenitors of granulocytes and macrophages the radiosensitivity decreases again as is evidenced by the high D_0 value for CFU-C of 0.51 Gy. This increase in radioresistance is probably due to specific changes in the cells caused by differentiation into the granulocytic/macrophage lineage. When stem cells differentiate into the erythroid lineage they are far more radiosensitive to both 1 MeV fission neutrons (BFU-E, $D_0 = 0.16$ Gy) (Schwartz et al., 1986) and X-rays (BFU-E, $D_0 = 0.69$ Gy) (Imai & Nakao, 1987).

The observed differences in radiosensitivity between the various HSC subsets may be due to intrinsic properties of these stem cell populations and/or to their spatial organization in the marrow. With X-rays, cells growing

close to the bone in a mouse femur (the more mature CFU-S-7 (Lord, 1972; Lord & Hendry, 1986) will receive a higher dose due to the emission of secondary short-range electrons from elements in the bone with a high atomic number. Because fission neutrons interact with hydrogen atoms this phenomenon does not occur with fission neutron irradiation. Rather, the observed dose close to the femur shaft may be somewhat lower due to a lower hydrogen content of the bone (Broerse & Barendsen, 1986).

The fact that with both types of radiation MRA[CFU-S-12] are far more radioresistant than CFU-S-7 indicates that differences in absorbed dose are too small to exert a substantial effect. Also differences in SLD repair between stem cell subpopulations, as have been observed after γ-irradiation (Ploemacher et al., 1992; Down et al., 1995), can only partly explain the observed differences in radiosensitivity, since there is almost no repair after fission neutron irradiation (Hall, 1988; Thames & Hendry, 1987). Our data therefore provide further evidence that differences in radiosensitivity between stem cell subpopulations are due to the different intrinsic characteristics of the various stem cell subpopulations and not due to their location in the bone marrow. The difference in SLD repair is not the only factor which accounts for the differences in inherent radiosensitivity between these subpopulations. Also other factors underlie the differences in intrinsic radiosensitivity. For example differential lesion tolerance, lesion repair accuracy and differences in chromatin structure have been considered as factors influencing cell radiosensitivity (Ward, 1990; McKay & Kefford, 1995).

The RBE value for the $LD_{50/30}$ of the CBA/H mice (age 11 - 17 weeks) was 2.3. This value is equal to the RBE value of 2.3 observed for 6 weeks old CBA/H mice (Huiskamp et al., 1986) and somewhat higher than the RBE of 1.9 for CBA/P mice (Davids, 1972). The RBE value for the different hemopoietic cell populations ranged from 2.2 to 2.6 and tended to be higher for the more primitive stem cell populations. The differences were however not significant. The RBE value for the CFU-C was slightly higher (RBE = 2.8).

Two classes of stem cells can be distinguished that are essential for successful long-term hemopoietic reconstitution: mature stem cells and/or committed progenitor cells (CFU-GM, CFU-S), which provide initial transient engraftment and PHSC, which produce delayed, but durable engraftment (Ploemacher & Brons, 1988b; Jones et al., 1990; Ploemacher et

Cell type	neutrons[a] D_0 value (Gy)	Correlation coefficient	X-rays[b] D_0 value (Gy)	Correlation coefficient	RBE[c]
MRA[CFU-S-12]	0.48 ± 0.05	-0.8684	1.18 ± 0.01	-0.9267	2.5 ± 0.3
MRA[CFU-C]	0.46 ± 0.01	-0.9408	1.13 ± 0.08	-0.9147	2.5 ± 0.2
SRA[CFU-C]	0.39 ± 0.01	-0.9745	0.88 ± 0.03	-0.9479	2.3 ± 0.1
MRA[cell]	0.50 ± 0.07	-0.9765	1.12 ± 0.04	-0.9618	2.2 ± 0.1
SRA[cell]	0.37 ± 0.01	-0.9865	0.91 ± 0.03	-0.9773	2.5 ± 0.1
CFU-S-12	0.36 ± 0.01	-0.9918	0.94 ± 0.03	-0.9903	2.6 ± 0.1
CFU-S-7	0.31 ± 0.01	-0.9899	0.71 ± 0.01	-0.9851	2.3 ± 0.1
CFU-C	0.51 ± 0.01	-0.9702	1.45 ± 0.04	-0.9737	2.8 ± 0.1

Table 4.1: Radiobiological characteristics of different hemopoietic cell populations exposed to graded doses of 1 MeV fission neutrons

[a]arithmetic mean ± standard error of the mean (SEM) from three to seven separate experiments.

[b]Data reproduced from Meijne et al., 1991.

[c]RBE values were obtained by dividing D_0 (X-rays) by D_0 (neutrons).

al., 1993). In lethally irradiated hosts, PHSC have to be transplanted together with early engrafting cells, which are capable of producing end cells within 1 or 2 weeks to allow the marrow ablated host to survive initial aplasia. The correlation between the radiosensitivity of CFU-S-7 and stem cells rendering survival at the LD_{50} (Van Bekkum, 1991) can be explained by assuming that transplanted CFU-S allow mice to survive initial aplasia, while endogenous radioresistant PHSC, which survive TBI, are responsible for the long-term hemopoietic reconstitution. The existence of a radioresistant stem cell subpopulation also explains the beneficial effect of supportive care after high doses TBI (MacVittie et al., 1991). Antibiotics, which combat infections, and blood/platelets transfusions help irradiated hosts to survive initial aplasia and allow surviving PHSC more time to produce functional end cells. Moreover growth factors may accelerate the recovery by PHSC (Gale & Butterine, 1990).

In summary our data indicate that there exists a subpopulation of primitive PHSC, which are intrinsically more radioresistant than CFU-S. These PHSC need more time to generate functional end cells than mature stem cells and are therefore not able to rapidly produce enough mature blood cells to allow irradiated hosts to survive the initial aplasia. Their existence, however, offers possibilities for the treatment of victims of radiation accidents with supportive care and without bone marrow transplantation.

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REFERENCES

Ainsworth, E.J., Larsen, R.M. Kendall, K., Leong, G.F., Krebs, J.S. and Mitchell, F.A. Recovery in the mouse after neutron-irradiation: evaluation of injury and recovery using split-dose lethality and repopulation of colony-forming units. Symposium on radiobiology, CONF-691106, pp. 435-449, Oak Ridge, Tennessie, 1969.

Bertoncello, I., Hodgson, G.S. and Bradley,T.R. Multiparameter analys of transplantable hemopoietic stem cells. I. The separation and enrichment of stem cells homing to marrow and spleen on the basis of rhodamine-123 fluorescence. Exp. Hematol. 13, 999-1006 (1985).

Broerse, J.J. and Barendsen, G.W. The importance of secondary particle equilibrium for neutron irradiations of cultured cells and intact organs. In Proceedings symposium on microdosimetry, Ispra, 1967 (H.G. Ebert, Eds.), Euratom, Brussels, EUR 3747, pp. 353-383, 1968.

Broerse, J.J. and Barendsen, G.W. Relative biological effectiveness of fast neutrons for effects on normal tissues. Curr. Top. Radiat. Res. Q. 8, 305-350 (1973).

Broerse, J.J. Influence of physical factors on radiation carcinogenesis in experimental animals. In: Low dose radiation. Biological bases of risk assessment (Baverstock, K.F. and Stather, J.W., Eds.), pp. 181-194, Taylor & Francis, London, 1989.

Carsten, A.L., Bond, V.P. and Thompson, K. The rbe of different energy neutrons as measured by the haemopoietic spleen-colony technique. Int. J. Radiat. Biol. 29, 65-70 (1976).

Carsten, A.L. Acute lethality. The hemopoietic syndrome in different species. In Response of different species to total body irradiation (J.J. Broerse, and T.J. MacVittie, Eds.) pp. 59-86. Martinus Nijhoff Publishers, Dordrecht, The Netherlands, 1984.

Handbook of histopathological and histochemical techniques. Third edition. Ed: Culling, C.F.A., Redwood Burn Limited, Trowbridge, p. 46 (1974).

Davids, J.A.G., Mos, A.P.J. and Oude, A. de. Fast-neutron facility for biological exposures in and Argonaut reactor: Design, tissue dosimetry and neutron spectrometry. Phys. Med. Biol. 14, 573-584 (1969)

Davids, J.A.G. Bone marrow syndrome in CBA mice exposed to fast neutrons of 1.0 MeV mean energy. Effect of syngeneic bone marrow transplantation. Int. J. Radiat. Biol. 17, 173-185 (1970).

Davids, J.A.G. Application of a fast-neutron facility in an Argonaut reactor for radiobiological research. In Radiobiological applications of neutron irradiation, pp. 27-40. International Atomic Energy Agency, Vienna, 1972.

Down, J.D., Boudewijn, A., Van Os, R, Thames, H.D. and Ploemacher, R.E. Variations in radiation sensitivity and repair among different hemopoietic stem cell subsets following fractionated irradiation. Blood 86, 122-127 (1995).

Gale, R.P. and Butterine, A. The role of hematopoietic growth factors in nuclear and radiation accidents. Exp. Hematol. 18, 958-964 (1990).

Hall, E.J., Novak, J.K., Kellerer, A.M., Rossi, H.H., Marino, S. and Goodman, J. RBE as a function of neutron energy. I. Experimental observations. Radiat. Res. 64, 245-255 (1975).

Hall, E.J. Radiobiology for the radiologist, 3rd ed., pp. 108-110. Lippincott, Philadelphia, 1988.

Hendry, J.H. and Lord, B.I. The analysis of the early and late response to cytotoxic insults in the hematopoietic cell hierarchy. In: Cytotoxic insult to tissue (Potten, C.S. and Hendry, J.H., Eds.), pp. 1-66. Churchill Livingstone, Edinburgh, 1983.

Hodgson, G.S. and Bradley, T.R. Properties of haemopoietic stem cells surviving 5-fluorouracil treatment: evidence for a pre-CFU-S cell? Nature 281, 381-382 (1979).

Hodgson, G.S. Bradley, T.R. and Radley, J.M. The organization of hemopoietic tissues as inferred from the effects of 5-fluorouracil. Exp. Hematol. 10, 26-35 (1982).

Hodgson, G.S. and Bradley, T.R. In vivo kinetic status of hematopoietic stem and progenitor cells as inferred from labelling with bromodeoxyuridine. Exp. Hematol. 12, 683-687 (1984).

Huiskamp, R., Davids, J.A.G. and Ewijk, W. van. The effect of graded doses of fission neutrons or X-rays on the lymphoid compartment of the thymus in mice. Radiat. Res. 105, 247-258 (1986).

Imai, Y. and Nakao, I. In vivo radiosensitivity and recovery pattern of the hemopoietic precursor cells and stem cells in mouse bone marrow. Exp. Hematol. 15, 890-895 (1987).

Jones, R.J. Wagner, J.E., Celano, P., Zicha, M.S. and Sharkis, S.J. Separation of pluripotent stem cells from spleen colony-forming cells. Nature 347, 188-189 (1990).

Jordan, C.T. and Lemischka, I.R. Clonal and systemic analysis of long-term hematopoiesis in the mouse. Genes Develop. 4, 220-232 (1990).

Krebs, J.S. and Jones, D.C. The LD_{50} and the survival of bone marrow colony forming cells in mice: Effect of rate of exposure to ionizing radiation. Radiat. Res. 51, 374-380 (1972).

Lord, B.I. and Hendry, J.H. The distribution of haemopoietic colony-forming units in the mouse femur, and its modification by X-rays. Br. J. Radiol. 45, 110-115 (1972)

Lord, B.I. The sensitivity of G_0-state haemopoietic spleen colony-forming cells to a stimulus for proliferation. Cell Tissue Kinet. 19, 305-310 (1986).

MacVittie, T.J., Monroy, R., Vigneulle, R.M., Zeman, G.H. and Jackson, W.E. The relative biological effectiveness of mixed fission-neutron-g radiation on the hematopoietic syndrome in the canine: Effect of therapy on survival. Radiat. Res. 128, 29-36 (1991).

Magli, M.C., Iscove, N.N. and Odartchenko, N. Transient nature of early haemopoietic spleen colonies. Nature 295, 527-529 (1982).

McCulloch, E.A. and Till, J.E. Proliferation of hemopoietic colony-forming cells transplanted into irradiated mice. Radiat. Res. 22, 383-397 (1964).

McKay, M.J. and Kefford, R.F. The spectrum of in vitro radiosensitivity in four human melanoma cell lines is not accounted for by differential induction or rejoining of DNA double strand breaks. Int. J. Radiat. Oncol. Biol. Phys. 31, 345-352 (1995).

Meijne, E.I.M., Winden - van Groenewegen, A.J.M. van der, Ploemacher, R.E., Vos, O., Davids, J.A.G. and Huiskamp, R. The effects of X-irradiation on hematopoietic stem cell compartments in the mouse. Exp. Hematol. 19, 617-623 (1991).

Mulder, A.H. and Visser, J.W.M. Separation and functional analysis of bone marrow cells separated by rhodamine fluorescence. Exp. Hematol. 15, 99-104 (1987).

Pietrzyk, M.E., Wolf, N.S. and Priestley, G.V. Cycling patterns of hemopoietic stem cell subpopulations in young and old BDF1 mice. Mech. Ageing Dev. 49, 79-86 (1989).

Ploemacher, R.E., Brons, N.H.C. and Leenen, P.J.M. Bulk enrichment of transplantable hemopoietic stem cell subsets from lipopolysaccharide-stimulated murine spleen. Exp. Hematol. 15, 154-162 (1987).

Ploemacher, R.E. and Brons, N.H.C. Cells with marrow and spleen repopulating ability, and cells forming spleen colonies on day 16, 12 and 8 are sequentially ordered on the basis of increasing rhodamine 123 retention. J. Cell. Physiol. 36, 531-536 (1988a).

Ploemacher, R.E. and Brons, N.H.C. Isolation of hemopoietic stem cell subsets from murine bone marrow. II. Evidence for an early precursor of day-12 CFU-S and cells associated with radioprotective ability. Exp. Hematol. 16, 27-32 (1988b).

Ploemacher, R.E. and Brons, N.H.C. Separation of CFU-S from primitive cells responsible for reconstitution of the bone marrow hemopoietic stem cell compartment following irradiation: evidence for a pre-CFU-S cell. Exp. Hematol. 17, 263-266 (1989).

Ploemacher, R.E., Van Os, R, Van Beurden, C.A.J. and Down, J.D. Murine hemopoietic stem cells with long-term engraftment and marrow repopulating ability are less radiosensitive to gamma radiation than are spleen colony forming cells. Int. J. Radiat. Biol. 61: 489-499 (1992).

Ploemacher, R.E., Van der Loo, J.C.M. , Van Beurden, C.A.J. and Baert, M.R.M. Wheat germ agglutinin affinity of murine hemopoietic stem cell subpopulations is an inverse function of their long-term repopulating ability in vitro and in vivo. Leukemia 7, 120-130 (1993).

Rosendaal, M., Hodgson, G.S. and Bradley, T.R. Organization of haemopoietic stem cells. The generation age hypothesis. Cell Tissue Kinet. 12, 17-29 (1979).

Schofield, R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells 426, 7-25 (1978).

Schwartz, G.N., Vigneulle, R.M. and MacVittie, T.J. Survival of erythroid burst-forming units and erythroid colony-forming units in canine bone marrow cells exposed in vitro to 1 MeV neutron radiation or X rays. Radiat. Res. 108, 336-347 (1986).

Fractionation in radiotherapy. Eds: Thames, H.D. and Hendry, J.H., London - New York - Philadelphia, pp. 53-99 (1987).

Till, J.E. and McCulloch, E.A. A direct measurement of the radiosensitivity of normal bone marrow cells. Radiat. Res. 14, 213-222 (1961).

Van Bekkum, D.W. Radiation sensitivity of the hemopoietic stem cell. Radiat. Res. 128, S4-S8 (1991).

Visser, J.W.M., Hogeweg, M.G.C., Vries, P. de, Bayer, J. and Ploemacher, R.E. Culture of purified pluripotent haemopoietic cells. In The biology of hematopoiesis (N. Dainiak, E.P. Cronkite, R. McCaffrey, and R.K. Shadduck, Eds.). pp. 1-8, Wiley-Liss Inc., New York, 1990.

Ward, J.F. The yield of DNA double-strand breaks produced intracellularly by ionizing radiation: a review. Int. J. Radiat. Biol. 57, 1141-1150 (1990)

Yuhas, J.M. and Storer, J.P. The effect of age on two modes of radiation death and on hemopoietic cell survival in mouse. Radiat. Res. 32, 596-606 (1967).

Yuhas, J.M. and Storer, J.B. On mouse strain differences in radiation resistance: Hematopoietic death and the endogenous colony-forming unit. Radiat. Res. 39, 608-622 (1969).

THE SENSITIVITY OF MURINE HEMOPOIETIC STEM CELL POPULATIONS TO X-RAYS AND 1 MEV FISSION NEUTRONS IN VITRO, AND UNDER HYPOXIC CONDITIONS IN VIVO

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ABSTRACT

The radiosensitivity of primitive hemopoietic stem cells, which 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. With 1 MeV fission neutron irradiation no significant difference in radiosensitivity of the cell populations is observed between normal in vivo irradiation and in vitro irradiation. With 300 kV X-rays a smallt decrease in radiosensitivity for all three cell populations is observed after in vitro irradiation compared to in vivo irradiation under normal oxic conditions. This is probably caused by slightly hypoxic conditions in the bone marrow cell suspensions during the in vitro irradiations. In vivo irradiation of MRA[CFU-C], CFU-S-7 and CFU-C with fission neutrons under hypoxic conditions led to a small decrease in radiosensitivity compared to in vivo irradiation under normal oxic conditions. The Oxygen Enhancement Ratio for fission neutrons (OER[neutrons]) varied from 1.2 for MRA[CFU-C] to 1.5 for CFU-C. After in vivo irradiation with 300 kV X-rays much higher OER values (OER[X-rays]) were observed. An OER of 1.8 was obtained for CFU-S and for MRA[CFU-C] and CFU-C an OER value of approximately 3 was observed. These data indicate that differences in oxygenation or in local dose due to disturbance of secondary particle equilibrium are not responsible for the observed differences in radiosensitivity between primitive stem cells (MRA[CFU-C]) and CFU-S after in vivo irradiation with X-rays.

INTRODUCTION

In a previous study we determined the radiosensitivity of various hemopoietic 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., 1996a). Primitive hemopoietic 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 & Bradley, 1979; Ploemacher & Brons, 1989) were found to be more resistant to ionizing irradiation than more mature stem

cells (CFU-S) (Meijne et al., 1991; Ploemacher et al., 1992). 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 tissues in the mammalian body may lead to inhomogeneities in dose-distribution, especially at the bone-soft tissue interface (Broerse & Barendsen, 1968a). Dependent on their location in the bone marrow some cell populations may therefore absorb a higher or lower dose than other cell populations. Another factor which may influence the observed radiosensitivity of the hemopoietic cell populations are local differences in oxygen tension (Broerse et al., 1968b; Allalunis et al., 1983; Allalunis-Turner & Chapman, 1986). To investigate to what extent these two factors influence the in vivo radiosensitivity of hemopoietic stem cell and progenitor cell populations we irradiated bone marrow cells in vitro and under hypoxic conditions in vivo.

MATERIALS AND METHODS

Mice

nbred 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 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 at the age of 20 weeks or more were used as recipients in the CFU-S assays and female mice aged 14-20 weeks were used as recipient in the MRA assays.

The experiments were conducted with permission of the experimental animal welfare commission (DEC) of the Netherlands Energy Research Foundation, as required by Dutch law.

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% gamma-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.

Whole body X-irradiation 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 & Blackett, 1981) mice were killed by CO_2 gassing and subsequently kept at room temperature for 12 minutes before starting the X-irradiations, or 30 minutes, 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 minutes. Immediately after irradiation both femurs were excised and placed in ice-cold Hanks (Gibco). In vitro irradiation 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.

Hemopoietic cell suspensions

Mice were killed with CO_2. 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 transferred to a Falcon tube and the larger bone particles were allowed to settle for 45 sec. The supernatant was sieved through a nylon filter (pore size 100 μm) and the cellularity determined with a Coulter counter.

CFU-S assay

CFU-S were assayed according to Till and McCulloch (Till et al., 1961). Recipient mice received 9 Gy of X-rays 1- 4 hours 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 of 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 10x magnification and their diameters measured with an eye piece micrometer.

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 x 10^4 to 1.5 x 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.

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 hemopoietic 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.

Experimental procedures and statistics

Statistical comparison of D_0 values was performed with a Chi-square test. Log-linear dose effect curves were obtained by least squares regression analysis. D_0 was obtained from the slope of these curves. The D_0 values 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.

RESULTS

In vitro radiosensitivity of hemopoietic cell populations

The survival curves for MRA[CFU-C], CFU-S-7 and CFU-C irradiated in vitro and in vivo with graded doses of 1 MeV fission neutrons or X-rays are shown in Figures 5.1, 5.2 and 5.3, respectively. From these survival curves D_0 values were calculated. The D_0 values are summarized in Table 5.1. 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 value decreased from 0.46 ± 0.01 Gy for in vitro irradiation to 0.40 ± 0.04 Gy for in vivo irradiation. This decrease in D_0 value 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. D_0 values obtained after in vitro irradiation of MRA[CFU-C], CFU-S-7 and CFU-C with 300 kV X-rays were higher for all three cell populations. The D_0 value of 1.25 Gy observed after in vitro irradiation of MRA[CFU-C] was 13% higher than the D_0 value of 1.11 Gy observed after in vivo irradiation. The D_0 values 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 the primitive MRA[CFU-C] stem cells were more resistant to ionizing irradiation 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.

Radiosensitivity of hemopoietic cell populations under hypoxic conditions.

The survival curves for MRA[CFU-C], CFU-S-7 and CFU-C irradiated in vivo under hypoxic condition are shown in Figures 5.4, 5.5 and 5.6. Total body irradiation (TBI) with 1 MeV fission neutrons under hypoxic condition led to a significant increase of D_0 values ($p < 0.001$) of all three cell populations. The D_0 value obtained for 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. Also the D_0 values for CFU-S-7 and CFU-C were higher after fission neutron irradiation under hypoxic

conditions compared to the normal in vivo irradiation. The D_0 value for CFU-S-7 increased from 0.31 ± 0.01 Gy for normal in vivo irradiation to

	D_0 value after in vivo irradiation (Gy)	D_0 value 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

Table 5.1: Radiobiological characteristics of hemopoietic stem and progenitor cell populations irradiated in vivo under normal conditions or in vitro with X-rays or 1 MeV fission neutrons.

	D_0 value in normal (air-breathing) mice (Gy)	D_0 value in hypoxic (asphyxiated) mice (Gy)	Oxygen Enhancement Ratio
1 MeV fission neutrons			
MRA[CFU-C]	0.46 ± 0.01	0.55 ± 0.02	1.2
CFU-S-7	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 ± 0.03	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

Table 5.2: Radiobiological characteristics of hemopoietic stem and progenitor cell populations irradiated in vivo under normal or under hypoxic conditions with X-rays or 1 MeV fission neutrons.

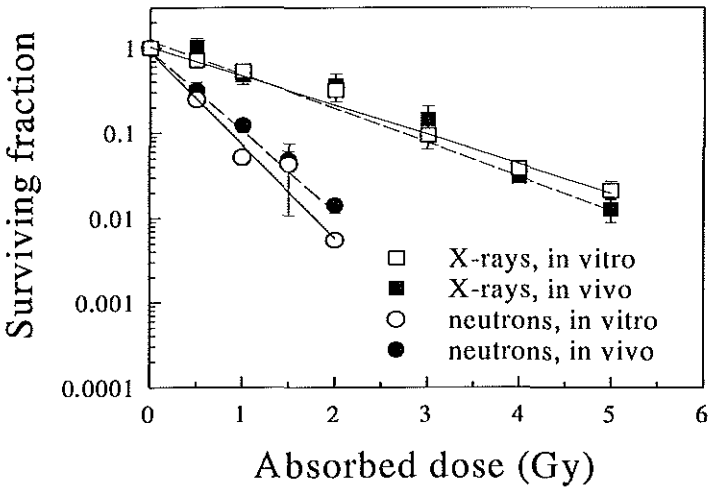

Figure 5.1: Dose response curves for MRA[CFU-C] irradiated in vitro (solid lines) or in vivo (dotted lines) with X-rays or fission neutrons.

0.43 ± 0.01 Gy for irradiation under hypoxic conditions. The D_0 values obtained for CFU-C were 0.51 ± 0.01 Gy and 0.74 ± 0.03 Gy for irradiation under normal in vivo and hypoxic conditions, respectively. The Oxygen Enhancement Ratio (OER) calculated for the three cell populations after fission neutron irradiation were small and ranged from 1.2 for MRA[CFU-C] to 1.5 for CFU-C.

Irradiation of the three hemopoietic 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 value 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 value for CFU-C increased from 1.50 ± 0.01 Gy to 4.33 ± 0.27 Gy. The calculated OER values 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 somewhat lower. The D_0 value increased from

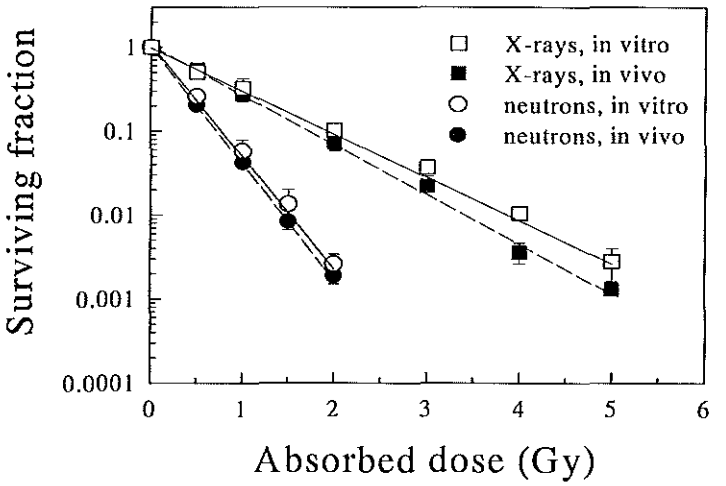

Figure 5.2: Dose response curves for CFU-S-7 irradiated in vitro (solid lines) or in vivo (dotted lines) with X-rays or fission neutrons.

0.74 ± 0.01 Gy for normal in vivo irradiation to 1.33 ± 0.07 Gy for irradiation under hypoxic conditions. The calculated OER value was 1.8. The results are summurized in Table 5.2.

DISCUSSION

In the present study we determined the radiosensitivity of hemopoietic stem cell and progenitor cell populations in vitro and in vivo under normal and hypoxic conditions in order to determine the effects of local differences in absorbed dose and oxygen tension on the radiosensitivity of hemopoietic cell populations in normal air breathing mice. 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

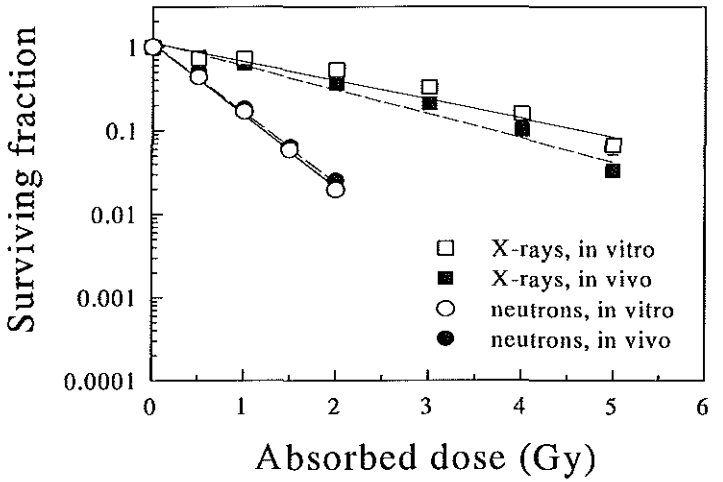

Figure 5.3: Dose response curves for CFU-C irradiated in vitro (solid lines) or in vivo (dotted lines) with X-rays or fission neutrons.

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 to the femur shaft than in the centre of the marrow spaces (Lord et al., 1975). 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 location of the MRA[CFU-C] has not yet been determined. Because they are primitive hemopoietic stem cells with negligible cycling activity, as evidenced by a low sensitivity for 5-FU (Hodgson & Bradley, 1979) and a low uptake of Rh123 (Ploemacher & Brons, 1988) it is more likely that they are located near the centre of the bone marrow, close to the primitive CFU-S, than close to the bone, where the mature CFU-S reside. Therefore we assumed that the MRA[CFU-C] are located near the centre of

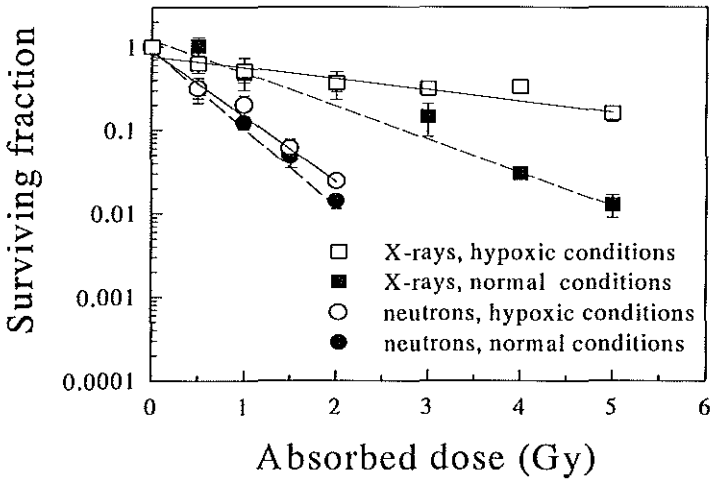

Figure 5.4: Dose response curves for MRA[CFU-C] irradiated in vivo under hypoxic (solid lines) or normal oxic conditions (dotted lines) with X-rays or fission neutrons.

the femur.

The above described variations in the spatial distribution of the various cell populations causes 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 & Barendsen, 1968). The range of recoil protons produced by 15 MeV neutrons is on average larger than 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 & Barendsen, 1968). 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

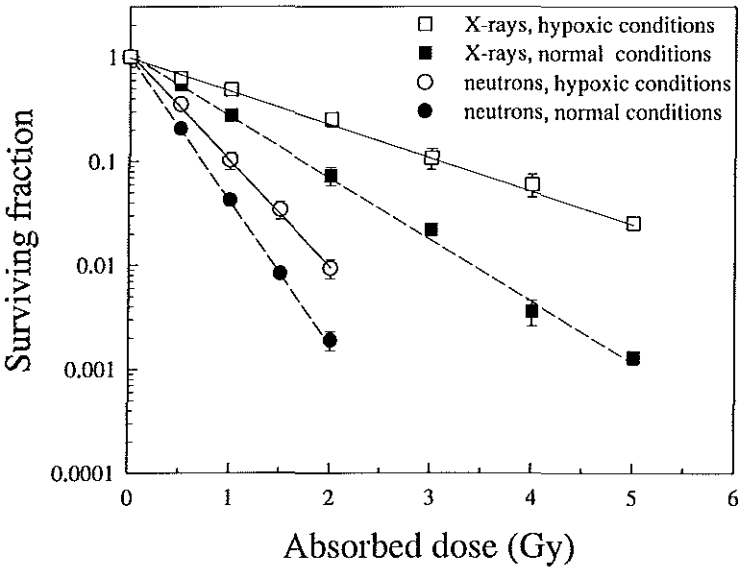

Figure 5.5: Dose response curve for CFU-S irradiated in vivo under hypoxic (solid lines) or normal oxic conditions (dotted lines) with X-rays or fission neutrons.

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 slightly lower dose compared to CFU-S irradiated in vitro. The D_0 value observed after in vitro irradiation of CFU-S was however not significant different form the D_0 value 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. The large differences in marginal and axial CFU-S concentration observed by Lord (1985) have not been observed by other authors (Maloney et al., 1978).

When mice are exposed to X-irradiation, the bone marrow cells close to the femur shaft receive a relatively higher ose owing to an excess of

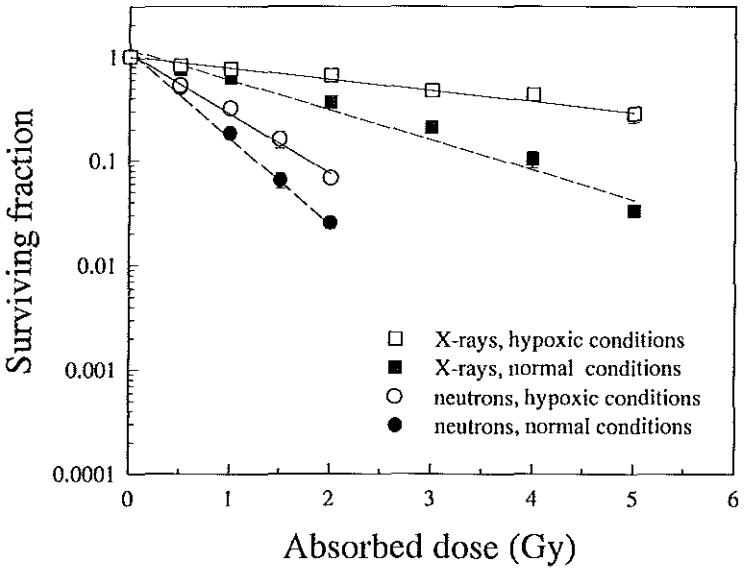

Figure 5.6: Dose response curve for CFU-C irradiated in vivo under hypoxic (solid lines) or normal oxic conditions (dotted lines) with X-rays or fission neutrons.

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 be 17% higher 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 (Broerse & Barendsen, 1968). For 300 kV X-rays identical percentages might be expected. The radiosensitivity of the hemopoietic cell populations irradiated in vitro in suspension was indeed lower (higher D_0 values) compared to cells irradiated within the animal. Surprisingly the increase in D_0 value 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 larger increase in D_0 value for CFU-S-7 irradiated in vitro would be expected. The OER for CFU-S-7 is however higher 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 is however higher than expected based on microdosimetric factors. Possibly more cells survived irradiation in vitro because the cells in suspension 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 influenced by the oxygenation level of the tissue. The OER values 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 value of 1.5 observed for human kidney cells after 1 MeV fission neutron irradiation (Broerse et al., 1968b). The OER[X-rays] values 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 locations in femora where the CFU-S-7 reside lower is than the oxygenation level at the positions of MRA[CFU-C] and CFU-C, since hypoxia increased the survival of CFU-S less than the survival of MRA[CFU-C] and CFU-C. The OER[X-rays] value of 1.8 for CFU-S is lower than the OER[γ-rays] of 2.4 observed by Millard and Blackett (1981) for CFU-S irradiated with γ-rays. Also the OER[X-rays] of 2.7 observed by Broerse & Barendsen (1973) after in vitro irradiation of CFU-S-9 was higher. The OER value of 2 observed by Blackett et al. (1974) and Hendry and Howard (1972) was however similar. On the other hand the OER[X-rays] value of 2.9 observed for CFU-C was higher than the OER[γ-rays] of 2.1 (Allalunis et al., 1983) and 1.3 (Millard & Blackett, 1981) observed for agar colony-forming units. It is however difficult to compare the D_0 and OER values of various investigators. Differences in mice strain, radiation source and conditions, days of colony culture, culture medium, colony stimulating factors used (Baird et al., 1990) and the level of hypoxia achieved may cause large differences in the observed radiosensitivity. Therefore it is very important to compare the radiosensitivity of different cell populations within one mice strain and within one institute.

In summary our data show that the radioresistance of primitive stem cells (MRA[CFU-C]) is not caused by their localization in the bone or by

hypoxia. Therefore intrinsic factors have to be responsible for the differences in radiosensitivity of the various stem cell subsets.

REFERENCES

Allalunis, M.J., Chapman. J.D. and Turner, A.R., Identification of a hypoxic population of bone marrow cells. Int. J. of Radiat. Oncol. Biol. and Phys. 9, 227-232 (1983).

Allalunis-Turner, J. and Chapman, J.D. The in vitro sensitivities to radiation and misonidazole of mouse bone marrow cells derived from different microenvironments. Int. J. Radiat. Biol. 49, 415-422 (1986).

Baird, M. C., Hendry, J. H. and Testa, N. G. Radiosensitivity increases with differentiation status of murine hemopoietic progenitor cells selected using enriched marrow subpopulations and recombinant growth factors. Radiat. Res. 123, 292-298 (1990).

Blackett, N. M., Wooliscroft, W. E., Fielden, E. M. and Lillicrap, S. C. Radiation modifying effect of the free radical norpseudopelletierene-N-Oxyl on normal bone marrow stem cells in vitro and in vivo. Radiat. Res. 58, 361-372 (1974).

Broerse, J.J. and Barendsen, G.W. The importance of secondary particle equilibrium for neutron irradiations of cultured cells and intact organisms. In: Proceedings of the symposium on microdosimetry, Ispra, Italy, 13-15 november, 1967. Edited by: H. G. Ebert. p. 353-383 (European Communities, Brussels) (1968a).

Broerse, J.J., Barendsen, G.W. and van Kersen, G.R. Survival of cultured human cells after irradiation with fast neutrons of different energies in hypoxic and oxygenated conditions. Int. J. Radiat. Biol. 13, 559-572 (1986b).

Broerse, J. J. and Barendsen, G. W. Relative biological effectiveness of fast neutrons for effects on normal tissues. Current Topics in Radiat. Res. Quart. 8, 305-350 (1973).

Davids, J. A. G., Mos, A. P. J. and de Oude, A. Fast-neutron facility for biological exposures in an Argonaut reactor: Design, tissue dosimetry and neutron spectrometry. Phys. Med. Biol. 14, 573-584 (1969).

Davids, J. A. G. Bone marrow syndrome in CBA mice exposed to fast neutrons of 1.0 MeV mean energy. Effect of syngeneic bone marrow transplantation. Int. J. Radiat. Biol. 17, 173-185 (1970).

Hendry, J. H. and Howard, A. The effect of anoxia on the response of haemopoietic colony-forming units in different proliferative states to γ-rays or D-T neutrons. Brit. J. Radiol. 45, 933-937 (1972).

Hodgson, G.S. and Bradley, T.R. Properties of haemopoietic stem cells surviving 5-fluorouracil treatment: evidence for a pre-CFU-S cell? Nature, 281, 381-382 (1979).

Hodgson, G. S., Bradley, T. R. and Radley, J. M. The organization of hemopoietic tissues as inferred from the effects of 5-fluorouracil. Exp. Hematol, 10, 26-35 (1982).

Lord, B. I., Testa, N. G. and Hendry, J. H. The relative spatial distribution of CFU_s and CFU_c in the normal mouse femur. Blood, 46, 65-72 (1975).

Lord, B. I. The sensitivity; of G_0-state haemopoietic spleen colony-forming cells to a stimulus for proliferation. Cell Tissue Kinet. 19, 305-310 (1986).

Maloney, M. A., Lamela, R. A., Dorie, M. J. and Patt, H. M. Concentration gradient of blood stem cells in mouse bone marrow - An open question. Blood, 521-525 (1978).

Meijne, E. I. M., van der Winden-van Groenewegen, R. J. M., Ploemacher, R. E., Vos, O., Davids, J. A. G. and Huiskamp, R. The effects of X-irradiation on hematopoietic stem cell compartments in the mouse. Exp. Hematol. 19, 617-623 (1991).

Meijne, E.I.M., Ploemacher, R.E. and Huiskamp, R. The sensitivity of murine hemopoietic stem cell populations for 1 MeV fission neutrons. Radiat. Res. Submitted for publication (1996a).

Millard, R. E. and Blackett, N. M. Radiosensitivity and recovery of two murine haemopoietic progenitor cell populations following gamma rays and neutrons. Acta Haematol. 66, 226-232 (1981).

Ploemacher, R. E. and Brons, N. H. C. Cells with marrow and spleen repopulating ability, and cells forming spleen colonies on day 16, 12 and 8 are sequentially ordered on the basis of increasing rhodamine 123 retention. J. Cell. Physiol. 36, 531-536 (1988).

Ploemacher, R. E. and Brons, N. H. C. Separation of CFU-S from primitive cells responsible for reconstitution of the bone marrow hemopoietic stem cell compartment following irradiation: evidence of a pre-CFU-S cell. Exp. Hematol. 17, 263-266 (1989).

Ploemacher, R.E., Van Os, R, Van Beurden, C.A.J. and Down, J.D. Murine hemopoietic stem cells with long-term engraftment and marrow repopulating ability are less radiosensitive to gamma radiation than are spleen colony forming cells. Int. J. Radiat. Biol. 61, 489-499 (1992).

Till, J. E. and McCulloch, E. A. A direct measurement of the radiation sensitivity of normal bone marrow cells. Radiat. Res. 14, 213-222 (1961).

THE EFFECTS OF SPLIT-DOSE X-RAY AND FISSION NEUTRON IRRADIATION ON DIFFERENT HEMOPOIETIC STEM CELL POPULATIONS

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ABSTRACT

The effect of split dose fast fission neutron and X-irradiation, separated by a 6 hours interval, on the radiosensitivity of primitive and mature hemopoietic stem cells was investigated. Split dose X-irradiation had a small sparing effect on the observed radiosensitivity of all cell populations investigated. This sparing effect was not observed after split dose neutron irradiation, thus excluding the contribution of repopulation. Delaying the assay of the bone marrow cells 24 hours did not change the sparing effect observed after X-irradiation. Decreasing the X-ray dose rate from 30 cGy/min to 5 cGy/min did not reveal a dose sparing effect for any of these cell types.

These data indicate that the difference in split-dose recovery between the different cell populations, as have been observed after γ-irradiation, is not the only factor responsible for the differences in radiosensitivity between these cell populations.

INTRODUCTION

The first quantitative assay for mouse hemopoietic stem cells, the spleen colony forming unit assay (CFU-S) was described by Till & McCulloch (1961). Since that time evidence has accumulated to indicate that the stem cell compartment is heterogeneous and can be divided in CFU-S giving rise to spleen colonies 7 to 9 days after bone marrow transplantation (CFU-S-7/9), CFU-S giving rise to colonies 11 to 13 days after bone marrow transplantation (CFU-S-11/13), cells with marrow repopulating ability (MRA) and cell responsible for long-term repopulating ability (LTRA) (Bertoncello et al., 1985; Ploemacher et al., 1988, 1993; Visser et al., 1990; Jones et al. , 1990). Long-term repopulation of irradiated recipients can be achieved with sorted cell fractions that exclude the majority of CFU-S (Ploemacher et al., 1989; Jones et al., 1990; Van der Loo, 1994), indicating that CFU-S are not a measure for pluripotent hemopoietic stem cells (PHSC). The former are however necessary for protection of lethally irradiated mice from hemopoietic death (Jones et al., 1990), indicating that both cell populations are important for bone marrow transplantation. The different hemopoietic stem cell populations have been shown to differ in their radiosensitivity in vivo for fission neutrons (Meijne et al., 1996a), X-rays

(Meijne et al., 1991) and γ-rays (Ploemacher et al., 1992). The observed differences in radiosensitivity could not be explained by differences in oxygen tension or local differences in absorbed dose due to secondary short-range electron emission from elements with a high atomic number in the bone (Meijne et al., 1996b). On the other hand, a higher split dose recovery was observed for the more radioresistant primitive MRA stem cells after γ-irradiation, indicating differences in early intracellular repair processes between the stem cell populations (Ploemacher et al., 1992). More extensive fractionation studies using the in vitro cobblestone forming cell (CAFC) assay also showed greater recovery in cell survival with increasing fraction number for primitive CAFC day-28 (MRA equivalent) and CAFC day-35 (LTRA equivalent) than for CAFC day-6 and CAFC day-12 (CFU-C and CFU-S-12 equivalents) (Down et al., 1995). An appreciable radiation dose-fractionation effect was also observed for long-term erythroid chimerism in a murine bone marrow transplantation model (Van Os et al., 1993). All these results indicate that primitive hemopoietic stem cells have a higher capacity to repair sublethal damage than the more mature CFU-S and colony forming cells in culture (CFU-C). However. these data are at variance with the observed differences in radiosensitivity after fission neutron irradiation and the almost exponential survival curves with very small or no shoulders, which would not predict the split-dose recovery observed after γ-irradiation. The split-dose experiments with γ-rays were carried out using a delayed assay. Survival of the different cell populations was assayed 24 hours after the first radiation dose to allow for any potential lethal damage to occur in situ (Thomas & Gould, 1982). Also in the more comprehensive fractionation studies with the CAFC assay, irradiated bone marrow was harvested at least 20 hours after the end of radiation treatment. Delaying the assay of irradiated marrow causes an increasing loss of CFU-S up to 16 hours after irradiation (Hendry & Howard, 1971). If this loss is different for the various cell populations it might influence the recovery of the various cell populations during split dose and thus the cell survival data.

In this study we determined the radiosensitivity of MRA, CFU-S-12, CFU-S-7 and in vitro clonable progenitors (CFU-C) after split dose irradiation with X-rays and fission neutrons using a direct assay of irradiated bone marrow cells and a delayed assay following single and split dose irradiation with X-rays. We also investigated the effect of dose protraction on the radiosensitivity of hemopoietic cells for X-rays.

MATERIALS AND METHODS

Animals

Inbred CBA/H mice (H-2k), bred at the Netherlands Energy Research Foundation, Petten, The Netherlands, were used throughout the experiments. 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 at the age of 12-14 weeks. Male or female mice at the age of >20 weeks or more were used as recipients in the CFU-S assays. In each experiment, all the recipients used were of the same sex.

Irradiation procedure

X-irradiations were performed with a Philips Müller X-ray tube, operating at 300 kV constant potential and 5 mA (HVL 2.1 mm Cu). The distance from the focus to the centre-line of the animals was 69 cm. Whole-body neutron irradiations were performed with fast fission neutrons from a ^{235}U-converter in the Low Flux Reactor at Petten as described previously (Davids et al., 1969). The mean energy of the neutron spectrum was 1 MeV.

Donor mice were irradiated with graded single doses of X-rays (30 cGy/min) or fission neutrons (10 cGy/min), or two doses separated by a 6 hours time interval. Femoral marrow was grafted from these mice into lethally irradiated (9 Gy X-rays) recipients immediately after the single doses, or immediately after the second of two doses.

The effect of delaying the assay was investigated in a second set of experiments. In this case bone marrow was removed from the femurs 24 hours after the first dose of X-rays.

Low dose rate X-irradiations (5 cGy/min) were performed by increasing the distance from the focus to the centre-line of the animals from 69 cm to 169 cm. Transplantation of bone marrow cells into lethally irradiated hosts was always performed within 4 hours after irradiation of the recipients.

Hemopoietic cell suspensions

After irradiation both femurs were excised from the donor mice and put on ice. 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), 100 IU/ml penicillin and 100 mg/ml streptomycin per femur.

Cell suspensions were put in Falcon tubes and the larger bone particles were allowed to settle for 45 sec. The supernatant was sieved through a nylon filter (pore size 100 μm).

CFU-S assay

CFU-S were assayed according to Till and McCulloch (1961). Recipient mice received 9 Gy 1-4 hours before injection of cell suspensions. This dose reduced endogenous CFU-S-7 to 0 and CFU-S-12 to < 0.04 colony per spleen. Cell suspensions were always made from at least three donor mice. Ten mice were used as recipient for the CFU-S-7 and CFU-S-12 assay. Because bone marrow cellularity decreases after irradiation in a dose dependent manner recipient mice were injected with femur equivalents instead of a number of bone marrow cells. Based on previous studies in which the radiosensitivity of the different cell populations was determined after in vivo irradiation the fraction of the femur content was estimated which had to be injected into a recipient to obtain a countable amount of colonies. Each recipient received 1/1500 of the femoral bone marrow cell content of unirradiated donors or up to 2/3 of the femoral bone marrow content of a 6 Gy irradiated donor in 0.2 ml - 0.5 ml Hanks' balanced salt solution (Gibco) by lateral tail vein injection. Seven or twelve days later the mice were killed, their spleens were excised and fixed in Telleyesniczky's solution. The macroscopic surface colonies were counted using a stereo-microscope at 10x magnification.

CFU-C assay

CFU-C were quantified in a semisolid (0.8% methylcellulose, Methocel, AP4 Premium; Dow Chemical) culture medium (alpha medium) containing 1% BSA, 10% fetal calf serum (FCS) and 10% Poke Weed Mitogen mouse spleen conditioned medium (PWM-MSCM). Bone marrow cells (1/3000 to 1/60 femoral content) 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% CO2 in air. Colonies (≥ 50 cells) were counted after 7 days of culture using an inverted microscope.

MRA

MRA[CFU-C] measures the regeneration of CFU-C in the hemopoietic tissue of an experimental animal after lethal irradiation and bone marrow reconstitution. Five lethally irradiated recipients were injected with one to

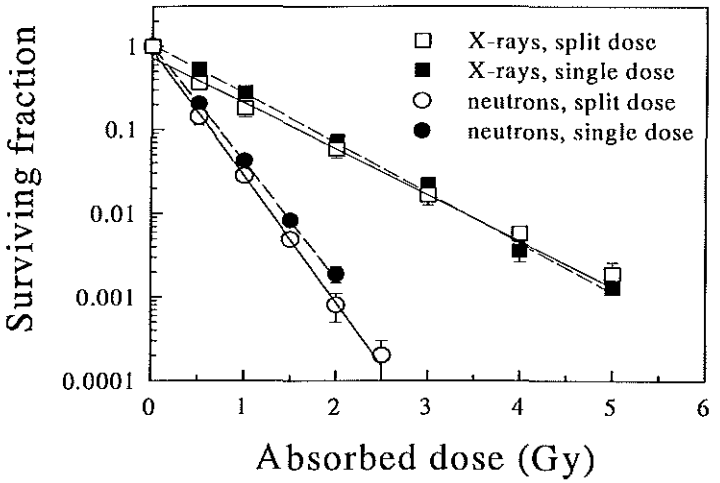

Figure 6.1: Survival curves for CFU-S-7 after in vivo irradiation with X-rays or fission neutrons given as a single dose (dotted lines) or two doses separated by a 6 hours interval (solid lines). The radiosensitivity of CFU-S-7 was assayed immediately after the first dose or after the second of two doses. Single dose survival curves were redrawn from Meijne et al. (1991 and 1996a). Solid lines represent the regression lines computed from four (X-rays) or two (fission neutrons) experiments using the linear model.

five times the femur equivalent used for the CFU-S-7 assay. Twelve days after transplantation, aliquots of their femoral marrow were assayed for the presence of CFU-C. MRA was expressed as the number of hemopoietic precursor cells (MRA[CFU-C]) generated over a 12-day period in one femur of a lethally irradiated recipient per 1/300 femoral bone marrow equivalent injected (Hodgson et al., 1982; Ploemacher & Brons, 1988; Meijne et al.,1991). 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.

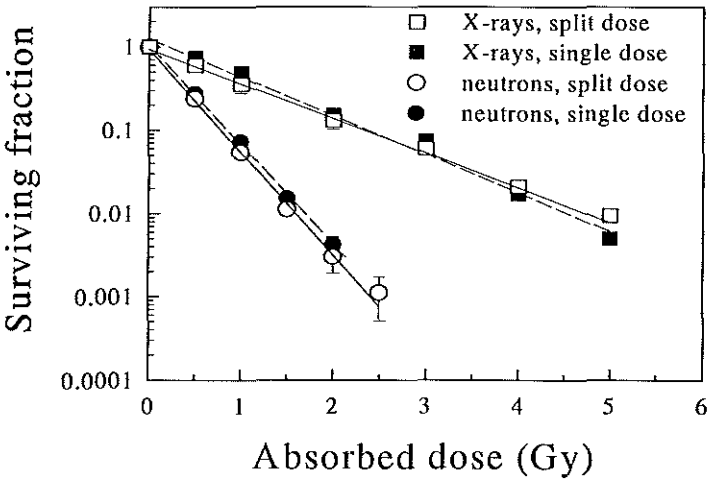

Figure 6.2: Survival curves for CFU-S-12 after in vivo irradiation with X-rays or fission neutrons given as a single dose (dotted lines) or two doses separated by a 6 hours interval (solid lines). The radiosensitivity of CFU-S-12 was assayed immediately after the first dose or after the second of two doses. Single dose survival curves were redrawn from Meijne et al. (1991 and 1996a). Solid lines represent the regression lines computed from four (X-rays) or two (fission neutrons) experiments using the linear model.

Statistical analysis

Log-linear dose effect curves were obtained by least squares regression analysis. D_0 values were obtained from the slope of these curves. Statistical comparison of D_0 values was performed with a Chi square test.

RESULTS

Effect of irradiation on femoral cellularity

Animal irradiated with graded doses X-rays or fast fission neutrons given as a single dose, were killed within 1 hour after irradiation. At this time point no differences in femoral cellularity were observed between control

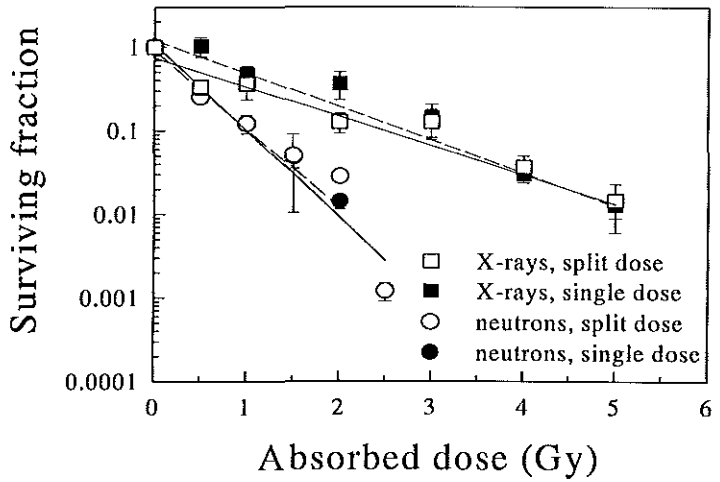

Figure 6.3: Dose response curves for stem cells that repopulate the irradiated bone marrow with in vitro clonable progenitors of granulocytes and macrophages (MRA[CFU-C]) after in vivo X-irradiation or fission neutron irradiation given as a single dose (dotted lines) or two doses separated by a 6 hours interval (solid lines). The radiosensitivity of MRA[CFU-C] was assayed immediately after the first dose or after the second of two doses. Single dose survival curves were redrawn from Meijne et al. (1991 and 1996a). Solid lines represent the regression lines computed from four (X-rays) or two (fission neutrons) experiments using the linear model.

and irradiated groups. Split dose irradiation with a 6 hours interval followed by immediate removal of the femora after the second fraction resulted in a decrease of the femoral cellularity to 81% of control values after 2.5 Gy fission neutron irradiation and 70% of control values after 5 Gy X-irradiation. Delaying removal of the femora 24 hours after the first fraction decreased the femoral cellularity to 35% after a single dose of 5 Gy X-rays and 31% after 2.5 + 2.5 Gy X-rays.

To investigate if this cell loss was caused by cell migration or cell death the number of viable nucleated BMC and CFU-C present in the femora of a mouse 24 hours after 3 Gy X-irradiation were compared with the number of cells present in a femur after storage in RPMI for 24 hours on ice.

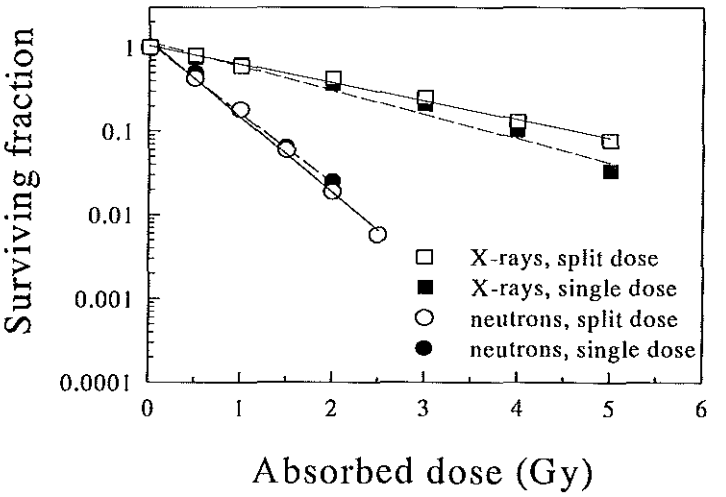

Figure 6.4: Survival curves for CFU-C after in vivo X-irradiation or fission neutron irradiation given as a single dose (dotted lines) or two doses separated by a 6 hours interval (solid lines). The radiosensitivity of CFU-C was assayed immediately after the first dose or after the second of two doses. Single dose survival curves were redrawn from Meijne et al. (1991 and 1996a). Solid lines represent the regression lines computed from four (X-rays) or two (fission neutrons) experiments using the linear model.

The effect of storage on unirradiated femora was also studied. The results are shown in Table 6.1. Delaying the removal of the femora from irradiated mice led to a dramatic decrease in the number of BMC and CFU-C. Storage for 24 hours on ice had no effect on the number of viable BMC as well as CFU-C in a femur, indicating that the decrease was primarily caused by cell migration and not to cell death.

Treatment femora	number viable BMC/femur	CFU-C per femur	CFU-C per 10^5 viable BMC
0 Gy, direct assay	$28.2\ 10^6$	68808	244
3 Gy, direct assay	$28.4\ 10^6$	17190	60
0 Gy, femurs 24 h on ice	$27.2\ 10^6$	69360	255
3 Gy, femurs 24 h on ice	$29.4\ 10^6$	17116	58
3 Gy, 24 h in mouse	$11.4\ 10^6$	5440	48

Table 6.1: Effect of delaying the assay of BMC after 3 Gy X-rays on the number of viable nucleated BMC and CFU-C.

Effect of split dose irradiation using the direct bone marrow assay

The effect of single or split dose irradiation with X-rays and fast fission neutrons, given as a single dose or two equal fractions separated by a 6 hours interval, on the radiosensitivity of HSC subsets is shown in Figure 6.1 - 6.4. The dose response curves observed after fast fission neutron and X-irradiation given as a single dose have been reported previously (Meijne et al., 1991, 1996a). All dose response curves observed after graded doses of X-rays and fast fission neutrons given as a single dose were exponential with no sign of a real shoulder.

X-rays	1 Fraction D_0 (Gy)	n	2 Fractions D_0 (Gy)	n	DRF
MRA[CFU-C]	1.11 ± 0.03	1.20	1.28 ± 0.08	0.74	1.15
CFU-S-12	0.94 ± 0.03	1.25	1.05 ± 0.02	0.91	1.12
CFU-S-7	0.73 ± 0.01	1.04	0.83 ± 0.02	0.72	1.14
CFU-C	1.50 ± 0.05	1.15	2.03 ± 0.04	1.05	1.35

Table 6.2: D_0 values (± 1 SEM) and extrapolation numbers (n) obtained after TBI with graded doses of X-rays given as a single dose or as two equal fractions separated by a 6 hours interval. Femoral bone marrow was grafted immediately after the first or second dose into lethally irradiated recipients.

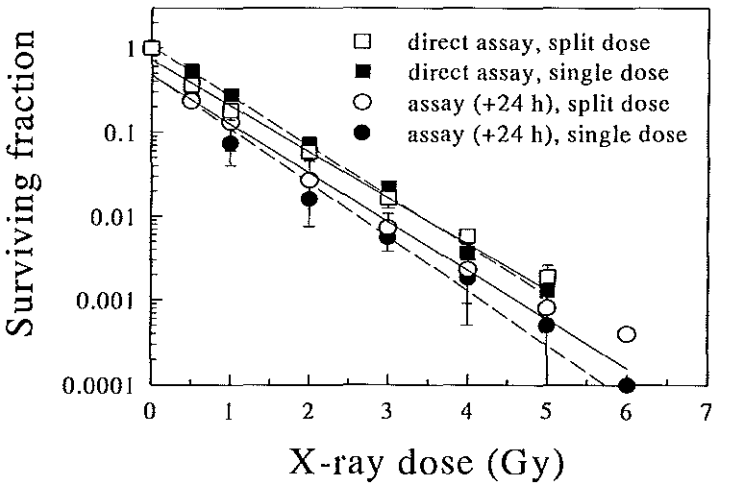

Figure 6.5: Survival curves for CFU-S-7 after in vivo irradiation with X-rays given as a single dose (dotted lines) or two doses separated by a 6 hours interval (solid lines). CFU-S-7 were assayed immediately after the first dose (squares) or with a delay of 24 hours after the first dose (circles). Survival curves obtained after delaying the assay 24 hours represent the regression lines computed from two (split dose) or one (single dose) experiments using the linear model.

Fission neutrons	1 Fraction D_0 (Gy)	n	2 Fractions D_0 (Gy)	n	DRF
MRA[CFU-C]	0.46 ± 0.01	0.92	0.42 ± 0.05	1.10	0.91
CFU-S-12	0.36 ± 0.01	1.05	0.33 ± 0.01	0.94	0.92
CFU-S-7	0.31 ± 0.01	1.01	0.29 ± 0.01	0.89	0.94
CFU-C	0.51 ± 0.01	1.10	0.48 ± 0.01	1.19	0.94

Table 6.3: D_0 values (± 1 SEM) and extrapolation numbers (n) obtained after TBI with graded doses of fast neutrons given as a single dose or as two equal fractions separated by a 6 hours interval. Femoral bone marrow was grafted immediately after the first or second dose into lethally irradiated recipients.

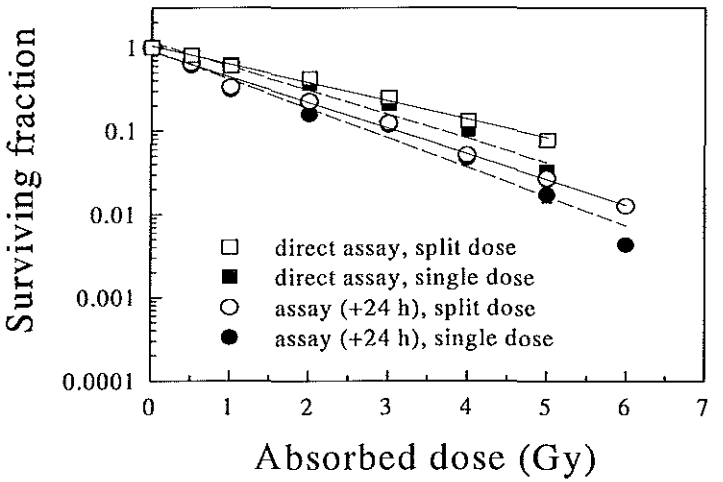

Figure 6.8: Survival curves for CFU-C after in vivo irradiation with X-rays given as a single dose (dotted lines) or two doses separated by a 6 hours interval (solid lines). CFU-C were assayed immediately after the first dose (squares) or with a delay of 24 hours after the first dose (circles). Survival curves obtained after delaying the assay 24 hours represent the regression lines computed from two (split dose) or one (single dose) experiments using the linear model.

X-rays	1 Fraction D_0 (Gy)	n	2 Fractions D_0 (Gy)	n	DRF
MRA[CFU-C]	1.02 ± 0.06	0.56	1.12 ± 0.14	0.41	1.10
CFU-S-12	0.76 ± 0.03	0.74	0.95 ± 0.06	0.55	1.25
CFU-S-7	0.68 ± 0.03	0.48	0.76 ± 0.03	0.47	1.12
CFU-C	1.26 ± 0.07	0.92	1.42 ± 0.05	0.81	1.13

Table 6.4: D_0 values (± 1 SEM) and extrapolation numbers (n) obtained after TBI with graded doses of X-rays given as a single dose or as two equal fractions separated by a 6 hours interval. Femoral bone marrow was grafted with a delay of 24 h after the first dose into lethally irradiated recipients.

resulting DRF values were comparable and varied from 1.10 for MRA[CFU-C] to 1.25 for CFU-S-12. However, only the sensitivity of CFU-S-12 after split-dose irradiation differed significantly from the sensitivity observed after single dose irradiation ($p < 0.01$).

Effect of low dose rate irradiation

The D_0 values obtained after irradiation of MRA[CFU-C], CFU-S-12, CFU-S-7 and CFU-C with graded doses of X-rays at a dose rate of 5 cGy/min are shown in Table 6.5. Lowering the dose rate from 30 cGy/min to 5 cGy/min had no sparing effect on the survival of the HSCs. CFU-C were the only cells which were slightly spared by lowering the dose rate to 5 cGy/min. Only the DRF value obtained for CFU-C (1.14) was above 1.

	D_0 (Gy) at a dose rate of 30 cGy/min	n	D_0 (Gy) at a dose rate of 5 cGy/min	n	DRF
MRA[CFU-C]	1.11 ± 0.03	1.20	1.07 ± 0.17	1.14	0.96
CFU-S-12	0.94 ± 0.03	1.25	0.81 ± 0.05	1.54	0.86
CFU-S-7	0.73 ± 0.01	1.04	0.71 ± 0.02	1.12	0.97
CFU-C	1.50 ± 0.05	1.15	1.71 ± 0.13	1.30	1.14

Table 6.5: D_0 values (± 1 SEM) and extrapolation numbers (n) obtained after TBI with graded doses of X-rays given at a dose rate of 30 or 5 cGy/min. Femoral bone marrow was grafted immediately after irradiation into lethally irradiated recipients.

DISCUSSION

The radiobiological characteristics of hemopoietic cells have been studied extensively. Primitive hemopoietic stem cells (MRA[CFU-C]) appeared to be less sensitive to ionizing radiation than more mature stem cell (CFU-S-7). On the other hand the most differentiated cells used in this study, CFU-C, which belong to the committed progenitor cell compartment, were most radioresistant (Meijne et al., 1991, 1996a; Ploemacher et al., 1992).

These data indicate that within the stem cell compartment the radiosensitivity of stem cells increases as a function of maturation. However, when stem cells leave the stem cell compartment and differentiate into committed progenitors their radiosensitivity changes as a function of their differentiation line, myeloid progenitors being far more radioresistant than erythroid progenitors (Imai & Nakao, 1987). In the present study we investigated the effect of split dose irradiation with a 6 hours interval on the radiosensitivity of MRA[CFU-C], CFU-S-12, CFU-S-7 and CFU-C. Data about the sparing effect of split-dose and fractionated irradiation on CFU-S-7/9 vary from no sparing effect (Tarbell et al., 1987; Ploemacher et al., 1992) to a significant increase in D_0 value. Using a fractionation scheme of two fractions per day, separated by 6 hours, for 3 days, Evans et al. (1988) observed an increase in D_0 value from 0.75 Gy for single dose to 1.09 Gy for fractionated irradiation. In studies showing a sparing effect of split-dose irradiation the repair capacity of CFU-S-7/9 was reported to be maximal after two doses given with an interval of 5 hours between the first and the second dose (Till & McCulloch, 1963; Hendry, 1973; Vavrova & Petyrek, 1988). In our studies we used a time interval of 6 hours between the two fractions, identical to the time interval used previously by Ploemacher et al. (1992). Separating the radiation dose with a 6 hours interval had a small sparing effect on the survival of all cell populations. The DRF value of 1.14 observed for CFU-S-7 using the direct assay was comparable to the DRF value of 1.16 observed for CFU-S-9 using split dose γ-irradiation with a 5 hours interval (Hendry & Howard, 1971). In contrast to the survival curves obtained after γ-irradiation by Ploemacher et al. (1992), no differences in sparing effect were observed between the various cell populations after X-irradiation.

After split dose neutron irradiation no sparing effect was observed, indicating that the small sparing effect observed after X-irradiation was due to early repair and not to repopulation. A lack of split-dose sparing for CFU-S-9 was also reported previously for D-T neutron irradiation (Hendry & Howard, 1971).

The previously reported differences in sparing effect observed for the various HSC subsets after γ-irradiation were obtained using a delayed assay. Hendry & Howard (1971) showed that the number of CFU-S-9 decreased to a larger extent after irradiation than total marrow cellularity (Hendry & Howard, 1971). Twenty-four hours after irradiation with 2 Gy X-rays about 75% of the CFU-S is lost from the bone marrow while the marrow cellularity is decreased with 60% (estimated values) (Hendry, 1973). In our study we

also found a somewhat larger loss for CFU-C than for bone marrow cellularity (68% and 60%, respectively). A significant delay in assessing the femora for surviving stem cells may therefore influence the obtained D_0 values and extrapolation numbers. To investigate if delaying the assay influenced the radiosensitivity we performed a second set of experiments in which the bone marrow cells were assayed 24 hours after the first dose. Delaying the assay did not increase the sparing effect of split dose X-irradiation, nor was a larger sparing effect observed for the more primitive MRA[CFU-C] compared to CFU-S. However, a decrease in extrapolation number was observed for all survival curves, a phenomena already described by Van Putten et al. (1969). This is caused by the radiation induced cell loss of bone marrow cells from the femur, which does not affect controls. Using a more extensive fractionation scheme (14 fractions, 1.2 Gy/fraction) Down et al. (1995) found a large dose sparing effect of fractionation for late appearing cobblestone area forming cells (CAFC day-35 and CAFC day-28, the equivalent of primitive stem cells responsible for LTRA and MRA, respectively) compared to early appearing CAFC day-12 (the equivalent of CFU-S-12). The differential dose-sparing effect of fractionated irradiation was attributed to a larger capacity of primitive CAFC to repair sublethal damage (SLD), since with increasing primitiveness decreasing α/β values were observed. For CAFC day-28 and day-35 an α/β ratio of about 4 Gy was observed. In contrast, a high α/β value of about 23 was observed for CAFC day-12. Also in the study of Van Os et al. (1993) using a murine erythroid transplantation model an extremely low α/β of 0.66 Gy was observed for primitive LTRA cells, indicating that these cells have higher repair capacity than CFU-S. Using in vivo stem cell assays it is possible to reach only about three decades of stem cell survival, owing to the initial numbers of stem cells present in the femur and the technical difficulties of injecting very concentrated cell suspensions. It is therefore possible that a differential split-dose effect would have been observed if in the present study a higher dose range could have been used. Depending on the mouse strain and radiation type a differential split-dose effect might just or just-not be observed in the dose range used for in vivo stem cell assays.

Dose protraction from 30 cGy/min to 5 cGy/min had no effect on the radiosensitivity of the cell populations. Decreasing the dose rate to 5 cGy/min may not be enough to observe a significant effect. Using a murine bone transplant model, dose sparing effects for LTRA cells were only observed at below 5 cGy/min (Van Os et al., 1993). A dose rate of 5 cGy/min

was the lowest dose rate which could be obtained with our radiation device without changing the quality of the beam.

In summary our data show that split dose irradiation with an interval of 6 hours between the first and the second fraction gave a slight dose sparing effect after X-irradiation. No sparing effect could be observed after split dose neutron irradiation. Delaying the assay of bone marrow cells decreased the extrapolation numbers, but did not influence the observed split dose sparing. The relative differences in radiosensitivity, observed for the various cell populations after single dose irradiation, were maintained after split dose irradiation. This suggests that previously reported differences in sublethal damage repair observed after γ-irradiation are not the only factors responsible for the observed differences in radiosensitivity between the various stem cell populations. Other factors such as apoptosis, lesion repair fidelity and lesion tolerance, might also play a role (McKay & Kefford, 1995).

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REFERENCES

Bertoncello, I., Hodgson, G.S., and Bradley, T.R. Multiparameter analysis of transplantable hemopoietic stem cells. I. The separation and enrichment of stem cells homing to marrow and spleen on the basis of rhodamine-123 fluorescence. Exp. Hematol. 13, 999-1006 (1985).

Davids, J.A.G. Bone marrow syndrome in CBA mice exposed to fast neutrons of 1.0 MeV mean energy. Effect of syngeneic bone marrow transplantation. Int. J. Radiat. Biol. 17, 173-185 (1970).

Davids, J.A.G., Mos, A.P.J. and Oude, A. de. Fast neutron facility for biological exposures in an Argonaut reactor. Design, tissue dosimetry and neutron spectrometry. Phys. Med. Biol. 14, 573-584 (1969).

Down, J.D., Boudewijn, A., Van Os, R., Thames, H.D. and Ploemacher, R.E. Variations in radiation sensitivity and repair among different hemopoietic stem cell subsets following fractionated irradiations. Blood 86, 122-127 (1995).

Evans, R.G., Wheatley, C.L. and Nielsen, J.R. Modification of radiation-induced damage to bone marrow stem cells by dose rate, dose fractionation, and prior exposure to cytoxan as judged by the survival of CFU-S: Application to bone marrow transplantation (BMT). Int. J. Radiat. Oncol. Biol. Phys. 14, 491-495 (1988).

Hendry, J.H. and Howard, A. The response of haemopoietic colony-forming units to single and split doses of gamma-rays or D-T neutrons. Int. J. Radiat. Biol. 19, 51-64 (1971).

Hendry, J.H. Differential split-dose radiation response of resting and regenerating haemopoietic stem cells. Int. J. Radiat. Biol. 24, 469-473 (1973).

Hodgson, G.S., Bradley, T.R., Martin, R.F., Summer, M. and Fry, P. Recovery of proliferating haemopoietic progenitor cells after killing by hydroxyurea. Cell Tissue Kinet. 8, 51-60 (1975).

Hodgson, G.S., Bradley, T.R. and Radley, J.M. The organization of hemopoietic tissue as inferred from the effects of 5-fluorouracil. Exp. Hematol. 10, 26-35 (1982).

Imai, Y. and Nakao, I. In vivo radiosensitivity and recovery pattern of the hemopoietic precursor cells and stem cells in the mouse bone marrow. Exp. Hematol. 15, 890-895 (1987).

Jones, R.J., Wagner, J.E., Celano, P., Zicha, M.S. and Sharkis, S.J. Separation of pluripotent haematopoietic stem cells from spleen colony-forming cells. Nature 347, 188-189 (1990).

McKay, M.J. and Kefford, R.F. The spectrum of in vitro radiosensitivity in four human melanoma cell lines is not accounted for by differential induction or rejoining of DNA double strand breaks. Int. J. Radiat. Oncol. Biol. Phys. 31, 345-352 (1995).

Meijne, E.I.M., Winden-van Groenewegen, R.J.M. van der, Ploemacher, R.E., Vos, O., Davids, J.A.G. and Huiskamp, R. The effects of X-irradiation on hematopoietic stem cell compartments in the mouse. Exp. Hematol. 19, 617-623 (1991).

Meijne, E.I.M., Ploemacher, R.E., Vos, O. and Huiskamp, R. The sensitivity of murine hemopoietic stem cell populations for 1 MeV fission neutrons. Radiat. Res. Submitted for publication (1996a).

Meijne, E.I.M., Ploemacher, R.E. and Huiskamp, R. The sensitivity of murine hemopoietic stem cell populations to X-rays and 1 MeV fission neutrons in vitro, and under hypoxic conditions in vivo. Int. J. Radiat. Biol. Submitted for publication (1996b).

Ploemacher, R.E. and Brons, N.H.C. Cells with marrow and spleen repopulating ability and forming spleen colonies on day 16, 12 and 8 are sequentially ordered on the basis of rhodamine-123 retention. J. Cell. Physiol. 136, 521-536 (1988).

Ploemacher, R.E. and Brons, R.H.C. Separation of CFU-S from primitive cells responsible for reconstitution of the bone marrow hemopoietic stem cell compartment following irradiation: evidence for a pre-CFU-S. Exp. Hematol. 17, 263-266 (1989).

Ploemacher, R.E., Van Os, R., Van Beurden, C.A.J. and Down, J.D. Murine haemopoietic stem cells with long-term engraftment and marrow repopulating ability are more resistant to gamma-radiation than are spleen colony forming cells. Int. J. Radiat. Biol. 61, 489-499 (1992).

Ploemacher, R.E., Van der Loo, J.C.M., Van Beurden, C.A.J. and Baert, M.R.M. Wheat germ agglutinin affinity of murine hemopoietic stem cell subpopulations is an inverse function of their long-term repopulating ability in vitro and in vivo. Leukemia 7, 120-130 (1993).

Tarbell, N.J., Amato, D.A., Down, J.D., Mauch, P.M. and Hellman, S. Fractionation and dose-rate effects in mice: A model for bone marrow transplantation in man. Int. J. Radiat. Oncol. Biol. Phys. 13, 1065-1069 (1987).

Thomas, F. and Gould, M.N. Evidence for the repair of potentially lethal damage in irradiated bone marrow. Radiat. Environ. Biophys. 20, 89-94 (1982).

Till, J.E. and McCulloch, E.A. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat. Res. 14, 213-222 (1961).

Till, J.E. and McCulloch, E.A. Early repair processes in marrow cells irradiated and proliferating in vivo. Radiat. Res. 18, 96-105 (1963).

Van der Loo, J.C.M., Van den Bos, C., Baert, M.R.M., Wagemaker, G. and Ploemacher, R.E. Stable multilineage hematopoietic chimerism in α-thalassemic mice induced by a bone marrow subpopulation that excludes the majority of day-12 spleen colony-forming units. Blood 83, 1769-1777 (1994).

Van Putten, L.M., Lelieveld, P. and Broerse, J.J. NIH, AEC, Mtg. In: Time and dose relationships in radiation biology as applied to radiotherapy. Carmel, B.N.L. 50230 (C-57), 162 (1969).

Van Os, R., Thames, H.D., Konings, A.W.T. and Down, J.D. Radiation dose-fractionation and dose-rate relationships for long-term repopulating hemopoietic stem cells in a murine bone marrow transplant model. Radiat. Res. 136, 118-125 (1993).

Vavrova, J. and Petyrek, P. Effect of short-term splitting and γ-ray doses on changes in haemopoietic stem cell number in bone marrow and on mortality of whole-body irradiated mice. Folia Biologica 34, 399-408 (1988).

Visser, J.W.M., Hogeweg, M.G.C., Vries, P. de, Bayer, J. and Ploemacher, R.E. Culture of purified pluripotent haemopoietic cells. In: The biology of hematopoiesis. Eds. Dainiak, N., Cronkite, E.P., McCaffrey, R., Wiley Liss, Academic Press, New York. pp. 1-8 (1990).

THE SENSITIVITY OF LONG-TERM REPOPULATING STEM CELLS FOR 300 KV X-RAYS AND 1 MEV FISSION NEUTRONS

ABSTRACT

The radiosensitivity of murine stem cells with long-term repopulating ability (LTRA) was determined for 300 kV X-rays and 1 MeV fission neutrons using an in vivo repopulation assay. Bone marrow cells from male donor mice were irradiated in vivo with graded doses of X-rays and fission neutrons and injected into sublethally irradiated female recipients. White blood cell chimerism at 4 months after transplantation was used to calculate the D_0 values for LTRA cells. LTRA cells were characterized by D_0 values of 1.09 ± 0.08 Gy for 300 kV X-rays and 0.47 ± 0.05 Gy for 1 MeV fission neutrons. These results are consistent with previous findings using sustained erythroid repopulating capacity of γ-irradiated recipient stem cells in normal mice and in vitro frequency analysis of LTRA cells, and confirm that primitive LTRA cells are less sensitive to ionizing radiation than colony forming units in the spleen (CFU-S). The apparent high radiosensitivity observed by Van den Bos (1994) for LTRA cells in partially chimeric α-thalassemic BALB/c mice may be the result of the selective advantage of normal red blood cell production in these mice rather than the existence of a more radiosensitive subpopulation of LTRA cells.

INTRODUCTION

The hemopoietic stem cell compartment in the bone marrow contains a hierarchically organized continuum of stem cell subsets ranging from pluripotent hemopoietic stem cells to in vitro clonable committed progenitors (Rosendaal et al., 1976). The different cell populations have been shown to be heterogeneous with respect to the time required for their clonal expansion (Magli et al, 1982; Jones et al., 1990; Ploemacher et al., 1991), sensitivity to cytotoxic drugs (Hodgson & Bradley, 1979; Down & Ploemacher, 1993; Down et al., 1994) and sensitivity to radiation (Meijne et al., 1991; Ploemacher et al., 1992; Down et al., 1995). The more mature cells within the stem cell compartment (colony forming units in the spleen (CFU-S) are responsible for the rapid but transient repopulation of the bone marrow following total body irradiation (TBI). Sustained long-term hemopoietic reconstitution is provided for by more primitive hemopoietic stem cells (marrow repopulating cells (MRA) and long-term repopulating cells (LTRA)) (Jones et al., 1990; Van der Loo et al., 1994). Using the MRA assay

and the in vitro cobblestone area forming cell (CAFC) assay D_0 values for MRA of 1.18 Gy for 300 kV X-rays (Meijne et al., 1991) and 1.25 - 1.4 Gy for γ-rays has been observed (Ploemacher et al., 1992). The disadvantage of the MRA-assay is that it is confined to a narrow window of repopulation in the bone marrow at 12-13 days after transplantation. The LTRA assay on the other hand measures the level of multilineage hemopoietic reconstitution over an extended period of time on the basis of the percentage endogenous and/or donor-cell repopulation.

Separation of hemopoietic stem cell subsets on the basis of wheat germ agglutinin (WGA) showed that the enrichment factors for primitive stem cells with MRA and LTRA were not fully comparable, suggesting that stem cells with LTRA are more primitive than stem cells with MRA (Ploemacher et al., 1993). The radiosensitivity of LTRA cells may therefore differ from the radiosensitivity of MRA cells. Studies using the in vitro CAFC-assay showed that CAFC day-35 (LTRA equivalent) are equally or even more radioresistant than are MRA cells (Down et al., 1995).

Two in vivo studies have been performed in which the radiosensitivity of in situ LTRA has been determined by long-term erythroid chimerism (Down & Van Os, 1993; Van Os, 1994; Van den Bos, 1994; Wagemaker, 1995). In these studies recipients were irradiated with graded doses of γ-rays followed by transplantation of increasing numbers of unirradiated BMC. By comparing the endogenous repopulation in comparison to the repopulation of transplanted BMC an estimate of the radiosensitivity of LTRA cells irradiated and growing in situ was obtained. The results of both studies were contradictory. Van Os calculated a D_0 value for LTRA of C57BL/6 (B6) mice of 1.3 Gy for γ-rays, while Van den Bos observed D_0 values of 0.68 ± 0.08 Gy for α-thalassemic LTRA and 0.59 ± 0.10 Gy for normal LTRA cells of partially chimeric α-thalassemic BALB/c mice. In the present study we determined the radiosensitivity of LTRA cells from CBA/H mice for 300 kV X-rays and 1 MeV fission neutrons by measuring white blood cell chimerism at 4 months after bone marrow transplantation (BMT) of irradiated donor cells. The obtained results are compared with the data obtained with γ-irradiation (Van Os, 1994; Van den Bos, 1994)

MATERIALS AND METHODS

Mice

Inbred CBA/H mice 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. Female mice aged 11-18 weeks were used as recipients for bone marrow transplantation (BMT).

Irradiation procedures

Three to six donor mice per dose group were irradiated with graded doses of 300 kV X-rays or 1 MeV fission neutrons. The design of the exposure facilities, tissue dosimetry and animal irradiation procedures have been described elsewhere (Davids et al., 1969; Meijne et al., 1992). Female recipients were sublethally irradiated with 7.2 Gy of X-rays 1- 4 hours before BMT.

LTRA

LTRA was determined by injection of male BM cells into sublethally irradiated syngeneic female recipients (5-8 mice per cell concentration). The preparation of the cell suspensions has been described elsewhere (Meijne et al., 1991). Three cell doses were used per dose point, ranging from 5 10^4 to 2 10^5 viable BMC per mouse for unirradiated control BM to 2.5 10^6 to 10^7 viable BMC per mouse for BMC irradiated with 5 Gy of X-rays. To assess chimerism, smears of blood taken from mouse tailtips were prepared from individual mice at 4 months (to determine LTRA) after BM transplantation. The slides were fixed in 100% methanol for 10 min at room temperature, air-dried and stored at - 20 °C until the in situ procedure. About 100 cells were scored in each blood smear. Control mice for both sexes were included. Male control mice almost always showed >90% positive cells, whereas female control mice always showed <1% positive cells.

Fluorescent in situ hybridization

Nucleated blood cell chimerism was determined by in situ hybridization (FISH) on blood smears of sex-mismatched chimeric mice using the murine Y chromosome-specific probe M34 (Singh et al., 1987a,

1987b). The probe (kindly provided by Dr. L. Singh, CCMB, Hyderabad, India) was labelled by nick-translation with biotin-16-dUTP (Boehringer-Mannheim). Total plasmid DNA was used for the in situ hybridization. FISH was performed using a modification of the protocol of Pinkel (Pinkel et al., 1986) as described previously (Van der Sluijs et al., 1993). In short, slides were pre-treated 7.5 min with 0.1 M HCl/0.05% Triton X-100 at 37 °C followed by 15 min incubation with 1% paraformaldehyde in PBS at room temperature and subsequently dehydrated in an ethanol series and air-dried. Five µl hybridization mix (2x SSC, 50% (v/v) deionized formamide, 10% (w/v) dextran sulphate, 0.1% (v/v) Tween-20, 0.5 mg/ml herring sperm DNA and 2.5 ng/ml biotinylated M34 Y-probe) was added under a plastic coverslip and target and probe DNA were denatured for 5-7 min at 80 °C followed by overnight hybridization at 37 °C in a 100% humidified moisture chamber. The next day the slides were subsequently washed in 2x SSC at room temperature, 50% formamide/2x SSC at 37 °C (twice), 2x SSC at 37 °C (twice) and 4x SSC/0.1% Triton at room temperature. After hybridization the slides were incubated with avidin-FITC (Vector). The fluorescence signal was amplified with a biotinylated goat-anti-avidin antibody (Vector) followed by a second incubation with avidin-FITC. The slides were then dehydrated in ethanol, dried, mounted in antifade medium and stored at - 20 ^{0}C until scoring for the percentage male and female cells.

Calculation of D_0 values

For each radiation dose, dose response relationships for donor bone marrow cell doses and the levels of white blood cell chimerism were fitted by least-squares linear-regression analysis following logit transformation of percent (P) engraftment (logit P = ln[P/100-P]) and logarithmic transformation of bone marrow cell input. From these curves the number of BMC required for 40% donor-type repopulation was determined. The number of unirradiated BMC required for 40% donor type repopulation was considered to correspond to 100% survival of LTRA cells. By dividing the required number of unirradiated BMC by the number of irradiated BMC required for 40% engraftment, the surviving fraction of LTRA for each radiation dose point was obtained. D_0 values were derived from the slope of the radiation dose-survival curves.

RESULTS

Donor mice were irradiated and killed within 1 h after irradiation. At this timepoint no difference in the total number of nucleated cells was observed between control and irradiated mice. The viability of the cell suspensions prepared from the donor mice ranged from 83% for the unirradiated control group to 89% for donor mice irradiated with 2 Gy X-rays. The mean viability of the cell suspensions was 87%.

For each radiation dose three groups of female recipient mice were transplanted with increasing numbers of male BMC. The percentage donor-type repopulation in the blood of the recipients transplanted with graded doses BMC, 4 months after transplantation, is shown in Figure 7.1. From these data the number of BMC required for 40% donor-type repopulation was determined.. The survival curves for LTRA cells observed after 300 kV X-irradiation and 1 MeV fission neutron irradiation is shown in Figure 7.2. LTRA cells were found to be relatively radioresistant and characterized by a D_0 value of 1.09 ± 0.08 Gy for X-rays and 0.47 ± 0.05 Gy for 1 MeV fission neutrons.

DISCUSSION

In this study we determined the radiosensitivity of cells with LTRA in CBA/H mice. LTRA cells were shown to be much more radioresistant for radiation than CFU-S of the same mouse strain (Meijne et al., 1991), thus supporting previous results obtained with the MRA assay and the CAFC assay (Ploemacher et al., 1992; Down et al., 1995). LTRA cells were characterized by D_0 values of 1.09 ± 0.08 Gy for X-rays and 0.47 ± 0.05 Gy for 1 MeV fission neutrons. These values correspond to the previously obtained D_0 values of 1.18 ± 0.01 Gy for X-rays and 0.48 ± 0.05 Gy for 1 MeV fission neutrons using MRA[CFU-S-12] as an endpoint indicating that MRA cells and LTRA cells do not differ in radiosensitivity. Two earlier in vivo studies have been published in which D_0 values for LTRA cells were reported (Down & Van Os, 1993; Van Os, 1994; Van den Bos, 1994). In contrast to our study in which white blood cell chimerism was used as an endpoint these studies used red blood cell chimerism as an endpoint. The results of both studies were contradictory. Van den Bos observed D_0 values for LTRA cells in α-thalassemic BALB/c mice comparable to the low D_0

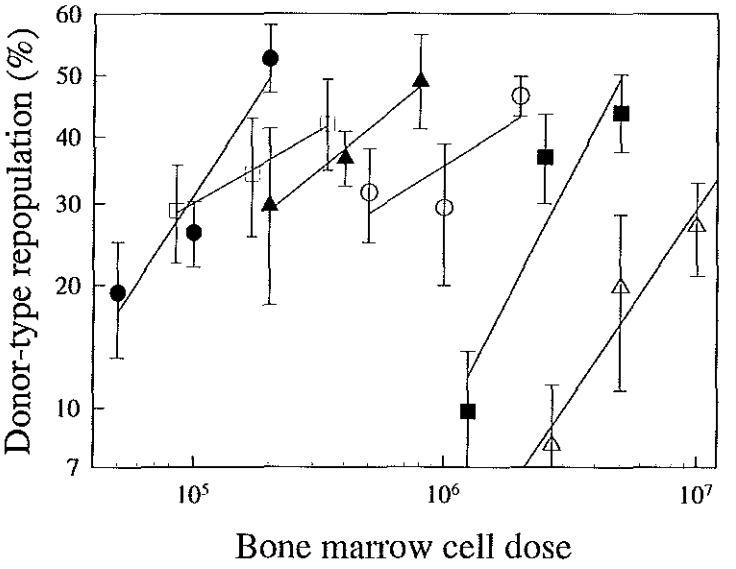

Figure 7.1: Logit regression analysis on the percentage of donor-type white blood cell repopulation,4 months after transplantation of male donor cells irradiated with graded doses of X-rays into sublethally irradiated female recipients.

values normally observed for CFU-S. Van Os on the other hand reported a higher D_0 value of 1.3 Gy for LTRA in B6 mice. White blood cell chimerism has been shown to correspond closely to immature hemopoietic cell chimerism. Red blood cell chimerism in α-thalassemic mice, however, always exceeded that of white blood cell chimerism (Van den Bos, 1994b). This is probably caused by the erythroid deficit in this mouse strain. In the study of Van Os, erythroid chimerism in congenically marked normal mice correlated reasonably well with engraftment in other tissues. This method therefore appears to suitable for estimating stable donor chimerism originating from LTRA cells (Van Os et al., 1992; 1994).

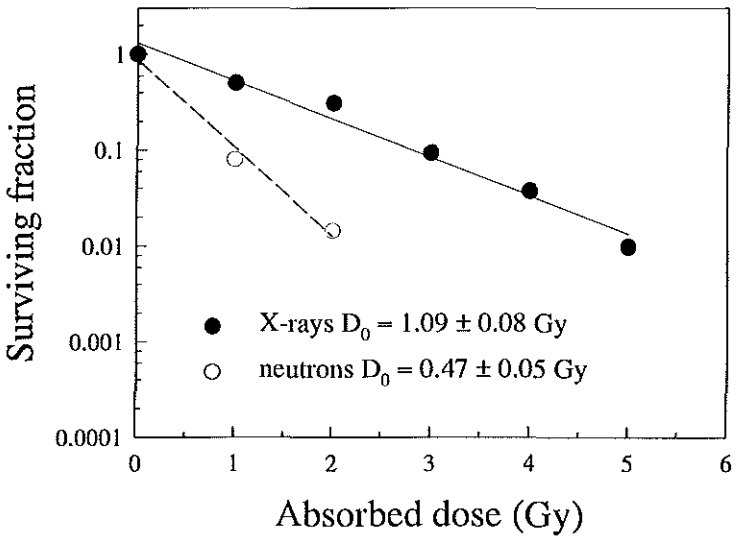

Figure 7.2: Determination of D_0 values for LTRA cells irradiated with X-rays and 1 MeV fission neutrons on the basis of the estimated number of BMC leading to 50 % donor-derived peripheral white blood cells 4 months after BMT.

When the radiosensitivity of LTRA cells is determined in situ the observed radiosensitivity may be influenced by other factors. Using this technique, recipient mice receive variable doses of radiation and thus differential radiation damage to the supporting hemopoietic microenvironment could be involved. Also, increasing number of unirradiated BMC are injected into recipients. When very high BM cell doses ($4 * 10^7$) are transplanted, up to 40% donor cells can be found in unirradiated recipients (Saxe et al, 1984; Wu & Keating, 1993; Van Os, 1994). This indicates that the homing fraction of the transplanted BMC might be influenced by the number of BM cells injected. By transplanting graded doses of irradiated BMC the number of viable LTRA transplanted per dose group is approximately the same, thus minimising differences in homing fraction. The fact that Van Os using an in situ assay observed a comparable D_0 value for LTRA as observed in the present study, indicates that in the dose

ranges used (radiation doses as well as cell doses) these two factors (i.e. microenvironmental damage and cell dose) do not significantly influence the outcome of the in situ assay. One of the explanations given by Van den Bos for the relatively low D_0 value observed for LTRA cells in BALB/c mice is the relatively high radiosensitivity of this mouse strain, since lower D_0 values for BALB/c derived CFU-S-12 compared to C57BL/6 derived CFU-S-12 have been observed (0.67 Gy and 0.98 Gy respectively) (Van den Bos, 1994). BALB/c mice ($LD_{50\,30}$ = 5.9 Gy) have been shown to be more sensitive to radiation than C57BL mice ($LD_{50\,30}$ = 7.6 Gy) (Hanson et al., 1987). In the same study Hanson found identical D_0 values for CFU-S-10 from BALB/c and C57BL/6 mice (0.89 Gy and 0.86 Gy, respectively), indicating that the observed differences in radiosensitivity did not result from differences in the radiosensitivity of CFU-S. Also the radiosensitivity of committed progenitors of both mice strains and syngeneic engraftment after TBI did not reveal differences in hemopoietic stem cell and progenitor radiosensitivity between B6 and BALB/c mice (Van Os, 1994). In general there is no correlation between animal survival and the radiosensitivity of hemopoietic stem cells and progenitor cells (Carsten, 1984; Van Os, 1994), thus emphasizing the complexity of the many factors which play a role in the survival of an animal in the LD_{50} range. The most likely explanation for the low D_0 values obtained for LTRA in partially chimeric α-thalassemic BALB/c mice is therefore that repopulation is influenced by the preferential growth of normal BMC.

REFERENCES

Carsten, A.L. Acute lethality. The hemopoietic syndrome in different species. In: Response of different species to total body irradiation (J.J. Broerse, and T.J. MacVittie, Eds.) pp. 59-86. Martinus Nijhoff Publishers, Dordrecht, The Netherlands, 1984.

Davids, J.A.G., Mos, A.P.J. and De Oude, A. Fast-neutron facility for biological exposures in an Argonaut reactor: Design, tissue dosimetry and neutron spectrometry. Phys. Med. Biol. 14, 573-584 (1969).

Davids, J.A.G. Bone marrow syndrome in CBA mice exposed to fast neutrons of 1.0 MeV mean energy. Effect of syngeneic bone marrow transplantation. Int. J. Radiat. Biol. 17, 173-185 (1970).

Down, J.D. and Van Os, R. Donor bone marrow engraftmen and host irradiation dose-response relationships for long-term hemopoietic repopulation. Int. J. Radiat. Biol. 63, 795 (abstract) (1993).

Down, J.D. and Ploemacher, R.E. Transient and permanent engraftment potential of murine hematopoietic stem cell subsets: differential effects of host conditioning with gamma radiation and cytotoxic drugs. Exp. Hematol. 21, 913-921 (1993).

Down, J.D., Boudewijn, A., Dillingh, J.H., Fox, B.W. and Ploemacher, R.E. Relationships between ablation of distinct haemopoietic cell subsets and the development of donor bone marrow engraftment following recipient pretreatment with different alkylating drugs. Brit. J. Cancer 70, 611-616 (1994).

Down, J.D., Boudewijn, A., Van Os, R., Thames, H.D. and Ploemacher, R.E. Variations in radiation sensitivity among different bone marrow hemopoietic cell subsets following fractionated irradiation. Blood 86, 122-127 (1995).

Hanson, W.R., Fry, FR.J.M., Sallese, A.R. and Ainsworth, E.J. Comparison of intestine and bone marrow radiosensitivity of the BALC/c and the C57BL/6 mouse strains and their B6CF1 offspring. Radiat. Res. 110, 340-352 (1987).

Hodgson, G.S. and Bradley, T.R. Properties of haematopoietic stem cells surviving 5 fluorouracil treatment: evidence for a pre-CFU-S cell? Nature 281, 381-382 (1979).

Jones, R.J., Wagner, J.E., Celano, P., Zicha, M.S. and Sharkis, S.J. Separation of pluripotent haematopoietic stem cells from spleen colony-forming cells. Nature 347, 188-189 (1990).

Magli, M.C., Iscove, N.N. and Odartchenko, N. Transient nature of early haematopoietic spleen colonies. Nature 295, 527-529 (1982).

Meijne, E.I.M., Van der Winden-van Groenewegen, R.J.M., Ploemacher, R.E., Vos, O., Davids, J.A.G. and Huiskamp, R. The effects of X-irradiation on hematopoietic stem cell compartments in the mouse. Exp. Hematol. 19, 617-623 (1991).

Meijne, E.I.M., Ploemacher, R.E., Vos, O. and Huiskamp, R. The effects of graded doses of 1 MeV fission neutrons or X-rays on the murine hematopoietic stroma. Radiat. Res. 131, 302-308 (1992).

Pinkel, D., Straume, T. and Gray, J.W. Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc. Natl. Acad. Sci. USA 83, 2934-2938 (1986).

Ploemacher, R.E., Van der Sluijs, J.P., Van Beurden, C.A.J., Baert, M.R.M. and Chan, P.L. Use of limiting-dilution type long-term marrow cultures in frequency analysis of marrow-repopulating and spleen colony-forming hematopoietic stem cells in the mouse. Blood 78, 2527-2533 (1991).

Ploemacher, R.E., Van Os, R., Van Beurden, C.A.J. and Down, J.D. Murine haemopoietic stem cells with long-term engraftment and marrow repopulating ability are more resistant to gamma-radiation than are spleen colony forming cells. Int. J. Radiat. Biol. 61, 489-499 (1992).

Ploemacher, R.E., Van der Loo, J.C.M., Van Beurden, C.A.J. and Baert, M.R.M. Wheat germ agglutinin affinity of murine hemopoietic stem cell subpopulations is an inverse function of their long-term repopulating ability in vitro and in vivo. Leukemia 7, 120-130 (1993).

Rosendaal, M., Hodgson, G.S. and Bradley, T.R. Haemopoietic stem cells are organized for use on the basis of their generation age. Nature 264, 68-69 (1976).

Saxe, D.F., Boggs, S.S. and Boggs, D.R. Transplantation of chromosomally marked syngeneic marrow cells into mice not subjected to hematopoietic stem cell depletion. Exp. Hematol. 12, 277-283 (1984).

Singh, L., Matsukuma, S. and Jones, K.W. Testis development in a mouse with 10% of XY cells. Dev. Biol. 122, 287-290 (1987a).

Singh, L., Matsukuma, S. and Jones, K.W. The use of Y-chromosome-specific repeated DNA sequences in the analysis of testis development in an XX/XY mouse. Development (supp.) 101, 143-149 (1987b).

Van den Bos, C. Partial hemopoietic chimerism in thalassemic recipients of normal bone marrow stem cells. Thesis for Erasmus University, Rotterdam, 1994.

Van den Bos, C., Kieboom, D., Van der Sluijs, J.P., Baert, M.R.M., Ploemacher, R.E. and Wagemaker, G. Selective advantage of normal erythrocyte production after bone marrow transplantation of a-thalassemic mice. Exp. Hematol. 22, 441-446 (1994).

Van der Loo, J.C.M., Van den Bos, C., Baert, M.R.M., Wagemaker, G. and Ploemacher, R.E. Stable multilineage hematopoietic chimerism in a-thalassemic mice induced by a bone marrow subpopulation that excludes the majority of day-12 spleen colony-forming units. Blood 83, 1769-1777 (1994).

Van der Sluijs, J.P., Van den Bos, C., Baert, M.R.M., Van Beurden, C.A.J. and Ploemacher, R.E. Loss of long-term repopulating ability in long-term bone marrow culture. Leukemia 7, 725-732 (1993).

Van Os, R., Konings, A.W.T. and Down, J.D. Radiation dose as a factor in host preparation for bone marrow transplantation across different genetic barriers. Int. J. Radiat. Biol. 61, 501-510 (1992).

Van Os, R. Radiobiological studies on target cell populations in murine bone marrow transplantation recipients. Thesis for State University Groningen, Groningen, 1994.

Wagemaker, G. Heterogeneity of radiation sensitivity of hemopoietic stem cell subsets. Stem Cells 13 (suppl. 1), 257-260 (1995).

Wu, D.D. & Keating, A. Hematopoietic stem cells engraft in untreated transplant recipients. Exp. Hematol. 21, 251-256 (1993).

THE EFFECT OF GRADED DOSES OF 1 MEV FISSION NEUTRONS OR X-RAYS ON THE MURINE HEMOPOIETIC STROMA

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ABSTRACT

The acute radiosensitivity in vivo of the murine hemopoietic stroma (HS) for 1 MeV fission neutrons and 300 kV X-rays was determined. Two different assays were used: 1) an in **vitro** clonogenic assay for fibroblast precursor cells (CFU-F) and 2) subcutaneous grafting of femora or spleens. The number of stem cells (CFU-S) or precursor cells (CFU-C), which repopulated the subcutaneous implants, was used to measure the ability of the stroma to support hemopoiesis. The CFU-F were the most radiosensitive, and the survival curves after neutron and X-irradiation were characterized by D_0 values of 0.75 and 2.45 Gy respectively. For regeneration of CFU-S and CFU-C in subcutaneously implanted femora, D_0 values of 0.92 and 0.84 Gy after neutron and 2.78 and 2.61 Gy after X-irradiation were found. The regeneration of CFU-S and CFU-C in subcutaneous implanted spleens was highly radioresistant as evidenced by D_0 values of 2.29 and 1.49 Gy for survival curves obtained after neutron irradiation, and D_0 values of 6.34 and 4.85 Gy after X-irradiation. The fission neutron RBE for all the cell populations was close to 3 and varied from 2.8 to 3.3. The higher RBE values observed for stromal cells , compared to the RBE of 2.1 reported previously for hemopoietic stem cells, indicate that stromal cells are relatively more sensitive to neutron irradiation than hemopoietic cells.

INTRODUCTION

Effective hemopoiesis requires a functional microenvironment, which provides stem cells with appropriate niches to proliferate and differentiate. Evidence has been obtained that stromal cells not only supply a supportive environment where cells can lodge and respond to external influences, but also play a determinative role in hemopoietic cell growth and differentiation by the production of growth factors, which, bound to heparan sulphate, can be presented in a biologically active form (Gordon et al., 1987; Roberts et al., 1988; Dexter et al, 1990). The stroma may also inhibit the differentiation of hemopoietic stem cells and control their self-renewal via cell surface-associated factors (restrictins) (Zipori, 1988, 1989). Summarizing, the available data suggest that there are qualitative differences between the stromal cells of different organs and even different niches within one organ. Such qualitative differences could lead to differences in radiosensitivity of

different stromal compartments, which, through sectorial failure of hemopoietic stromal cell functions, could have an impact on hemopoiesis. In the last decade, various studies have been conducted in which the radiosensitivity of the stroma after γ- or X-irradiation has been determined. In contrast, little is known about the effects of fission neutron-irradiation on the hemopoietic stroma. In the present study we have determined the radiosensitivity of the hemopoietic stroma for neutrons and X-rays using two different assays that measure the proliferative ability of different stromal cells.

The fibroblast colony forming unit (CFU-F) assay is an in vitro clonal assay for fibroblastoid precursor cells from the hemopoietic microenvironment (Friedenstein et al., 1970; Piersma et al., 1985). In a second stromal assay, the regenerative capacity of subcutaneously implanted femora and spleens was measured after implantation into syngeneic hosts. Since the stroma of the implanted organs is mainly of donor origin (Friedenstein et al, 1968) and the hemopoietic cells which grow in these implants are host-derived (Fried et al, 1973; Hotta et al., 1983), the number of spleen colony forming units (CFU-S) and granulocyte-macrophage colony forming unit (CFU-GM) detected in the regenerated implants at 8 weeks after implantation is considered to provide a measure of stromal regeneration (Fried et al., 1976).

The biological effectiveness of two types of radiation can be compared by determining the relative biological effectiveness (RBE). The RBE of fission neutrons compared with X-rays is the ratio of the absorbed dose of X-rays to the absorbed dose of neutrons required to produce the same biological effect. Neutron RBE data are relevant for radiation protection, fast-neutron therapy and treatment after accidental exposure to fast neutrons. Since the replacement of the T65 dosimetry for atomic bomb survivors by the DS86 dosimetry system, the potential for studying the effect of high-LET neutron irradiation in humans has been greatly reduced. Thus risk estimation for neutron irradiation in humans must be based on experimental studies in animals. Comparison of RBEs for fast neutrons of various mean energies shows that those for fast fission neutrons of 1 MeV mean energy are highest for each biological effect investigated (Broerse & Barendsen, 1973). Therefore RBE data for fast fission neutrons of 1 MeV mean energy can be regarded as the upper limit for neutron RBE data for each biological effect.

In the present study, we have investigated whether the qualitative differences in stromal microenvironments lead to differences in

radiosensitivity. In addition, we determined the neutron RBE values for the different hemopoietic stromal compartments.

MATERIALS AND METHODS

Mice

Inbred CBA/P mice 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 mice were irradiated or sham-irradiated with graded doses of X-rays or neutrons at the age of 12-14 weeks. Male mice aged 20 weeks or more were used as implant recipients (Schofield et al., 1986), and male or female mice were used as recipients in the CFU-S assays.

Irradiation procedures

The animals 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 fast neutrons at a dose rate of 0.1 Gy/min. Forty mice could be irradiated simultaneously. The absorbed doses are given as neutron centre-line doses; they do not include the 9% γ-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.

Whole body X-irradiation 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. Twenty animals could be irradiated simultaneously. During irradiation the animals were confined in polycarbonate tubes, which were mounted in a rotating disk of perspex.

Hemopoietic cell suspensions

Immediately after irradiation, donor mice were killed by CO_2 gassing and the spleen and both femurs of six experimental mice per group were removed. Bones were freed of fat, muscles and tendons and placed in ice-cold Hank's solution (Gibco) containing penicillin and streptomycin. The

spleens and the left femurs were used for subcutaneous ectopic implantation. The right femurs were 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 suspensions were put in a Falcon tube, and the larger bone particles were allowed to settle for 45 sec. Then the cell suspensions were passed twice through a 23 gauge-needle to obtain a single cell suspension.

CFU-F assay

CFU-F were quantified in a semisolid (0.8% methylcellulose, Methocel, AP4 Premium; Dow Chemical) culture medium (alpha medium) containing 20% fetal calf serum (FCS). Appropriate concentrations of bone marrow cells were plated in 35-mm culture dishes (Costar) in 1 ml of final culture medium. Triplicate cultures were incubated at 33 °C in a humidified atmosphere containing 10% CO_2 in air. After 10 days the cultures were washed with phosphate buffered saline (PBS, pH 7.3), fixed with methanol for 10 min and stained with 10% Giemsa. Colonies containing at least 50 fibroblastoid cells were counted using an inverted microscope.

Ectopic implantation method

Before implantation, the epiphyses of the femora were removed, and five small incisions were made in the splenic hilus to ensure optimal regeneration. Because the regeneration of subcutaneously implanted spleens is dependent on the total spleen volume of the recipient (Metcalf, 1964; Tavassoli, 1975) , host mice receiving spleens were irradiated with 5 Gy X-rays 1 day before implantation to stimulate splenic regeneration. Irradiation of host mice had no effect on femoral regeneration (Chamberlin, 1974; Chertkov, 1979). Host mice were anaesthetized with Avertin. One or two small incisions were made into the skin, after which a small 1.5 cm hole was made between the skin and the peritoneum using a blunt-tipped forceps. The spleen and femurs were placed subcutaneously as far as possible from the incision. The incision was closed with two sutures and histoacryl tissue adhesive. One spleen of two femora were implanted in each recipient mouse. Eight weeks after implantation the implants were removed and the number of CFU-S and CFU-C per implanted organ was assayed.

CFU-S assay

CFU-S were assayed according to Till and McCulloch (1961). Recipient mice received 9 Gy X-rays 1-4 hours before injection of cell suspensions into the lateral vein. This dose reduced the number of endogenous CFU-S-7 to 0. Aliquots of the ectopically transplanted femora and spleens were injected intravenously into 10 lethally irradiated recipients. 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 10x magnification.

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). Aliquots of the ectopically transplanted spleens and femora 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.

Regression analysis and statistics

Log-linear dose-effect curves were obtained by least-squares regression analysis. D_0 values were obtained from the slopes of these curves. Values for α and β were obtained using the linear-quadratic (LQ) model (Stata; release 2.05). Significance of the correlation coefficients was tested with a t-test according to procedures described by Sokal and Rohlf (1981). Values for all parameters in the tables are expressed as the arithmetic mean ($\pm$1 SEM) from three separate experiments except for Spl-impl[CFU-S] and Spl-impl[CFU-C], where only two experiments were performed. Eight dose groups were used for each experiment.

RESULTS

The effect of fission neutron and X-irradiation on CFU-F survival

The dose survival curves of CFU-F for fission neutrons and X-rays are shown in Figure 8.1. CFU-F are clearly more sensitive to fission neutrons than to X-rays. A D_0 value of 0.75 ± 0.04 Gy was calculated for neutron-irradiation, while after X-irradiation a D_0 value of 2.45 ± 0.37 Gy was obtained. The X-ray survival curve showed only a small shoulder indicating limited repair of sublethal damage. After fission neutron irradiation a steep dose-response curve was observed without any shoulder. An RBE value of 3.3 ± 0.5 Gy was obtained for the CFU-F by dividing the D_0 (X-rays) by the D_0 (neutrons) (Table 8.1). The X-ray data points were also fitted to the linear-quadratic (LQ) model. The α and β values obtained for the X-ray survival curve were 0.18 Gy^{-1} and 0.026 Gy^{-2}, respectively. The calculated α/β value was 7.1 Gy (Table 8.2).

The effect of fission neutron and X-irradiation on femoral marrow regeneration

The number of CFU-S (Fem-impl[CFU-S]) or CFU-C (Fem-impl[CFU-C]) present in a femur 8 weeks after subcutaneous implantation into a syngeneic host was used to quantify the stromal regenerative capacity following irradiation. Irradiation induced a dose-dependent decrease of Fem-impl[CFU-S] (Figure 8.2). Marrow CFU-S regeneration appeared to be more sensitive to neutron irradiation than to X-irradiation. After neutron irradiation, a D_0 value of 0.92 ± 0.05 Gy was obtained while after X-irradiation a D_0 value of 2.78 ± 0.41 Gy was found. Compared to the femoral CFU-F survival following irradiation, the Fem-impl[CFU-S] was slightly more radioresistant. The RBE value calculated for Fem-impl[CFU-S] was 3.0 ± 0.5. The α, β and α/β values obtained for Fem-impl[CFU-S] after analyzing the data points for X-ray survival with the LQ model were 0.20 Gy^{-1} for α, 0.036 Gy^{-2} for β and 6.1 Gy for α/β.

The capacity of femur implants to support CFU-C also appeared to be more sensitive to neutrons than the X-irradiation Figure 8.3. Survival curves obtained for Fem-impl[CFU-C] after neutron and X irradiation indicated D_0 values of 0.84 ± 0.05 Gy and 2.61 ± 0.43 Gy, respectively. The neutron RBE value was 3.1 ± 0.5. The α and β values obtained for Fem-impl[CFU-C]

Cell type	neutrons[a] D_0 value (Gy)	Correlation coefficient	X-rays D_0 value (Gy)	Correlation coefficient	RBE[b]
CFU-F	0.75 ± 0.04	-0.9750 ($p < 0.001$)[c]	2.45 ± 0.37	-0.8993 ($p < 0.001$)	3.3 ± 0.5
Fem-impl[CFU-S]	0.92 ± 0.05	-0.9548 ($p < 0.001$)	2.78 ± 0.41	-0.8696 ($p < 0.001$)	3.0 ± 0.5
Fem-impl[CFU-C]	0.84 ± 0.05	-0.9529 ($p < 0.001$)	2.61 ± 0.43	-0.8758 ($p < 0.001$)	3.1 ± 0.5
Spl-impl[CFU-S]	2.29 ± 0.33	-0.9102 ($p < 0.001$)	6.34 ± 1.35	-0.6047 ($p < 0.05$)	2.8 ± 0.7
Spl-impl[CFU-C]	1.49 ± 0.10	-0.9521 ($p < 0.001$)	4.85 ± 1.60	-0.6847 ($p < 0.001$)	3.3 ± 1.1

Table 8.1: Radiobiological characteristics of the stromal cell populations after 1 MeV fission neutron or X-irradiation.

[a]Arithmetic mean ± standard error of the mean (SEM) from three separate experiments except for Spl-impl[CFU-S] and Spl-impl[CFU-C] for which only two experiments were performed.

[b]RBE values were obtained by dividing D_0 (X-rays) by D_0 (neutrons).

[c]Significance of the correlation was tested with a t-test according to procedures described by Sokal & Rohlf (21).

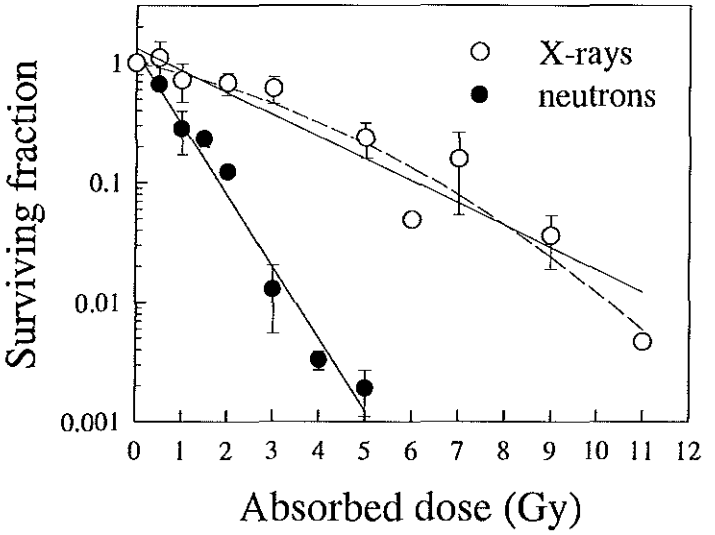

Figure 8.1: Survival curves of CFU-F after in vivo fission neutron irradiation (closed circles) or X-irradiation (open circles). Each solid line represents the regression line computed from three experiments using the linear model. The dotted line represents the fit of the LQ model. Mean absolute number of CFU-F per 10^5 unirradiated bone marrow cells: 11 CFU-F.

after X-irradiation were 0.26 Gy^{-1} and 0.029 Gy^{-2}, respectively. The calculated α/β value was 9.0 Gy

The effect of fission neutron and X-irradiation on splenic regeneration

The effects of neutron or X-irradiation on the regenerative capacity of ectopically implanted spleens ((Spl-impl[CFU-S]) and Spl-impl[CFU-C]) is shown in Figure 8.4 and Figure 8.5. As in femora, neutrons are more effective than X-rays in decreasing the regenerative capacity of the splenic stroma. Spl-impl[CFU-S] were observed to be far more radioresistant for both neutrons and X-rays than were the Fem-impl[CFU-S]. Survival curves following neutron irradiation had a D_0 value of 2.29 ± 0.33 Gy, while for X-irradiation a D_0 value of 6.35 ± 1.53 Gy was observed. X-ray doses between

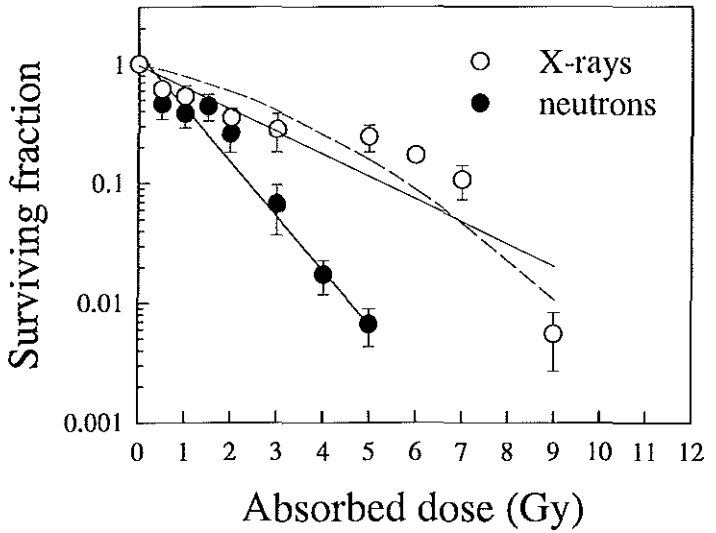

Figure 8.2: Dose-response curves for the ability of subcutaneous implanted femoral stroma to support CFU-S after fission neutron irradiation (closed circles) or X-irradiation (open circles). Each solid line represents the regression line computed from three experiments using the linear model. The dotted line represents the fit of the LQ model. Averaged control numbers: 660 CFU-S per unirradiated femoral implant.

1 and 5 Gy elicited a higher CFU-S content than that observed in unirradiated grafts. This was not the case after neutron irradiation, where a steep dose response curve was observed.

The effect of neutron or X-irradiation on the Spl-impl[CFU-C] is shown in Figure 8.5. The survival curves show a pattern similar to that for CFU-S regeneration. After neutron irradiation a D_0 value of 1.49 ± 0.10 Gy was found, compared to a D_0 value of 4.85 ± 1.60 Gy after X-irradiation, indicating that Spl-impl[CFU-C] are more radiosensitive than Spl-impl[CFU-S]. The RBE values obtained for the regeneration of splenic implants did not differ from the RBE values obtained for the regeneration of femoral

implants. The RBE values for Spl-impl[CFU-S] and Spl-impl[CFU-C] were 2.8 ± 0.7 and 3.3 ± 1.1, respectively.

	α (Gy^{-1})[a]	β (Gy^{-1})[a]	α/β (Gy)	Correlation coefficient
CFU-F	0.18 ± 0.09	0.026 ± 0.011	7.1	-0.9063 ($p < 0.001$)[b]
Fem-impl[CFU-S]	0.20 ± 0.11	0.036 ± 0.016	6.1	-0.9051 ($p < 0.001$)
Fem-impl[CFU-C]	0.26 ± 0.12	0.029 ± 0.016	9.0	-0.9075 ($p < 0.001$)

Table 8.2: α and β from the linear-quadratic model after X-irradiation.
[a]α and β values ± SEM are listed from three experiments.
[b]Significance of the correlation coefficients was tested with a t-test according to procedures described by Sokal & Rohlf (1981).

RBE values

The D_0 values for CFU-F, Fem-impl[CFU-S], Fem-impl[CFU-C], Spl-impl[CFU-S], and Spl-impl[CFU-C] are summarized in Table 8.1. The RBE values for the different stromal cell populations were found to vary from 2.8 to 3.3.

DISCUSSION

The hemopoietic stroma is extremely resistant to irradiation in situ. In vivo, little damage of stroma has been detected after exposures below 10 Gy X-rays (Knospe et al., 1988). Also, established confluent stromal layers in long-term bone marrow cultures do not exhibit any morphological changes of decreased viability and still are able to support hemopoiesis after irradiation with doses up to 50 Gy γ-rays (Zuckerman et al., 1986). The high radioresistance of the stromal cells under these conditions is due to their very slow turnover, which prevents the expression of radiation-induced damage during mitosis and allows them more time to repair radiation-induced damage than is the case with rapidly dividing emopoietic cells. However,

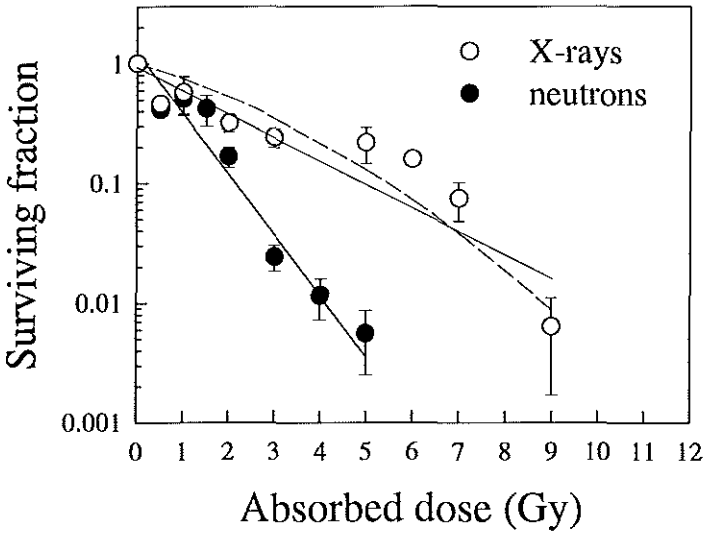

Figure 8.3: Dose-response curve for the ability of subcutaneous implanted femoral stroma to support CFU-C after fission neutron irradiation (closed circles) or X-irradiation (open circles). Each solid line represents the regression line computed from three experiments using the linear model. The dotted line represents the fit of the LQ model. Averaged control numbers: 6938 CFU-C per unirradiated femoral implant.

radiation damage may become overt even months after irradiation when stromal cells enter mitosis (Ploemacher et al., 1983). Acute radiation damage can be measured only by assays that induce stromal cells to proliferate. The CFU-F assay (Friedenstein et al., 1974) quantifies a single stromal precursor cell type. However, the hemopoietic microenvironment is a complex structure that is composed of many different cell types, and the measurement of CFU-F numbers as an indication of stromal integrity may lead to erroneous interpretations (Nikkels et al, 1987a). In the present investigation, we have determined the acute radiosensitivity of the hemopoietic stroma using a nonclonal functional in vivo assay. The extent to which proliferation and maintenance of hemopoietic stem cells is supported in subcutaneously

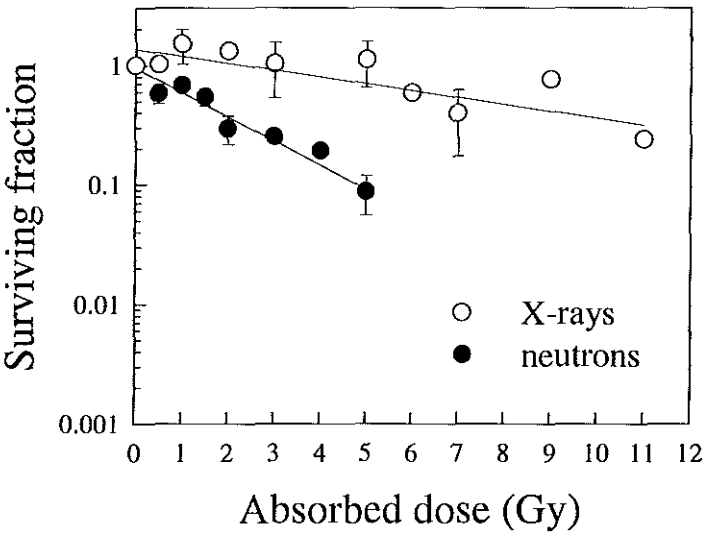

Figure 8.4: Dose-response curve for the ability of subcutaneous implanted splenic stroma to support the growth of CFU-S after fission neutron irradiation (closed symbols) or X-irradiation (open symbols). Each line represents the regression line computed from two experiments. Averaged control numbers: 269 CFU-S per unirradiated splenic implant.

implanted organs is determined by the integrity of a complex of stromal cell types. The response of the stroma to busulfan (Nikkels et al., 1987a) and the recovery pattern of the stroma after gamma radiation (Molineux et al., 1987, Nikkels et al., 1987b) have been observed to differ depending on the assay, indicating that these assays measure different stromal components. Endothelial cells may represent a predominant component in the regeneration of ectopic implants (Nikkels et al., 1987a, Molineux et al., 1987).

In the present investigation, we show that the acute radiosensitivity of marrow stromal progenitor cells for neutrons and X rays is much lower than that of hemopoietic stem or precursor cells (Imai & Nakao, 1987; Meijne et al., 1991). The CFU-F survival curve was characterized by D_0 values of 0.75 and 2.45 Gy for fission neutrons and X-rays, respectively. The latter D_0 value

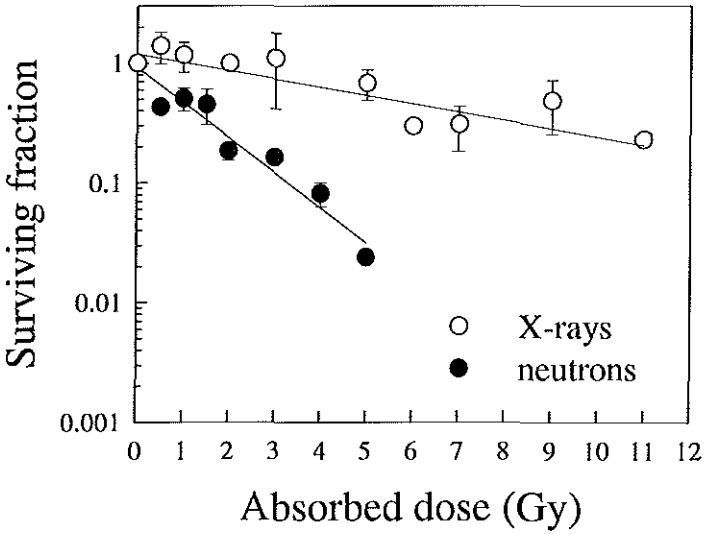

Figure 8.5: Dose-response curve for the ability of subcutaneous implanted splenic stroma to support the growth of CFU-C after fission neutron irradiation (closed circles) or X-irradiation (open circles). Each line represents the regression line computed from three experiments. Averaged control numbers: 669 CFU-C per splenic implant.

is in good agreement with the D_0 of 2.57 Gy obtained by Imai (1987) and 1.7 - 2.6 Gy obtained by Wilson (1974) following in vivo X-irradiation. The regenerative capacity of the femoral stroma (Fem-impl[CFU-S, CFU-C]) was less radiosensitive than the CFU-F. For Fem-impl[CFU-S], a D_0 of 2.78 Gy was derived, which is consistent with the D_0 value of 3.25 Gy obtained after transplantation of in vivo γ-irradiated bone marrow under the kidney capsule (Chertkov, 1979). The regenerative capacity of the splenic stroma was even more radioresistant for neutrons and X-rays. The RBE value for all stromal cell populations was close to three, which is comparable to the RBE of 3.2 obtained for intestinal-crypt stem cells, but much higher than the RBE of 2.1 for hemopoietic stem cells (Davids, 1973). The higher RBE value indicates that stromal cells are relatively more sensitive than hemopoietic cells to

neutron irradiation . In contrast to X-rays, almost all the damage caused by 1 MeV fission neutrons is irreparable and thus lethal. This means that the regeneration of the stromal cells after neutron irradiation takes place practically without sublethal damage repair (SLD repair) (Broerse & Barendsen, 1973; Barendsen, 1971) or potentially lethal damage repair (Hall, 1988). The higher RBE values obtained for stromal cells compared to hemopoietic cells are in agreement with the observation that stromal cells, in contrast to CFU-S, demonstrate a dose-rate-dependent radiosensitivity (Molineux et al., 1987; Hendry et al., 1984) and support the thought that stromal cells have a higher ability for SLD repair than hemopoietic cells. Repair of PLD enhances survival of stromal cells if several hours are allowed to elapse between irradiation in situ and removal of the cells from the host to assess their reproductive integrity (Hall, 1988; Hendry, 1983). However, in our experiments femora and spleens were removed several minutes after irradiation. This interval is so short that in the case of the CFU-F assay we do not think PLD repair can play an important role. For the ectopic implantation assays a second period exists wherein PLD repair is possible: the period between the implantation of the organs and their revascularization. The RBE value determined for Fem-impl[CFU-S] and Spl-impl[CFU-S], however, is not significantly different from the RBE value obtained for the CFU-F, indicating that PLD repair does not play a prominent role in our experiments.

The sensitivity of a tissue to low-LET radiation can be described using several models. The survival data for CFU-F and femoral-implants after X-irradiation were also fitted to the LQ model. The LQ model assumes that the mean number of lethal events can be described by $\alpha D + \beta D^2$ and is composed of a linear term, where the number of lethal events is proportional to dose, and a quadratic term, where two sublesions interact to produce a lethal event (Thames et al., 1987). Using this model, the α/β value for the stromal cell populations was estimated to range from 6.1 to 9.0 Gy. In general the α/β value is high (>6 Gy) for early responding, fast-proliferating tissues and low for late-responding tissues. Under the experimental conditions used in this study, the stromal cells therefore react as early responding tissue. However, β values derived from acute single-dose curves are prone to large statistical errors and are not as accurate as α/β values derived from data for fractionated exposures (Peacock, 1988). High LET radiation produces almost only lethal events. The quadratic term is therefore equal to zero and the mean number of lethal events is only described by αD.

The regeneration pattern of Spl-impl differed from that of Fem-impl. X-ray doses between 1 and 5 Gy elicited a higher CFU content than was observed in unirradiated spleen grafts. This can be explained by assuming that the dose-effect curves are determined by two different processes, i.e., increasing damage to the stroma with increasing dose and repair of radiation damage, which might be influenced by improved primary survival conditions due to elimination of non-stromal cells. The quick disappearance of irradiated hemopoietic cells from the implants may be of particular relevance for splenic implant regeneration. The angiogenic potential of splenic tissue is negligible. Spl-impl undergo almost complete necrosis because penetration of these implants by capillaries from surrounding tissues does not occur within the first 3 days. The viability of surviving cells within the first days is probably maintained by perfusion of the required nutrients from the surrounding subcutaneous tissue (Tavassoli et al., 1973). Thus rapid elimination of non-stromal cells may be essentially favourable under conditions such as these where nutrient supply is limited. Eventually, regeneration of the spleen implant ensues in the outer zone from a thin peripheral shell of surviving reticular cells, which regenerates into splenic tissue with a microscopic structure indistinguishable from the original structure (Tavassoli et al., 1973). The dose-effect curve for the regenerative capacity of the spleen after neutron irradiation shows no overshoot, since there is almost no repair after exposure to fission-neutron radiation. Bone marrow tissue, on the other hand, has considerable angiogenic potential. Within 12 h after implantation there is a spate of angiogenesis which leads to revascularization and the regenerative process (Tavassoli, 1984).

In conclusion, our data indicate that the acute radiosensitivity of stromal cell populations, as measured by ectopic organ implantation and CFU-F assays, is much higher than their in situ radiosensitivity. Also, under the conditions used in this study, in which stromal cells are induced to proliferate immediately after irradiation, they are still much more radioresistant for neutron or X- irradiation than hemopoietic cells, as is shown by the high D_0 values for the stromal cell populations. The RBE values calculated for the stromal cell populations, which ranged from 2.8 to 3.3, were higher than the RBE values obtained for hemopoietic cells, indicating that the stromal cells are relatively more sensitive than hemopoietic cells to neutron irradiation .

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LITERATURE

Barendsen, G.W. Cellular responses determining the effectiveness of fast neutrons relative to X-rays for effects on experimental tumours. Eur. J. Cancer 7, 181-190 (1971).

Broerse, J.J. and Barendsen, G.W. Relative biological effectiveness of fast neutrons for effects on normal tissues. Curr. Top. Radiat. Res. Q. 8, pp. 309-350, North-Holland, Amsterdam, 1973.

Chamberlin, W., Barone, J., Kedo, A. and Fried, W. Lack of recovery of murine hematopoietic stromal cells after irradiation-induced damage. Blood 44, 385-392 (1974).

Chertkov, J.L. and Gurevitch, O.A. Radiosensitivity of progenitor cells of the hematopoietic microenvironment. Radiat. Res. 79, 117-186 (1979).

Davids, J.A.G. Mos, A.P.J. and Oude, A. de. Fast-neutron facility for biological exposures in an Argonaut reactor: Design, tissue dosimetry and neutron spectrometry. Phys. Med. Biol. 14, 573-584 (1969).

Davids, J.A.G. Bone marrow syndrome in CBA mice exposed to fast neutrons of 1.0 MeV mean energy. Effect of syngeneic bone marrow transplantation. Int. J. Radiat. Biol. 17, 173-185 (1970).

Davids, J.A.G., Acute effects of 1 MeV fast neutrons on the haemopoietic tissues, intestinal epithelium and gastric epithelium in mice. In Advances in Radiation Research (J.F. Duplan and A. Chapiro, Eds.), Biology and Medicine, Vol. 2, pp. 565-576, Gordon and Breach, New York, 1973.

Dexter, T.M., Heyworth, C.M., Spooncer, E. and Ponting, I.L.O. The role of growth factors in self-renewal and differentiation of haemopoietic stem cells. Phil. Trans. R. Soc. Lond. B 327, 85-98 (1990).

Fried, W., Husseini, S. Knospe, W.H. and Trobaugh, F.E. Studies on the source of hematopoietic tissue in the marrow of subcutaneously implanted femurs. Exp. Hematol. 1, 29-35 (1973).

Fried, W., Chamberlin, W., Kedo, A. and Barone, J. Effects of radiation on hematopoietic stroma. Exp. Hematol. 4, 310-314 (1976).

Friedenstein, A.J., Petrakova, K.V., Kurolesova, A.I. and Frolova, G.P. Heterotopic transplants of bone marrow. Transpl. 6, 230-247 (1968).

Friedenstein, A.J., Chailakhyan, R.K. and Lalykina, K.S. The development of fibroblast colonies in monolayer cultures of guinea pig bone marrow and spleen cells. Cell Tissue Kinet. 3, 395-403 (1970).

Friedenstein, A.J., Chailakhyan, R.K., Latsinik, N.V., Panasuyk, A.F. and Keiliss-Borok, I.V. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. Transplantation 17, 331-340 (1974).

Gordon, M.Y., Riley, G.P., Watt, S.M. and Greaves, M.F. Compartmentalization of a haemopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. Nature 326, 403-405 (1987).

Hall, E.J. Radiobiology for the radiologist. 3rd edition, Lippincott, Philadelphia, 108-110 (1988).

Hendry, J.H. and Lord, B.I. The analysis of the early and late response to cytotoxic insults in the haemopoietic cell hierarchy. In Cytotoxic insults to tissue: effects on cell lineages (C.S. Potten and J.H. Hendry, Eds.), pp. 1-66, Churchill Livingstone, Edinburgh, 1983.

Hendry, J.H., Wang, S.B. and Testa, N.G. Greater sparing of stromal progenitor cells than of haemopoietic stem cells in y-irradiated mouse marrow using low dose-rates. Biomed. Pharmacother. 38, 356-358 (1984).

Hotta, T., Murate, T., Utsumi, M., Hirabayashi, N. and Yamada, H. Origins of hemopoietic and stromal cells in subcutaneous femur implants. Exp. Hematol. 11, 107-113 (1983).

Imai, Y. and Nakao, I. In vivo radiosensitivity and recovery pattern of the hematopoietic precursor cells and stem cells in mouse bone marrow. Exp. Hematol. 15, 890-895 (1987).

Knospe, W.H. Long-term bone marrow damage after irradiation. In Hematopoiesis: long-term effects of chemotherapy and radiation (N.G. Testa and R.P. Gale, Eds.) pp. 93-130, Marcel Dekker Inc., New York, 1988.

Metcalf, D. Restricted growth capacity of multiple spleen grafts. Transplantation 2, 387-392 (1964).

Meijne, E.I.M., Winden-Groenewegen, R.J.M. van der, Ploemacher, R.E., Vos, O., Davids, J.A.G. and Huiskamp, R. The effects of X-irradiation on hematopoietic stem cell compartments in the mouse. Exp. Hematol. 19, 617-623 (1991).

Molineux, G., Testa, N.G., Hendry, J.H. and Schofield, R. The radiation sensitivity of the haemopoietic microenvironment-effect of dose rate on ectopic ossicle formation. Radiother. Oncol. 10, 157-161 (1987).

Nikkels, P.G.J., Jong, J.P. de and Ploemacher, R.E. Long-term effects of cytostatic agents on the hemopoietic stroma: A comparison of four different assays. Leuk. Res. 11, 817-825 (1987a).

Nikkels, P.G.J., Jong. J.P. de and Ploemacher, R.E. Radiation sensitivity of hemopoietic stroma: Long-term partial recovery of hemopoietic stromal damage in mice treated during growth. Radiat. Res. 109, 330-341 (1987b).

Peacock, J.H., Cassoni, A.M., McMillan, T.J. and Steel, G.G. Radiosensitive human tumour cell lines may not be recovery deficient. Int. J. Radiat. Biol. 54, 945-953 (1988).

Piersma, A.H., Brockbank, K.G.M., Ploemacher, R.E., Vliet, E. van, Brakel-van Peer, C.M.J. and Visser, W.J. Characterization of fibroblastic stromal cells form murine bone marrow. Exp. Hematol. 13, 237-243 (1985).

Ploemacher, R.E., Brockbank, K.G.M., Brons, N.H.C. and De Ruiter, R. Latent sustained injury of murine hemopoietic organ stroma induced by ionizing radiation. Haematology 68, 454-468 (1983).

Roberts, R., Gallagher, J., Spooncer, E., Allen, E.D., Bloomfield, F. and Dexter, T.M. Heparan sulphate bound growth factors: A mechanism for stromal cell mediated haemopoiesis. Nature 332, 376-378 (1988).

Schofield, R. Standardization of procedures for ectopic marrow grafting: I. Influence of sex of recipient. Exp. Hematol. 14, 66-71 (1986).

Sokal, R.R. and Rohlf, F.J. Biometry, The principles and practice of statistics in biological research, 2nd edition, eds: Freeman, W.H., New York, 1981.

Tavassoli, M., Ratzan, R.J. and Crosby, W.H. Studies on regeneration of heterotopic splenic autotransplants. Blood 41, 701-709 (1973).

Tavassoli, M. Limitation of splenic growth as studied by heterotopic splenic implants. Blood 46, 631-635 (1975).

Tavassoli, M. Hemopoiesis in ectopically implanted bone marrow. In Long-term bone marrow culture (D.G. Wright and J.S. Greenberger, Eds.), pp. 31-54, Alan R. Liss, Inc., New York, 1984.

Thames, H.D. and Hendry, J.H. Fractionation in radiotherapy. Taylor & Francis, London, pp. 187-189 (1987).

Till, J.E. and McCulloch, E.A. A direct measurement of the radiation sensitivity of normal bone marrow cells. Radiat. Res. 14, 212-222 (1961).

Wilson, F.D., O'Grady, L., McNeill, C.J. and Munn, S.L. The formation of bone marrow derived fibroblastic plaques in vitro: preliminary results contrasting these populations to CFU-C. Exp. Hematol. 2, 343-354 (1974).

Zipori, D., Kalai, M. and Tamir, M. Restrictins: Stromal cell associated factors that control cell organization in hemopoietic tissues. Nat. Immun. Cell Growth Regul. 7, 185-192 (1988).

Zipori, D. Stromal cells from the bone marrow: Evidence for a restrictive role in regulation of hemopoiesis. Eur. J. Haematol. 42, 225-232 (1989).

Zuckerman, K.S., Prince, C.W., Rhodes, R.K. and Ribadeneira, M. Resistance of the stromal cells in murine long-term bone marrow cultures to damage by ionizing radiation. Exp. Hematol. 14, 1056-1062 (1986).

GENERAL DISCUSSION

The hemopoietic system is a very diverse and complex system and the disclosure of its functions and regulation mechanisms have been the subject of research for many decades. Hemopoiesis is maintained throughout life by a population of relatively few pluripotent hemopoietic stem cells (PHSC) that have the capacity to generate the progeny of all the different blood cell lineages as well as well as the ability to self renew (at least at the stem cell compartment level). Within the hemopoietic system self renewal is unique to PHSC. As PHSC give rise to mature stem cells their pluripotentiality gradually decreases until they differentiate into progenitors, which are committed to a particular line of cell development. Committed progenitor cells only possess limited self-replicative capacity, which serves to amplify their numbers prior to entering the maturation stages. This cascade of proliferation and differentiation enables the hemopoietic system to produce large amounts of functional blood cells by a limited number of PHSC. It is regulated by a large amount of cytokines which can interfere in the hemopoietic process at each stage and can increase or decrease the production of each cell type according to the need. Along the maturation pathway proliferation potential and the requirement for multiple growth factor stimulation decreases and the turnover rate increases.

Hemopoietic cells belong to the most radiosensitive cells of the mammalian body. Radiation doses in the mid-lethal range (LD_{50} ranges from 2.4 Gy for goats to 10 Gy for Mongolian Gerbrils (Carsten, 1984) lead to death due to the bone marrow syndrome (Davids, 1973; Carsten, 1984). The bone marrow syndrome is associated with granulocytopenia, thrombocytopenia and lymphopenia. Granulocytopenia is usually considered to be the injury of prime importance. Anaemia occurs as a result of haemorrhages, and hence is associated with thrombocytopenia. Anaemia is of less consequence in humans, but increases in importance in monkeys, dogs and guinea pigs, while being most important in mice and rats. In most species death occurs within 30 days with the exception of humans where significant numbers of death may occur between 25 and 60 days after exposure. Protection against death due to the bone marrow syndrome could be obtained by injection of bone marrow cells (Jacobson et al., 1951) and was later shown to be determined by the number of surviving mature hemopoietic stem cells (Ploemacher & Brons, 1988; Van Bekkum, 1991). Much radiobiological research has been focused on the bone marrow syndrome, because it is the only radiation syndrome where therapy (BMT and or supportive care) may lead to survival. Using the LD_{50} for total body

irradiation by X-rays, autologous or syngeneic bone marrow rescue numbers and estimates of total bone marrow cell numbers in different species, D_0 values between 0.5 and 0.75 Gy were derived for hemopoietic stem cells responsible for short-term hemopoietic reconstitution in the LD_{50} dose range (Vriesendorp & Van Bekkum, 1980; Van Bekkum, 1991).

The first quantitative assay to measure the numbers of hemopoietic stem cells was described by Till & McCulloch (Till & McCulloch, 1961). They discovered that bone marrow cell suspensions, which protected lethally irradiated recipients from hemopoietic death, contained cells capable of forming colonies on the spleens (CFU-S) of lethally irradiated mice. Originally the CFU-S assay was performed by irradiating bone marrow cells in the recipient and removing the spleens of the recipients 9 days after irradiation (McCulloch & Till, 1962). In this case the radiation response to the CFU-S is measured after transplantation into recipient mice. The lethal dose to the recipient mice is delivered in two fractions, the second of which is the test dose delivered to the transplanted cells. The magnitude of the second dose is variable when a survival curve for the transplanted CFU-S is to be obtained. A second method consists of irradiating bone marrow cells in donor mice and injecting the irradiated donor cells into recipients that had received a standard radiation treatment (Hendry & Howard, 1971). Using either of these assays the radiosensitivity of CFU-S has been determined. The radiosensitivity of CFU-S-7/9 fitted with the D_0 values calculated on the basis of dose survival relationships in the LD_{50} range and therefore CFU-S-7/9 have been considered as a measure of the radiosensitivity of PHSC, by many investigators for a large number of mice strains. Over the past decade, however, it has become clear that the stem cell compartment is heterogeneous and can be divided into several subpopulations, which can be physically separated using a variety of cell sorting protocols. These studies showed that short-term and long-term repopulating ability are the characteristics of different cell populations. CFU-S have a function in short-term hemopoietic reconstitution (Ploemacher & Brons, 1988; Sprangrude et al., 1988; Van Van Bekkum, 1991) but were shown to be incapable of long-term hemopoietic repopulation (Jones et al., 1990; Van der Loo et al., 1994) and therefore cannot be considered to be PHSC.

Studies on the heterogeneity of the hemopoietic stem cell compartment in the mouse have shown that at least 7 different stem cell populations can be identified and categorized by functional characteristics. These different stem cell populations, which may partly or significantly overlap, can be globally

ordered on be basis of their primitivity. In this thesis the radiosensitivity of these seven hemopoietic stem cell populations was determined for 300 kV X-rays and 1 MeV fission neutrons and investigated which factors might be responsible for the observed differences in radiosensitivity. The stem cell populations investigated were: a) cells with long-term repopulating ability (LTRA) b) primitive stem cells, which are able to repopulate the bone marrow of lethally irradiated recipients with CFU-S (MRA[CFU-S-12]) or in vitro clonable progenitors of granulocytes and macrophages (MRA[CFU-C]) c) cells capable of repopulating the spleen of lethally irradiated recipients with CFU-C (SRA[CFU-C]) d) cells forming spleen colonies 12 days after bone marrow transplantation (CFU-S-12) and e) cells forming spleen colonies 7 days after bone marrow transplantation. In addition the radiosensitivity of in vitro clonable progenitors of granulocytes and macrophages (CFU-C), cells belonging to the committed progenitor cell compartment, was determined.

In these studies bone marrow cells were irradiated in donor mice. This method has the advantages that a) the recipients receive a standard radiation treatment b) the response of cells in their natural environment is assayed (Hendry & Howard, 1971) and c) the same donor cell suspension can be used to determine the radiosensitivity of all cell populations assayed. CFU-S-7, which can be considered as belonging to the same cell population as CFU-S-9 (Magli et al., 1982), were used as representatives of the early appearing CFU-S and CFU-S-12 as representatives of the late appearing CFU-S in order to achieve an as large as possible difference in the CFU-S populations assayed.

LTRA cells and MRA showed the highest degree of radioresistance for X-irradiation (Chapter 3, 7) as well as fission neutron irradiation (Chapter 4,7), whereas stem cells differentiating into CFU-S-7 became progressively more radiosensitive, indicating that primitive hemopoietic stem cells are less sensitive to ionizing radiation than mature stem cells. MRA[CFU-C] were characterized by a D_0 value of 1.13 Gy after X-irradiation, which is comparable to the D_0 value of 1.25 Gy observed for this cell population after γ-irradiation (Ploemacher et al., 1992). The D_0 values observed for LTRA cells and MRA[CFU-S-12] after X-irradiation (1.09 Gy and 1.18 Gy, resp.) as well as fission neutron irradiation (0.47 Gy and 0.48 Gy, resp.) were comparable. Whether primitive stem cells have both LTRA and MRA or that LTRA and MRA are characteristics of two distinct cell populations remains a question for further research. Cell sorting studies in which primitive

hemopoietic stem cells were sorted on the basis of their affinity for the lectin wheat germ agglutinin showed that the enrichment factors for primitive stem cells with LTRA and MRA were not fully comparable, thus suggesting that LTRA cells represent a more primitive cell population (Ploemacher et al., 1993). Studies in which the cytotoxic effects of 7 different chemotherapeutic drugs on stem cells were investigated using the CAFC assay showed only after treatment with 5-FU a consistent and significant difference in cytotoxic effect on LTRA cells and MRA cells (Down & Ploemacher, 1993; Down et al., 1994). Until now attempts to completely separate LTRA from MRA cells have been unsuccessful. The currently available data indicate that LTRA cells and MRA cells are closely related and are equally sensitive to a number of cytotoxic agents. CFU-S-7 were found to be the most radiosensitive of the cell populations investigated. CFU-S were characterized by a D_0 value of 0.31 Gy for 1 MeV fission neutrons and 0.71 Gy for 300 kV X-rays.. The latter value is in agreement with the mean D_0 value of 0.77 Gy reported by Hendry (1983) for CFU-S-9 after 200-300 kV X-irradiation obtained by pooling several experiments of various authors. CFU-C were the most radioresistant cells. The neutron RBE values obtained for the various stem cell populations ranged from 2.2 to 2.6 and were comparable to the RBE of 2.3 obtained for the $LD_{50/30}$ of the same mouse strain. Subsequently it was investigated which factors might be responsible for the observed differences in radiosensitivity. The spatial distribution of the various hemopoietic cell populations across the femoral axis has been shown to differ (Lord et al., 1975). Local differences in oxygen tension as well as local dose differences in absorbed dose due to perturbation of the secondary particle equilibrium at the bone bone-marrow interface might therefore influence the radiosensitivity of the cell populations after in vivo irradiation. In totally hypoxic cells, low-LET radiation doses must be raised by a factor 3 to achieve the same cell killing effect as obtained in fully oxygenated cells. With neutron radiation the oxygen effect is not so prominent because high LET radiation damages its targets mostly directly and not through intervention of radicals. Although the comparable RBE values for the different cell populations did not point to differences in oxygen tension, sparing effects generated by hypoxia might be compensated by a local higher dose due to the disturbance of the secondary particle equilibrium and also the interference of other unknown factors could not be excluded. In chapter 5 the radiosensitivity of the various cell populations after in vitro irradiation and in vivo radiation under hypoxic conditions were determined. As expected the

oxygen enhancement ratio observed after neutron irradiation was small and ranged from 1.2 for MRA[CFU-C] to 1.45 for CFU-C. The OERs observed after X-irradiation were much higher for MRA[CFU-C] and CFU-C than for CFU-S indicating a lower oxygen concentration at the niches occupied by CFU-S, thus excluding hypoxia as a reason of the lower radiosensitivity of the primitive MRA[CFU-C]. The differences in radiosensitivity between the various subpopulations observed after in vitro radiation were similar to in vivo radiation indicating that local dose differences due to disturbance of the secondary particle equilibrium only had a minor effect on the observed radiosensitivity. In chapter 6 the effect of split-dose irradiation on the hemopoietic cell populations is described. As already expected from the exponential survival curves and small shoulders observed after single dose irradiation no large sparing effects were observed after split-dose irradiation. Dose recovery factors (DRF) varying from 1.12 for CFU-S-12 to 1.35 for CFU-C were obtained. Fractionated neutron irradiation had no sparing effect at all as evidenced by DRF values slightly below one. These data show that under the conditions used in these experiments no significant differences are observed in the split dose recovery of the various cell populations. These data were in contradiction with the higher split dose recovery obtained for MRA[CFU-C] after γ-irradiation (Ploemacher et al., 1992). Using a more extensive fractionation scheme (14 doses of 1.2 Gy at alternate 6 and 18 hours intervals) the same authors observed low α/β values of 4.0 Gy for CAFC day-35 (LTRA equivalent) and 4.3 Gy for CAFC day-28 (MRA equivalent) and high α/β values of 22.9 Gy for CAFC day-12 (CFU-S-12 equivalent) and 9.9 Gy for CAFC day-6 (CFU-C equivalent) (Down et al., 1995). Control experiments showed little evidence of proliferative repopulation for all cell populations investigated up to one week after a single dose of 6 Gy and hence the differential dose-sparing effect of fractionation among CAFC subsets appears mostly to be attributable to the influence of sublethal damage repair. In the latter experiments the bone marrow cells were assayed with a delay of 24 hours after the first radiation dose. To investigate if delaying the assay might be responsible for this difference the fractionation experiments were repeated using the delayed assay protocol. Delaying the assay had no effect on the split dose recovery observed. Instead somewhat lower D_0 values and much lower extrapolation numbers were observed. This is the result of delaying the assay (Van Putten, 1970) and is caused by a dramatic loss of CFU from the femur within the first 16 hours after irradiation (Hendry & Howard, 1971). Fractionation

studies on survival of C57Bl/6 mice showed extensive dose-sparing effects. A dose of 10 Gy given as a single dose or in 2.5-Gy increments at 2 hours intervals was fatal to 100% of the mice. The same dose administered in 2.5-Gy increments at an 22 to 24-hours interval caused no fatalities and resulted in an similar CFU-S depression, which however was followed by an exponential increase in CFU-S over the ensuing 12 days. These data indicate that fractionation also allows substantial repair of the mechanisms controlling proliferation of CFU-S and other cells responsible for short-term survival (Cronkite et al, 1994). The nature of these mechanisms remains a question for further research. Altogether the split dose experiments showed small dose sparing effects after X-irradiation for all subpopulations, indicating that differences in early split-dose repair are not responsible for the observed differences in radiosensitivity after 300 kV X-rays.

In Chapter 8 the effect of 300 kV X-rays and 1 MeV fission neutrons on proliferating stromal cells was determined. Two assays were used: a) the fibroblastoid colony forming unit assay and b) subcutaneous implantation of femora and spleens. Ectopic organ implantations can be considered as the in vivo equivalent and the forerunner of long-term bone marrow cultures. Stromal cells were less sensitive to ionizing radiation than hemopoietic cells. D_0 values of 0.75 Gy and 2.45 Gy, for neutrons and X-rays respectively, were obtained for the most sensitive stromal cell population (CFU-F) investigated. The regeneration of subcutaneous implanted spleen with CFU-S was even more radioresistant as evidenced with D_0 values of 6.34 Gy for X-rays and 2.29 Gy for fission neutrons. These data show that also proliferating stromal cells are much more radioresistant to ionizing radiation than hemopoietic cells. The RBE values for stromal cells ranged from 2.8 to 3.3, indicating that stromal cells are relatively more sensitive to neutron irradiation than hemopoietic cells.

In summary the experiments described in this thesis show the existence of a stem cell population which is less sensitive to X-rays and 1 MeV fission neutrons than CFU-S. These primitive hemopoietic stem cells were also shown to be less sensitive to γ-rays. The existance of two types of stem cell populations, one capable of rapid proliferation and responsible for the rapid production of mature stem cells and another responsible for long-term repopulation explains the good results obtained with supportive care in monkey and dog models (Gerritsen et al., 1988; Wielenga et al. 1989; Wagemaker, 1995; MacVittie et al., 1991). The difference in radiosensitivity of the various cell populations is not caused by local hypoxia, the position of

these cells relative to the bone or differences in split-dose repair. Other characteristics, as yet undefined, must therefore be responsible for the observed differences in radiosensitivity. Apoptosis, differences in chromatin structure, lesion repair fidelity and lesion tolerance all might play a role (Ward, 1990, McKay & Kefford, 1995). On the other hand different stromal niches may have a different impact on cell survival.

These results have important consequences for victims of radiation accidents, since prolonged radiation-induced pancytopenia is not due to lack of surviving stem cells, but to a lack of time to reconstitute the hemopoietic system with sufficient numbers of functional blood cells. Optimal supportive care (consisting of gastrointestinal decontamination, fluid and electrolyte substitution, treatment of infections, thrombocyte and whole blood transfusion and treatment with combinations of growth factor) might be sufficient to abridge and decrease the pancytopenic period and possibly replace bone marrow transplantation, especially after radiation accidents when there usually is no time to find suitable donors. Growth factor treatment should not only aim at accelerated production of functional blood cells but also at acceleration of stem cell regeneration.

REFERENCES

Carsten, A.L. Acute lethality. The hemopoietic syndrome in different species. In: Response to different species to total body irradiation. Eds. Broerse, J.J. and MacVittie, T.J. Martinus Nijhoff Publishers, Dordrecht, pp. 59-86 (1984).

Cronkite, E.P., Inoue, T. and Bullis, J.E. Influence of radiation fractionation on survival of mice and spleen colony-forming unit. Radiat. Res. 138, 266-271 (1994).

Davids, J.A.G. Acute effects of 1 MeV fast neutrons on the haematopoietic tissues, intestinal epithelium and gastric epitheliumin mice. In: Advances in radiation research, Eds. Duplan, J.F. and Chapiro, A., Gordon & Breach, New York, vol 2, pp. 565-576 (1973).

Down, J.D. and Ploemacher, R.E. Transient and permanent engraftment potential of murine hematopoietic stem cell subsets: differential effects of host conditioning with gamma radiation and cytotoxic drugs. Exp. Hematol. 21, 913-921 (1993).

Down, J.D., Boudewijn, A., Dillingh, J.H., Fox, B.W. and Ploemacher, R.E. Relationships between ablation of distinct haematopoietic cell subsets and the development of donor bone marrow engraftment following recipient pretreatment with different alkylating drugs. Br. J. Cancer 70, 611-616 (1994).

Down, J.D., Boudewijn, A., Van Os, R., Thames, H.D. and Ploemacher, R.E. Variations in radiation sensitivity among different bone marrow hemopoietic cell subsets following fractionated irradiation. Blood 86, 122-127 (1995).

Hendry, J.H. and Howard, A. The response of haemopoietic colony-forming units to single and split doses of γ-rays or D-T neutrons. Int. J. Radiat. Res. 19, 51-64 (1971).

Hendry, J.H. and Lord, B.I. The analysis of the early and late response to cytotoxic insults in the haemopoietic cell hierarchy. In: Cytotoxic insult to tissue. Effect on cell lineages. Eds: Potten, C.S. and Hendry, J.H., Churchill Livingstone, New York, pp. 1-66 (1983).

Jones, R.J., Wagner, J.E., Celano, P., Zicha, M.S. and Sharkis, S.J. Separation of pluripotent haemopoietic stem cells from spleen colony forming cells. Nature 347, 188-189 (1990).

Jacobson, L.O., Simmons, E.L., Marks, E.K. and Eldredge, J.H. Recovery from radiation injury. Science 133, 510 (1951).

Lord, B.I., Testa, N.G. and Hendry, J.H. The relative spatial distribution of CFU-S and CFU-C in the normal mouse femur. Blood 46, 65-72 (1975).

Magli, M.C., Iscove, N.N. and Odartchenko, N. Transient nature of early haemopoietic spleen colonies. Nature 295, 527-529 (1982).

MacVittie, T.J., Monroy, R., Vigneulle, R.M., Zeman, G.H. and Jackson, W.E. The relative biological effectiveness of mixed fission-neutron-g radiation on the hematopoietic syndrome in the canine: Effect of therapy on survival. Radiat. Res. 128, 29-36 (1991).

McCulloch, E.A. and Till, J.E. The sensitivity of cells from normal mouse bone marrow to gamma-radiation in vitro and in vivo. Radiat. Res. 16, 822-832 (1962).

McKay, M.J. and Kefford, R.F. The spectrum of in vitro radiosensitivity in four human melanoma cell lines is not accounted for by differential induction or rejoining of DNA double strand breaks. Int. J. Radiat. Oncol. Biol. Phys. 31, 345-352 (1995).

Ploemacher, R.E. and Brons, N.H.C. Isolation of hemopoietic stem cell subsets from murine bone marrow: I. Radioprotective ability of purified cell suspensions differing in the proportion of day-7 and day-12 CFU-S. Exp. Hematol. 16, 21-26 (1988).

Ploemacher, R.E., Os. R. van, Beurden, C.A.J. van and Down, J.D. Murine haemopoietic stem cells with long-term engraftment and marrow repopulating ability are more resistant to gamma-radiation than are spleen colony forming cells. Int. J. Radiat. Biol. 61, 489-499 (1992).

Ploemacher, R.E., Van der Loo, J.C.M., Van Beurden, C.A.J. and Baert, M.R.M. Wheat germ agglutinin affinity of murine hemopoietic stem cell subpopulations is an inverse function of their long-term repopulating ability in vitro and in vivo. Leukemia 1, 120-130 (1993).

Spangrude, G.J., Heimfeld, S. and Weissman, I.L. Purification and characterization of mouse hematopoietic stem cells. Science 241, 58-62 (1988).

Till, J.E. and McCulloch, E.A. A direct measurement of the radiation sensitivity of normal bone marrow cells. Radiat. Res. 14, 213-222 (1961).

Van Bekkum, D.W. Radiation sensitivity of the hemopoietic stem cell. Radiat. Res. 128, S4-S8 (1991).

Van der Loo, J.C.M., Van den Bos, C., Baert, M.R.M., Wagemaker, G. and Ploemacher, R.E. Stable multilineage hematopoietic chimerism in a-thalassemic mice induced by a bone marrow subpopulation that excludes the majority of day-12 spleen colony-forming units. Blood 83, 1769-1777 (1994).

Van Putten, L.M. In: Time and dose relationships in radiation biology as applied to radiotherapy. Eds. Bond, V.P., Suit, H.D. and Marcial, V. BNL report no. 50203 (C-57), Biology and Medicine, TID-4500, pp. 162-166 (1970).

Wagemaker, G. Heterogeneity of radiation sensitivity of hemopoietic stem cell subsets. Stem Cells 13 (suppl. 1), 257-260 (1995).

Ward, J.F. The yield of DNA double-strand breaks produced introcellularly by ionizing radiation: a review. Int. J. Radiat. Biol. 57, 1141-1150 (1990).

Wielenga, J.J., Van Gils, F.C.J.M., Wagemaker, G. The radiosensitivity of primate haemopoietic stem cells based on in vivo measurements. Int. J. Radiat. Biol. 55, 1041 (1989).

SUMMARY

Most mature blood cells have a limited lifespan varying from several days (granulocytes) to several months (erythrocytes) or even years (memory T-cells). To compensate for the daily loss of blood cells and to ensure proper functioning of the hemopoietic system new blood cells are produced continuously. An avarage human male produces every day 2×10^{11} red cells and 2×10^{9} granulocytes, a number which can be increased quickly after depletion of hemopoietic cells by cytotoxic agents like for example radiation. Mature cells of all blood cell lineages are produced by a small population of primitive pluripotent hemopoietic stem cells (PHSC) by a process called hemopoiesis. Under normal conditions only a few PHSC actively contribute to hemopoiesis. Most stem cells are quiescent and are triggered to proliferate later during the life span of an individual or function as reserve capacity in case of accidental depopulation of the hemopoietic system. Mouse studies have shown that a single primitive stem cell can participate in blood cell formation for over 2½ years. To be able to produce the large numbers of mature cells necessary each day by only a limited number of stem cells the hemopoietic system is organized hierarchically. With each cell division, cells gradually loose their primitiveness and differentiate from primitive PHSC through mature stem cells and committed progenitor cells into functional end cells of one of the blood cell lineages. The large number of cell divisions necessary to develop from a primitive stem cell to a functional blood cell function as amplification and control steps, thus enabling the system to produce many cells of the required cell type from a single PHSC. Over the past decade, it has become clear that also the stem cell compartment is heterogeneous and can be divided into cells with long-term repopulating ability (LTRA cells), cells with marrow repopulating ability (MRA cells), cells with spleen repopulating ability (SRA cells), cells forming spleen colonies 12 days after bone marrow transplantation (BMT) into a lethally irradiated recipient (CFU-S-12) and cell forming spleen colonies 7 days after BMT (CFU-S-7). All these different cell populations can be physically separated using a variety of cell sorting protocols and have been shown to differ in their sensitivity for a number of cytotoxic drugs.

In this thesis the radiosensitivity of these different stem cell subpopulations was determined for 300 kV X-rays and fast fission neutrons with a mean energy of 1 MeV and it was investigated which factors might be responsible for the observed differences in radiosensitivity. LTRA and MRA cells showed the highest degree of radioresistance for X-irradiation (Chapter 3,7) as well as fission neutron irradiation (Chapter 4,7), whereas cells

differentiating into CFU-S-7 became progressively more radiosensitive. The neutron RBE values obtained for the various stem cell populations ranged from 2.2 to 2.6 and were comparable to the RBE of 2.3 obtained for the LD_{50} of the same mouse strain. It has been suggested by some authors that the spatial distribution of the various hemopoietic cell populations across the femoral axis differs. Local differences in oxygen tension as well as local differences in absorbed dose due to perturbation of the secondary particle equilibrium at the bone - bone marrow interface might therefore influence the radiosensitivity of the cell populations after in vivo irradiation. Although the comparable RBE values for the different cell populations did not point to differences in oxygen tension, hypoxia might be compensated by a local higher dose due to the disturbance of the secondary particle equilibrium. In chapter 5 the radiosensitivity of the various cell populations after in vitro irradiation and in vivo radiation under hypoxic conditions was determined. As expected the oxygen enhancement ratio (OER) observed after neutron irradiation was small and ranged from 1.2 for MRA[CFU-C] to 1.5 for CFU-C. The OER observed after X-irradiation were much larger and were higher for MRA[CFU-C] and CFU-C than for CFU-S indicating a lower oxygen concentration at the niches for CFU-S, thus excluding hypoxia as a reason of the lower radiosensitivity of the primitive MRA[CFU-C]. The differences in radiosensitivity between the various subpopulations observed after in vitro radiation were similar to in vivo radiation indicating that local dose differences due to disturbance of the secondary partical equilibrium only had a minor effect on the observed radiosensitivity. In chapter 6 the effect of fractionated irradiation on the hemopoietic cell populations is described. As already expected from the exponential survival curves and small shoulders observed after single dose irradiation no large sparing effects were observed after fractionated irradiation. Dose reduction factors (DRF) varying from 1.12 for CFU-S-12 to 1.35 for CFU-C were obtained. Fractionated neutron irradiation had no sparing effect at all as evidenced by DRF values slightly below one. These data show that under the conditions used in these experiments no significant differences are observed in the split dose recovery of the various cell populations. To investigate if a delay between irradiation of donor mice and removal of bone marrow cells from the donors for assaying the radiosensitivity of the various cell populations might influence the results, the fractionation experiments were repeated using the delayed assay protocol. Delaying the assay had no effect on the split dose recovery

observed. Instead somewhat lower D_0 values and much lower extrapolation numbers were observed.

In Chapter 8 the effect of 300 kV X-rays and 1 MeV fission neutrons on proliferating stromal cells was determined. Two assays were used: a) the fibroblastoid colony forming unit assay and b) subcutaneous implantation of femora and spleens. Ectopic organ implantations can be considered as the in vivo equivalent and the forerunner of long-term bone marrow cultures. Stromal cells were less sensitive to ionizing radiation than hemopoietic cells. D_0 values of 0.75 Gy and 2.45 Gy, for neutrons and X-rays respectively, were obtained for the most sensitive stromal cell population (CFU-F) investigated. The regeneration of subcutaneous implanted spleen with CFU-S was even more radioresistant as evidenced with D_0 values of 6.34 Gy for X-rays and 2.29 Gy for fission neutrons. These data show that also proliferating stromal cells are much more radioresistant to ionizing radiation than hemopoietic cells. The RBE values for stromal cells ranged from 2.8 to 3.3, indicating that stromal cells are relatively more sensitive to neutron irradiation than hemopoietic cells.

NEDERLANDSE SAMENVATTING

Het bloedcelvormende systeem is een van de meest dynamische en gecompliceerde systemen van het menselijk lichaam. Bloed bevat verschillende soorten cellen die ieder een speciale functie hebben en alle essentieel zijn voor het in stand houden van het organisme. De vier meest bekende bloedcellen zijn de erythrocyten (rode bloedcellen), die verantwoordelijk zijn voor de opname en het transport van zuurstof en stikstof, de granulocyten en lymfocyten (die behoren tot de grote groep witte bloedcellen), die betrokken zijn bij de verdediging van het lichaam tegen infecties en zorgen voor de vernietiging van lichaamsvreemde stoffen die het lichaam binnen dringen en de thrombocyten (bloedplaatjes) die verantwoordelijk zijn voor de bloedstolling en er voor zorgen dat we bij een verwonding niet doodbloeden, maar dat er een korst op de wond gevormd wordt waardoor het bloeden stopt. De verschillende bloedcellen hebben elk slechts een beperkte levensduur die varieert van enkele dagen (granulocyten) tot enkele maanden (erythrocyten) of zelfs jaren (sommige lymfocyten). Om er toch voor te zorgen dat er altijd voldoende bloedcellen aanwezig zijn moet er iedere dag een groot aantal bloedcellen geproduceerd worden. Elke dag produceert een mens bijvoorbeeld 2 x 10^9 rode bloedcellen en 2 10^{11} granulocyten. Het proces dat zorgt voor de vorming van bloedcellen wordt hematopoiëse genoemd en vindt plaats in het beenmerg. Een kleine groep primitieve hematopoiëtische stamcellen is verantwoordelijk voor de produktie van bloedcellen. Door de vorming van een grote hierargische organisatie is het bloedcelvormende systeem in staat met een kleine groep primitieve stamcellen heel veel rijpe bloedcellen te maken. De uitrijping van primitieve stamcellen tot functionele bloedcellen vindt plaats via een groot aantal celdelingen. Bij iedere celdeling wordt het aantal cellen met een factor twee vermenigvuldigt. Doordat voor de uitrijping van een primitieve stamcel tot een functionele rijpe bloedcel heel veel celdelingen nodig zijn, kunnen vanuit één stamcel miljarden functionele bloedcellen gevormd worden. Het lichaam is in staat de vorming van rijpe bloedcellen te sturen met behulp van bloedhormonen. Indien bijv. extra rode bloedcellen nodig zijn, zorgt het lichaam met behulp van het hormoon erythropoiëtine ervoor, dat cellen zich uitrijpen tot erythrocyten. Op dit moment zijn er meer dan 30 verschillende bloedcelhormonen ontdekt die de uitrijping van primitieve stamcellen op verschillende niveau's kunnen beïnvloeden en de rijping van bloedcellen in verschillende richtingen kunnen sturen. Slechts enkele stamcellen zijn voldoende om het beenmerg van een bestraalde muis of patiënt te repopuleren en het individu gedurende zijn gehele verdere leven van

voldoende bloedcellen te voorzien. Het bijzondere aan primitieve stamcellen is, dat zij als enige cellen in staat zijn twee dochtercellen te vormen die precies of bijna precies dezelfde vermenigvuldigingscapaciteit hebben als de moedercel. Een primitieve stamcel heeft hierdoor de mogelijkheid twee nieuwe stamcellen te vormen of twee iets rijpere cellen die de richting van rijping tot functionele bloedcel zijn ingeslagen. Onderzoek aan muizenstamcellen heeft aangetoond dat één stamcel langer dan twee jaar in staat is om voor de productie van nieuwe rijpe bloedcellen te zorgen. Gezien de enorme vermenigvuldigingscapaciteit van stamcellen en de grote diversiteit van bloedcellen voor de produktie waarvan zij verantwoordelijk zijn, is het niet moeilijk het grote belang van stamcellen in te zien. Gedurende tientallen jaren is men daarom op zoek geweest naar methoden om primitieve stamcellen te kunnen identificeren en vervolgens te isoleren. De grote moeilijkheid van dit onderzoek was dat er slechts weinig stamcellen zijn en dat het uiterlijk van deze stamcellen niet duidelijk afwijkt van dat van lymfocyten. De eerste techniek waarmee men dacht primitieve hematopoiëtische stamcellen te kunnen aantonen stamt uit 1961. Toen werd ontdekt dat wanneer onrijpe beenmergcellen intraveneus werden ingespoten in letaal bestraalde muizen 7 tot 12 dagen na injectie witte knobbels op de milt van deze muizen zichtbaar waren. De cellen die in staat waren deze knobbels te vormen, werden functioneel aangeduidt als milt-kolonie vormende eenheden of wel (in het engels) colony-forming unit in the spleen (CFU-S). Later onderzoek naar de eigenschappen van deze cellen toonde aan dat de kolonies die zeven dagen na inspuiting van beenmergcellen op de milt van een muis zichtbaar zijn, afkomstig zijn van andere cellen dan de kolonies die 12 dagen na inspuiting van beenmergcellen op de milt zichtbaar zijn. Kolonies gevormd door CFU-S die in staat zijn om al 7 dagen na intraveneuze injectie een kolonie te vormen (CFU-S-7 genoemd) verdwenen na enkele dagen en nieuwe kolonies verschenen die door andere cellen gevormd werden. Deze cellen zijn iets primitiever en hebben twaalf dagen nodig om een kolonie op de milt te vormen en worden daarom functioneel aangeduid als CFU-S-12. Gedurende de laatste tien jaar is duidelijk geworden dat CFU-S niet de meest primitieve stamcellen zijn maar dat er eigenlijk een heel stamcelcompartiment bestaat, waarin verschillende celtypen onderscheiden kunnen worden. Deze verschillende stamcellen kunnen gerangschikt worden naar primitiviteit op grond van hun mitotisch verleden. Omdat al deze celtypen niet onderscheiden kunnen worden aan de hand van hun uiterlijke kenmerken, zijn ze vernoemd naar de functionele

eigenschap waarmee ze bepaald worden. De meest onrijpe stamcellen zijn de lange-termijn repopulerende stamcellen (in het engels cells with long-term repopulating ability (LTRA cellen)). Deze cellen worden functioneel gedefinieerd door hun vermogen bij te dragen aan de vorming van alle typen bloedcellen voor tenminste 4 maanden. Deze cellen kunnen bepaald worden door mannelijk cellen in te spuiten in vrouwelijke muizen en met behulp van een specifieke Y-probe (een stukje DNA dat alleen aan het mannelijke Y-chromosoom plakt en vervolgens met een kleurstof gekleurd kan worden) te kijken hoeveel cellen in het bloed van het ingespoten (mannelijke) beenmerg afkomstig zijn en hoeveel bloedcellen van de ontvanger (het vrouwtje) afkomstig zijn. LTRA cellen rijpen tot stamcellen die het vermogen hebben het beenmerg van bestraalde muizen te repopuleren met CFU-S of cellen die buiten het lichaam in kweekbakjes (in vitro) kunnen groeien (in het engels colony-forming units in culture genoemd ofwel CFU-C). Deze cellen worden in het engels 'cells with marrow repopulating ability' genoemd (afhankelijk van het feit of ze CFU-S cellen vormen of CFU-C spreekt men van MRA[CFU-S] of MRA[CFU-C]). Deze MRA cellen zijn dus niet in staat om direct een kolonie op de milt te vormen maar zorgen ervoor dat het beenmerg van een letaal bestraalde muis (de eerste ontvanger) met cellen bevolkt wordt die dat wel kunnen. Wanneer nu dit beenmerg in een tweede bestraalde muis wordt gespoten, zijn 12 dagen na injectie kolonies op de milt zichtbaar. Het aantal kolonies in deze tweede ontvanger is dus een maat voor het aantal MRA[CFU-S-12] cellen in de oorspronkelijke celsuspensie. Wanneer het beenmerg van de eerste ontvanger niet in een tweede bestraalde ontvanger getransplanteerd wordt maar in een kweekbakje wordt gekweekt is het aantal kolonies dat na een aantal dagen in het kweekbakje zichtbaar is een maat voor het aantal MRA[CFU-C] in het oorspronkelijke beenmerg. MRA cellen rijpen vervolgens uit tot SRA cellen, die het vermogen hebben de milt te repopuleren met CFU-S of CFU-C (SRA[CFU-S] of SRA[CFU-C]). Het aantal SRA[CFU-C] kan op dezelfde manier bepaald worden als het aantal MRA[CFU-C], alleen worden dan de miltcellen van de eerste ontvanger in plaats van de beenmergcellen in vitro gekweekt. SRA[CFU-C] rijpen vervolgens tot CFU-S-12 welke zich weer tot CFU-S-7 ontwikkelen. Op dit moment kunnen er dus minstens 6 verschillende stamcelsubpopulaties onderscheiden worden. Alleen LTRA cellen en MRA cellen kunnen beschouwd worden als echte primitieve stamcellen. Het doel van het onderzoek dat beschreven is in dit proefschrift was de stralingsgevoeligheid van al deze celpopulaties in vivo (in de muis) te bepalen voor 300 kV

röntgenstraling and 1 MeV splijtingsneutronen. De vorming van bloedcellen is echter niet alleen afhankelijk van de kwaliteit van de stamcellen, maar ook van het beenmergstroma dat onder andere zorgt voor de produktie van bloedhormonen en voor een omgeving waarin de stamcellen goed kunnen functioneren. Daarom is tevens de stralingsgevoeligheid van het stroma voor beide stralingssoorten onderzocht. 1 MeV splijtingsneutronen behoren tot de categorie hoge-LET straling. Dit houdt in dat deze stralingssoort veel ionisaties veroorzaakt over een korte afstand. Dit heeft tot gevolg dat in vergelijking met röntgenstraling bij een gelijke dosis minder cellen worden getroffen, maar dat de schade per cel groter is. Hierdoor hebben cellen die getroffen zijn door 1 MeV neutronenstraling minder mogelijkheden stralingsschade te herstellen en wordt het schadelijke effect van neutronenstraling minder of anders beïvloedt door externe factoren.

LTRA cellen en MRA cellen bleken minder gevoelig te zijn voor zowel röntgenstraling (hoofdstuk 3 en 7) als neutronenstraling. Wanneer deze cellen uitrijpten tot CFU-S-7 nam hun stralingsgevoeligheid sterk toe. De relatieve biologische effectiviteit (RBE waarde) van neutronenstraling lag voor alle stamcelpopulaties tussen de 2,2 en 2,6, wat aangeeft dat neutronenstraling per eenheid van dosis een factor 2,2 tot 2,6 schadelijker is dan röntgenstraling. Vervolgens is onderzocht waardoor dit verschil in stralingsgevoeligheid veroorzaakt kon worden. Door sommige onderzoekers is gesuggereerd dat de verschillende stamcelpopulaties zich op verschillende plaatsen in het beenmerg bevinden. Hierdoor zou de gemeten stralingsgevoeligheid beïnvloed kunnen worden door secondaire effecten zoals verschillen in zuurstofconcentratie en/of kleine verschillen in lokale geabsorbeerde dosis op de grensvlakken van verschillende weefsels. Daartoe werden de verschillende stamcelsubpopulaties onder anoxische condities (condities met een lage zuurstofspanning) bestraald en werden tevens beenmergcellen uit femurs van muizen geïsoleerd die vervolgens in reageerbuizen (in vitro) bestraald werden. Een lokaal lagere zuurstofspanning kan de schadelijke effecten van röntgenstraling verminderen doordat er in aanwezigheid van zuurstof meer radicalen gevormd worden. Radicalen zijn zeer schadelijk voor de cel waardoor in de schade per eenheid van dosis in de aanwezigheid van zuurstof hoger is. Omdat de schade per geraakte cel na bestraling met neutronen veel groter is, heeft de aanwezigheid van zuurstof weinig effect op de uiteindelijke schade veroorzaakt door neutronenstraling. Zoals verwacht had bestraling onder anoxische condities slechts een klein sparend effect op de stralingsschade veroorzaakt door neutronenstraling. Om een evengrote

stralingsschade als te bereiken als onder normale onstandigheden moest een 1,2 tot 1,5 grotere stralingsdosis gegeven worden. Voor bestralingen met röntgenstraling was het sparende effect veel groter en varieerde van 1,8 voor CFU-S-7 tot 3,0 voor MRA[CFU-C]. Het sparende effect voor CFU-S-7 was kleiner dan voor MRA[CFU-C]. Dit houdt in dat onder normale omstandigheden de zuurstofconcentratie ter plekke van CFU-S-7 lager is dan van MRA[CFU-C]. De grotere stralingsgevoeligheid van CFU-S-7 wordt dus niet veroorzaakt door een lokaal hogere zuurstofconcentratie. Indien de celpopulaties in vitro bestraald werden was het verschil in stralingsgevoeligheid vergelijkbaar met de in vivo situatie. Locale verschillen in dosis op de grensvlakken van verschillende weefsels zijn dus ook niet verantwoordelijk voor de verschillen in stralingsgevoeligheid van de verschillende celpopulaties. Een derde mogelijke factor welke de stralingsgevoeligheid van de verschillende celpopulaties kan beïnvloeden is een verschil in het vermogen van de verschillende stamcellen om stralingsschade te herstellen. Om dit te onderzoeken werd de totale dosis in twee fracties gegeven met een interval van 6 uur. In de 6 uur tussen de eerste en de tweede fractie kunnen de stamcellen de stralingsschade, veroorzaakt door de eerste fractie, repareren. Bestraling van van de celpopulaties in twee fracties had een klein sparend effect op alle celpopulaties. De gevonden verschillen in stralingsgevoeligheid worden dus niet veroorzaakt door verschil in het zogenaamde 'split-dose' herstel. Dit werd ook niet verwacht omdat dezelfde verschillen in stralingsgevoeligheid ook na neutronenbestraling gevonden werden en de schade aan geraakte cellen na neutronenbestraling zo groot is dat er vrijwel geen herstel van stralingsschade kan plaatvinden. Bestraling van de stamcelpopulaties met neutronenstraling in twee fracties had dan ook helemaal geen sparend effect. Tenslotte werd het effect van röntgen- en neutronenstraling op het stroma onderzocht. Het is reeds bekend dat stromale cellen die in rust zijn heel erg ongevoelig zijn voor straling. Over de stralingsgevoeligheid van stromale cellen die weer gaan delen is echter vrij weinig bekend. Met behulp van twee technieken werd de stralingsgevoeligheid van delende stromale cellen bepaald. Delende stromale cellen bleken minder gevoelig te zijn voor straling dan hematopoiëtische cellen. De gevonden RBE waarde voor stromale cellen was echter hoger wat aangeeft dat stromale cellen relatief gevoeliger zijn voor neutronenstraling dan hematopoiëtische cellen.

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ABBREVIATIONS

5FU	5-fluorouracil
a/ß	alpha beta ratio, describing the shape of the survival curve in the linear-quadratic model
Ag	antigen
AML	acute myeloid leukaemia
BFU-E	erythroid burst-forming unit
BSA	bovine serum albumin
CAFC	cobblestone area-forming cell(s)
CFU-C	colony-forming unit in culture
CFU-C	erythroid colony-forming unit
CFU-F	fibroblastoid colony-forming unit
CFU-GM	granulocyte macrophage colony-forming unit
CFU-S	spleen colony-forming unit
CFU-S-7	spleen colony-forming unit forming a spleen colony 7 days after transplantation into a lethally irradiated recipient
CFU-S-12	spleen colony-forming unit forming a spleen colony 12 days after transplantation into a lethally irradiated recipient
ConA	concanavalin A: a mitogen for T-cells
ConA-MSCM	concanavalin A mouse spleen conditioned medium
D_0	absorbed dose of ionizing radiation which reduces the surviving fraction of a cell population with a factor e^{-1} (0.37 or 37%)
DNA	deoxyribonucleic acid
DSB	double strand break
FCS	fetal calf serum
Gy	gray (unit of absorbed radiation dose), 1 Gy = 1 Joule per kg = 100 rad
HDR	high dose rate
Hprt	hypoxanthine-guaninephosphoribosyl-transferase
$LD_{50/30}$	radiation dose leading to death of 50% of the irradiated animals within 30 days after exposure, death is caused by the bone marrow syndrome
LDR	low dose rate

LET	linear energy transfer of the ionzing particles, the total energy transfer per unit of track length, expressed in keV per micro-meter
LTRA	long-term repopulating ability
MRA[CFU-C]	marrow repopulating ability defined as the number of secondary CFU-C in the bone marrow after 12 days, per 10^5 injected cells
MRA[CFU-S-12]	marrow repopulating ability defined as the number of secondary CFU-S-12 in the bone marrow after 12 days, per 10^5 injected cells
n	extrapolation number, point of extrapolation of the exponential part of a survival curve on the Y-axis
PBS	phosphate-buffered saline
PHSC	pluripotent hemopoietic stem cell(s)
PWM-MSCM	pokeweed mitogen-stimulated mouse spleen conditioned medium
RBE	relative biological effectiveness, in this study D_R/D_N, where D_R and D_N represent the absorbed dose of X-rays, respectively fission neutrons, which cause the same biological effect.
Rh123	rhodamine-123
Sca	stem cell antigen
SEM	standard error of the mean
SLDR	sublethal damage repair
SSB	single strand break
STRA	short-term repopulating ability
TBI	total body irradiation
WGA	wheat germ agglutinin

CURRICULUM VITAE

Emma Irmgard Maria Meijne was born in Amsterdam, The Netherlands, on June 1, 1960. After completion of secondary school (gymnasium b) in 1978 she worked for a year at different companies after which she started to study Medical Biology at the Free University in Amsterdam. In 1987 she obtained her masters degree (cum laude) with graduation subjects in oncology, tumour-immunology and cellular-biology. Between March 1987 and December 1991 she worked as a Ph.D. student at the department of Radiobiology and Radioecology (RBO) of the Netherlands Energy Research Foundation (ECN) in Petten to study the effects of X-rays and fission neutrons on the hemopoietic system. From June 1992 she is employed at the department RBO, ECN-Radiation Technology and involved in contract research investigating the effects of natural radioactivity in industrial processes and fundamental research investigating the mechanisms of radiation induced acute myeloid leukaemia. For the latter project she worked from November 1993 to May 1994 as a guest researcher at the National Radiological Protection Board in Chilton, United Kingdom.

PUBLICATIONS

Meijne, E.I.M., Van der Winden-van Groenewegen, R.J.M., Ploemacher, R.E., Vos, O., Davids, J.A.G. and Huiskamp, R. The effects of x-irradiation on the hematopoietic stem cell compartments in the mouse. Exp. Hematol. 19, 617-623 (1991).

Meijne, E.I.M., Ploemacher, R.E., Vos, O. and Huiskamp, R. The effects of graded doses of 1 MeV fission neutrons of X-rays on the murine hematopoietic stroma. Radiat. Res. 131, 302-308 (1992).

Meijne, E.I.M., Ploemacher, R.E., Vos, O. and Huiskamp, R. The sensitivity of murine hemopoietic stem cell populations for 1 MeV fission neutrons. Radiat. Res., submitted.

Meijne, E.I.M., Ploemacher, R.E., and Huiskamp, R. The sensitivity of murine hemopoietic stem cell populations to x-rays and 1 MeV fission neutrons in vitro, and under hypoxic conditions in vivo. Int. J. Radiat. Biol., submitted.

Meijne, E.I.M., Ploemacher, R.E. and Huiskamp, R. The effects of split-dose X-ray and fission neutron irradiation on different hemopoietic stem cell populations. Int. J. Radiat. Biol., submitted.

Meijne, E.I.M., Silver, A.R.J., Bouffler, S.D., Morris, D.J., Winter-van Kampen, E., Spanjer, S., Huiskamp, R. and Cox, R. The role of telomeric sequences in murine radiation-induced myeloid leukaemia. Genes Chrom. Cancer, in press.

Clark, D., Meijne, E.I.M., Bouffler, S.D., Huiskamp, R., Skidmore, C., Cox, R. and Silver, A.R.J. Microsatellite analysis of recurrent chromosome 2 deletions in acute myeloid leukaemia induced by radiation in F1 hybrid mice. Genes Chrom. Cancer, in press.

Bouffler, S.D., Meijne, E.I.M., Huiskamp, R. and Cox, R. Chromosomal abnormalities in neutron-induced acute myeloid leukaemias in CBA/H mice. Radiat. Res., accepted for publication.