MURINE HEMATOPOIETIC STEM-CELLS WITH LONG-TERM ENGRAFTMENT AND MARROW REPOPULATING ABILITY ARE MORE RESISTANT TO GAMMA-RADIATION THAN ARE SPLEEN COLONY-FORMING CELLS

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Murine haemopoietic stem cells with long-term engraftment and marrow repopulating ability are more resistant to gamma-radiation than are spleen colony forming cells

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Abstract. The radiation sensitivity of various subsets in the haemopoietic stem cell hierarchy was defined using a limiting dilution type long-term bone marrow culture technique that was previously shown to allow quantification of cells with spleen colony-forming potential (day-12 CFU-S) and *in vivo* marrow repopulating ability (MRA). Primitive stem cells that generate new *in vitro* clonable colony-forming cells (CFU-C) in the irradiated marrow (MRA) and have long-term repopulation ability (LTRA) *in vitro* (cobblestone area forming cell, CAFC day-28) had D_0 values of 1·25 and 1·38 Gy, respectively. A lower D_0 was found for the less primitive CFU-S day-12, CAFC day-12 and cells with erythroid repopulating ability (0·91, 1·08 and 0·97 Gy, respectively). CFU-S day-7 were the most radiosensitive (D_0 equalling 0·79 Gy), while CFU-C and CAFC day-5 were relatively resistant to irradiation (D_0 1·33 and 1·77 Gy). Split-dose irradiation with a 6 h interval gave dose sparing for stem cells with MRA and even more with *in vitro* LTRA, less for CFU-S day-12 and CAFC day-10 and none for CFU-S day-7. The cell survival data of the specified stem cell populations were compared with the ability of a fixed number of B6-Gpi-1^a donor bone marrow cells to provide for short- and long-term engraftment in single- and split-dose irradiated congenic B6-Gpi-1^b mice. Serial blood glucose phosphate isomerase (Gpi) phenotyping showed less chimerism in the split as compared to the single radiation dose groups beyond 4 weeks after transplant. Radiation dose-response curves corresponding to stable chimerism at 12 weeks for single and fractionated doses revealed appreciable split-dose recovery (D_2-D_1) in the order of 2 Gy. This was comparable to D_2-D_1 estimates for MRA and late-developing CAFC (1·27 and 1·43 Gy, respectively), but differed from the poor dose recovery in cells corresponding to the committed CFU-S day-7/12 and CAFC day-10 population (0·14-0·33 Gy). These data are together consistent with differential radiosensitivity and repair in the haemopoietic stem cell hierarchy, and provide a cellular basis for explaining the dose-sparing effect of fractionated total-body irradiation conditioning on long-term host marrow repopulation.

1. Introduction

The radiobiological characteristics of spleen colony-forming stem cells (CFU-S) in the murine bone marrow, especially those that produce early colonies at 7-9 days, have been extensively studied. These haemopoietic stem cell (HSC) subsets are relatively sensitive to radiation, characterized by steep cell-survival curves with slopes (D_0) that have been reported to range from 0·6 to 1·2 Gy (Till and McCulloch 1961, Hendry and Lord 1983). The dose-survival curves of CFU-S commonly show a small or absent shoulder and CFU-S are not spared by protracted or fractionated irradiation to implicate a reduced sublethal damage (SLD) repair (Fu *et al.* 1975, Glasgow *et al.* 1983, Peacock *et al.* 1986, Tarbell *et al.* 1987). Over the past decade, however, evidence has accumulated for extensive heterogeneity in the haemopoietic stem cell compartment. The applicability of the CFU-S assay as a measure of the genuine HSC, even when late-appearing colonies are being measured at around day 12, has been challenged since CFU-S can be physically separated from more primitive pre-CFU-S which repopulate the bone marrow, thymus or spleen (Bertoncello *et al.* 1985, Mulder *et al.* 1985, Ploemacher and Brons 1988a,b, Visser *et al.* 1990, Jones *et al.* 1990).

An appreciable dose-sparing effect of fractionated or protracted total-body irradiation (TBI) conditioning on subsequent long-term repopulation of host cells has been recently found in murine models of syngeneic or allogeneic bone marrow chimerism, and this contrasts with predictions obtained from postirradiation CFU-S survival (Down *et al.* 1991). The inability of bone marrow CFU-S survival to correlate with engraftment in the recipient demonstrates the need for a more careful study of the host target cell population that is responsible for donor marrow engraftment. In bone marrow transplantation (BMT), the formation of a permanent bone marrow chimera requires engraftment of primitive stem cells with the highest self-renewal at the expense of the analogous radiation-sterilized host stem cell population. Radiation-induced engraftment, especially syngeneic, may therefore compare better with the ablation of early host stem cells residing in the pre-CFU-S compartment.

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We have recently developed a miniaturized long-term bone marrow culture system (micro-LTBMC) that allows enumeration of these murine stem cells with long-term repopulating ability (LTRA) using limiting dilution analysis of cobblestone area forming cell (CAFC) frequencies (Ploemacher *et al.* 1989). The kinetics of clonal amplification in these cultures appear to reflect the primitiveness of HSC subsets (Ploemacher *et al.* 1989). Linear regression analysis of *in vivo* and *in vitro* data, obtained with a variety of physically sorted bone marrow cell populations, has indicated that the frequency of CFU-S day-12 (CFU-S-12) and MRA cells can be quantified with this CAFC-assay (Ploemacher *et al.* 1991). Thus, the frequency of cells giving rise to cobblestone areas around day 10 in micro-LTBMC (CAFC day-10) correlates with that of CFU-S-12, while enrichments for CAFC day-28 reflect *in vivo* MRA of a cell suspension. In the present study we have taken the opportunity to assess the radiosensitivity and split-dose recovery of a series of HSC subsets representing a cross-section through the stem cell hierarchy using this *in vitro* technique for close comparisons with parallel radiobiological experiments using *in vivo* cell survival assays and *in situ* competitive repopulation after TBI and syngeneic BMT.

2. Materials and methods

2.1. *Animals*

Male inbred (CBA × C57BL)F1 mice, 12–30 weeks old, were either bred in the Central Animal Department of the Erasmus University or purchased from the Medical Biological Laboratory ITRI (Rijswijk, The Netherlands). Animals were maintained under clean conventional conditions, and standards were in compliance with The Netherlands Experiments on Animal Act (1977) and the European Convention for the Protection of Vertebrate Animals used for Experimental Purposes (Strasbourg, 18 March 1986). The drinking water was acidified to pH 2·8. Donor mice for bone marrow irradiation experiments were always 12 weeks of age.

Male C57BL/6JIco (B6-Gpi-1^b) mice were obtained from the Central Animal Facilities of the University of Groningen. The C57BL/6J-Gpi-1^a (B6-Gpi-1^a) strain, congenic for the glucose-phosphate isomerase gene, was originally obtained from Jackson Laboratories (Bar Harbor, ME) and then produced in the Radiobiology Department in Groningen. These congenic mice have shown no evidence for histoincompatibility with the original C57BL/6J strain (Voralia *et al.* 1987).

2.2. *Irradiation*

Donor animals were irradiated in groups of five within a cylindrical Perspex chamber (21 cm diameter, 3·5 cm deep) with continuous air flow and positioned between two opposing ^{137}Cs sources (Gamma Cell 40, Atomic Energy of Canada, Ottawa, Canada) at a dose rate of 1·06–1·08 Gy/min. The dose-rate across the irradiation field varied by less than 5%. To assess the radiation sensitivity of the various HSC subsets with fractionated irradiation, donor mice were given doses of total-body irradiation as a single fraction or as two equal fractions with a 6 h interval. The bone marrow was removed from the femurs and tibias 24 h after the first radiation dose to allow for any potential lethal damage that may occur *in situ* (Thomas and Gould 1982) and without the occurrence of significant repopulation (McCulloch and Till 1964). Since CFU-S and, to a lesser extent, total marrow cellularity decreases after irradiation (Hendry and Howard 1971), the surviving fractions were calculated on the basis of HSC yield per hind limb and not as a proportion of the infused cells. Recipient mice for the CFU-S-7, CFU-S-12 and MRA assays received a potentially lethal dose of 9·27–9·30 Gy. For the bone marrow chimerism studies, 5-month-old male B6-Gpi-1^b recipient mice were γ-irradiated using a ^{60}Co source (Siemens-Gammatron3 Unit) at a dose rate of 42 cGy/min with either single doses (2–8 Gy) or two fractions separated by a 6 h interval (4–10 Gy total dose). At 3·5–4 h after irradiation treatment 10^7 nucleated bone marrow cells from the femurs and tibia of B6-Gpi-1^a donor mice were injected intra-orbitally under ether anaesthesia.

2.3. *Colony assays*

Bone marrow cells (BMC) were prepared by cleaning femurs and tibiae from muscles and tendons and grinding them in a mortar using PBS with 5% foetal calf serum. The cell suspensions were sieved over a nylon filter (mesh size 100 μm).

The CFU-S-7 and CFU-S-12 content of cell suspensions was determined by injecting the appropriate dilutions into a lateral tail vein of seven or eight lethally irradiated mice per experimental group between 2 and 4 h after irradiation. Seven to 10 control irradiated mice that did not receive cells

were included for all observation days in each experiment. Seven and 12 days later their spleens were excised, fixed in Telleyesniczky's solution, and the macroscopic surface colonies counted. No endogenous spleen colonies were found in the latter mice on day 7, while on day 12 an average of 0·2 colony per spleen was observed.

Quantitation of CFU-C (including CFU-M, CFU-G and CFU-GM) was performed using a semisolid (0·8% methylcellulose; Methocel AP4 Premium, Dow Chemical, Rotterdam, The Netherlands) culture medium (α-modification of DMEM) in a fully humidified incubator at 37°C and 5% CO_2. The cultures contained 10% pokeweed mitogen-stimulated mouse spleen-conditioned medium (MSCM), 20% horse serum (HS) and 1% BSA. Colonies were counted on day 7 of culture with an inverted microscope. In these experiments an average of 250 ± 30 (mean $\pm$ 1 SEM) CFU-C was found per 10^5 unirradiated nucleated cells plated.

2.4. *Marrow and spleen repopulating ability (MRA and SRA)*

The MRA and SRA describe the ability of a cell suspension to generate new progenitor cells in the bone marrow or spleen of lethally irradiated recipient mice over a period of 12 days, a period which is determined by the survival time of fatally irradiated mice not grafted with BMC. MRA and SRA are measured by injection of irradiated or control BMC into five lethally irradiated mice per group, and assaying different aliquots of their femoral marrow and spleen 12 days later for the presence of CFU-C. MRA and SRA were expressed as the number of CFU-C detected in one femur or spleen, calculated in terms of one organ equivalent injected, irrespective of the number of cells contained in that organ (Ploemacher and Brons 1988c). As an example, if an infused cell dose equalling 0·1 femur would generate 1000 CFU-C in one recipient's femur in a 12-day period, then the MRA[CFU-C] is 10^4. Control irradiated mice were included in each experiment and their endogenous CFU-C number per femur (50–200) or spleen (none) on day 12 was used to correct experimental data.

2.5. *Erythroid repopulating ability (ERA)*

The ERA characterizes a cell suspension with respect to its ability to produce reticulocytes. Similar to the MRA and SRA tests, fatally irradiated recipients were either not injected, or were infused with BMC from irradiated or control marrow donors. Twelve days later blood was collected by heart puncture under ether anaesthesia, total erythrocyte numbers determined using a Coulter Counter apparatus, and the percentage of reticulocytes determined using FACScan technology after staining the blood with acridine-orange. ERA was expressed as the absolute number of reticulocytes per ml of blood evoked per 10^5 nucleated cells injected. Reticulocyte number in non-infused control irradiated mice were used to correct the data.

2.6. *Micro-LTBMC*

LTBMC were produced and maintained as previously described (Ploemacher *et al.* 1989). LTBMC medium consisted of α-medium complemented with 10% foetal calf serum (Gibco, Breda, The Netherlands) and 10% horse serum (Boehringer, Mannheim, Germany), 0·5 mg/ml human transferrin (Hoechst-Behring, Amsterdam, The Netherlands), 10^{-5} mol/l hydrocortisone sodium succinate (Sigma, St Louis, MO, USA) and 10^{-4} mol/l β-mercaptoethanol (Merck, Amsterdam, The Netherlands). Briefly, flat-bottomed microtitre plates (Costar, Badhoevedorp, The Netherlands) were inoculated with $5\text{–}10 \times 10^5$ non-sieved BMC in 0·2 ml of LTBMC-medium per well and an adherent stromal layer was grown in 9–11 days at 33°C, 10% CO_2 and 100% humidity. The layers were then heavily irradiated (20 Gy ^{137}Cs γ-rays) in order to eliminate haemopoietic activity without affecting the ability of the stroma to support haemopoiesis (Zuckerman *et al.* 1986). One day later the medium was changed and various dilutions of fresh sorted or unsorted BMC were overlaid between 1 day and 6 weeks following irradiation to allow limiting dilution analysis of the precursor cells forming haemopoietic clones under the stromal layers in these cultures. A limiting dilution assay of a particular cell suspension included the use of eight to 12 dilution steps differing by a factor of 2–3, and 15–20 wells per concentration. Cultures were fed weekly by changing half of the medium. Between 3 and 28 days after overlay all wells were inspected daily, and scored as positive if at least one phase-dark haemopoietic clone (cobblestone area, CA, containing five up to more than 10^5 cells) was observed. The frequency of cobblestone area-forming cells (CAFC) was then calculated using the maximum-likelihood solution (Fazekas de St Groth 1982). We have previously demonstrated that a relationship exists between the primitiveness of the HSC population forming clones in a LTBMC

on the one hand, and the time delay before clonal expansion starts and the duration of clonal expansion on the other (Ploemacher *et al.* 1989, 1991). Thus, day-28 CAFC (CAFC-28) represent a primitive HSC subset detected in this system and their frequencies correlate with the MRA[CFU-C] of a cell suspension (coefficient of determination r^2 in a linear regression study is 0·79 using 53 observations) while CAFC-10 reflect CFU-S-12 frequencies *in vitro* (r^2 0·92 using 55 observations). The frequencies of CAFC-28 do not correlate with those of CFU-S-12 (r^2 0·39 using 51 observations), nor does the CAFC-10 frequency correlate with MRA[CFU-C] (r^2 0·25 using 53 observations).

2.7. *Calculation of* D_0 *and of* D_2–D_1 *values*

Surviving fractions relative to corresponding unirradiated controls were based on total yields per femur. Log-linear dose–effect curves were obtained by least-squares regression analysis with exclusion of the 0 Gy data. D_0 values were obtained from the slope of the curves. Statistical tests of D_0 values was performed with a chi-square test. Significance of the difference between CAFC data for single and fractionated irradiations was done using Student's *t*-test. The dose-sparing of split-dose irradiation was estimated on the basis of the difference in dose between the single dose (D_1) at 4 Gy and the split dose (D_2) to produce the same level of cell kill.

2.8. *Erythroid chimerism assay*

To determine the level of donor-type chimerism 20 μl of blood was taken from the orbital vein of recipient mice under ether anaesthesia at various time intervals after BMT. Erythrocytes were washed and then lysed by adding 180 μl distilled water. The haemolysate was stored at −20°C and later used for electrophoresis on cellulose acetate strips as described by Ansell and Micklem (1986). Gpi-1^a (donor) and Gpi-1^b (host) were separated during a 45-min run at 250 V on a semi-Micro-II-Chamber Gelman Sciences, Ann Harbor, MI). The bands were stained using a modified method of DeLorenzo and Ruddle (1969). The staining solution contained 5 mg MTT, 5 mg NADP, 40 mg $MgCl_2$, 15 mg fructose-6-phosphate, 500 μl phenazine methosulphate (2·5 mg/ml), 10 μl glucose-6-phosphate dehydrogenase and 60 ml Tris-HCl (pH 8·0) containing 25% polyethylene glycol. After staining, the strips were fixed in 5% acetic acid and the proportion of donor-type Gpi was calculated using a scanning densitometer (LKB, Uppsala, Sweden). The sensitivity of this assay was within 10% as determined from controlled mixing experiments.

3. Results

3.1. *Effect of high numbers of irradiated BMC on apparent CAFC frequency*

Femurs irradiated with 2–6 Gy have only about 40% of normal cellularity, yet the bone marrow is far more severely depleted for haemopoietic precursor cells. The measurement of such low stem cell frequencies using the limiting dilution type micro-LTBMC technique required that the stromal feeder layers were overlaid with a series of eight to 12 dilutions starting with $\frac{1}{10}$ of an irradiated femur content per well. This irradiated aliquot contained 50 times more nucleated cells, and many more erythrocytes, than is used for CAFC quantification in unirradiated bone marrow where the highest cell dose per well equals $\frac{1}{1000}$ part of a femur content. In order to detect a possible effect of these high irradiated cell numbers on the cloning efficiency of CAFC we set up a CAFC test in parallel, starting the dilution series with $\frac{1}{1000}$ of a normal femur with or without $\frac{1}{10}$ of the femoral content of a mouse that had received 12 Gy of TBI 24 h before. Figure 1 shows that the frequency curves for the various CAFC day-types are quite similar with a small tendency of inhibited clonal expression at 3–4 weeks of culture in the presence of the irradiated cells. Since the number of CAFC-28 correlates with the MRA of a cell suspension (Ploemacher *et al.* 1989, 1991), these data indicate that the CAFC assay might slightly underestimate MRA cells relative to CFU-S-12, which in turn correlate with CAFC-10/12.

3.2. *CAFC survival*

Intact animals received either a single dose of γ-rays at −24 h, or a split dose at 24 and 18 h before they were killed. A typical example of a CAFC frequency curve during 4 weeks of culture is presented in Figure 2. It appears that a single or split dose of 4 Gy γ-radiation affected clone formation around week 2 more than it did on week 1 or 4. In other words, CAFC-14 have lower survival than have CAFC-5 and CAFC-28 following this radiation dose. Survival curves of 4 CAFC day-types are presented in Figure 3. The CAFC content per femur was calculated for irradiated and for control mice,

and the survival expressed as fraction of the unirradiated control value. Radiation cell survival curves were made for all CAFC day-types and D_0 values calculated from the slopes using linear regression analysis (Figure 4). All curves showed a straight exponential decrease in CAFC survival following increasing total dose. It appeared that the less primitive CAFC, which form clones early after inoculation on the stromal feeders, are less radiosensitive than are CAFC-10-14 (D_0 values of 2·3 and 1·1 Gy, respectively). With increasing primitiveness of the HSC subsets towards CAFC-28 we observed a gradual increase in radioresistance to D_0 values of about 1·4 Gy. Split-dose recovery amplified the differential radiosensitivity of all CAFC subsets measured (Figures 4 and 5) with high D_0 and D_2–D_1 doses for the primitive CAFC-28 (1·85 and 1·43 Gy, respectively). Two-sample analysis of the difference between the split- and single-dose irradiations indicated a significance level between 0·97 and 0·91 for day 13 and later time points. Since the presence of the many irradiated cells per well led to a tendency of decreased CAFC-28 frequencies (Figure 1), the apparent low relative radiosensitivity of CAFC-28 may be underestimated in this case.

3.3. *Survival of other HSC subsets*

We have also determined the radiosensitivity of a series of *in vivo* assayable HSC subsets and CFU-C under similar conditions as used for CAFC. D_0 values for single and split dose irradiation are summarized in Table 1, where the various HSC populations, which may overlap, have been tentatively ordered according to increasing primitiveness in the haemopoietic stem cell hierarchy (Ploemacher and Brons 1988c, Rosendaal *et al.* 1979, Hodgson and Bradley 1984). The least primitive, *in vitro* clonable, CFU-C showed relatively low radiosensitivity (D_0 1·33 Gy). CFU-S-7 appeared most affected by irradiation (D_0 0·79 Gy), while the most primitive MRA cells again had relatively low radiosensitivity (D_0 1·25 Gy). CFU-S-12 (D_0 0·91 Gy) were less sensitive to the effects of γ-radiation than were CFU-S-7, but both HSC subsets were the least spared by a split-dose regimen (D_2–D_1 0·14 and 0·33 Gy, respectively). The more primitive MRA and SRA cells, that repopulated the bone marrow and spleen with new CFU-C, showed greater sparing when the dose was fractionated (D_2–D_1 1·27 and 1·42 Gy, respectively).

3.4. *Effect of split-dose TBI on development of blood chimerism*

Pretransplant conditioning with 6 Gy as a single dose and as two fractions of 3 Gy on the subsequent development of donor-type chimerism in the blood is given in Figure 6. This shows an increase in the level of chimerism with time until 10 weeks. After 10 weeks mixed chimerism remained stable out to 20 weeks post-transplant. Separation of the TBI treatment with a 6 h interval had the effect of inducing less donor blood chimerism. The difference between single-and split-dose TBI became more pronounced at later time points. In Figure 7 the radiation dose–response curves for stable engraftment at 12 weeks post-BMT are given. Here fractionation

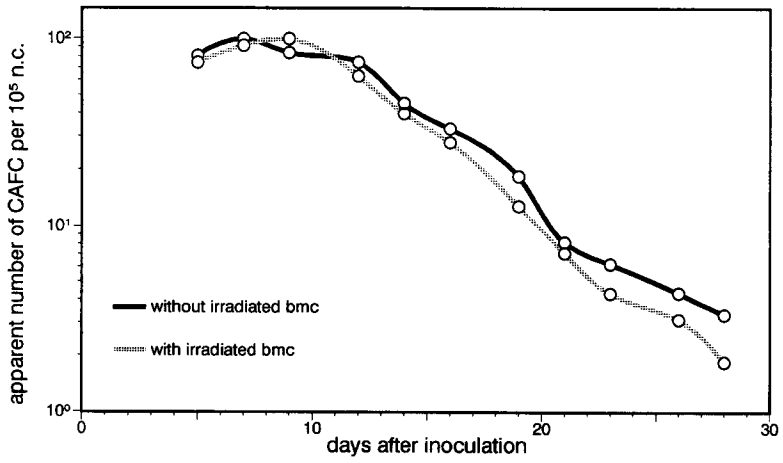

Figure 1. Effect of high numbers of irradiated bone marrow cells on the cloning efficiency of cobblestone area forming cells (CAFC) in limiting dilution type micro-LTBMC. Limiting dilution was started with 0·001 normal femur content per well in the presence or absence of 0·1 femur content of donor mice that had received 12 Gy TBI 1 day before.

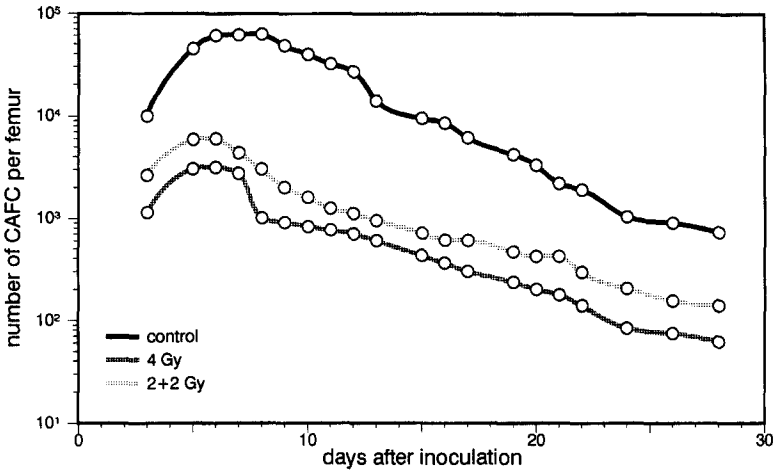

Figure 2. Gamma-radiation (4 Gy)-induced reduction of the CAFC frequency in mouse bone marrow varies with the ability of stem cells for long-term repopulation of stomal feeders *in vitro*.

shifted the response curve to higher radiation doses and enabled a determination of the extent of dose recovery (D_2–D_1) from split-dose (D_2) when compared with single-dose (D_1) TBI conditioning. To achieve the same level of engraftment as a single dose of 4 Gy, an extra 2 Gy was required when TBI was given in two equal fractions.

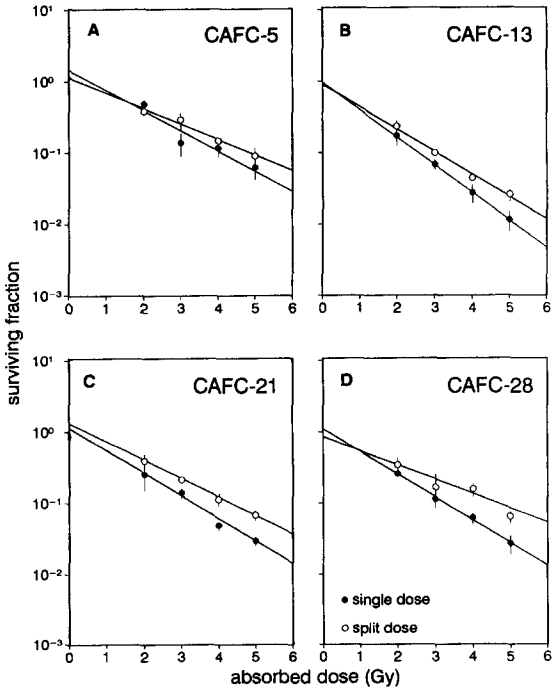

Figure 3. Single and split-dose survival curves for CAFC-5 (**A**), CAV3-13 (**B**), CAFC-21 (**C**) and CAFC-28 (**D**). Each curve represents the regression line calculated using data from five separate experiments. Bars denote 1 SEM. Mean absolute frequencies per 10^5 unirradiated cells were: CAFC-5, 90; CAFC-13, 92; CAFC-21, 12; CAFC-28, 3.

4. Discussion

This study was directed towards understanding the processes governing normal haemopoietic stem cell ablation after total-body irradiation and their importance in determining the success of BMT. Our data show that the survival of the primitive MRA and *in vitro* LTRA cells, and accordingly recipient type repopulation of the haemopoietic system following high-dose single or fractionated irradiation using a Gpi-mismatched chimaerism model, is importantly larger than previously estimated on the basis of radiation data for CFU-S. The data also suggest that the dose sparing with fractionated irradiation increases as a function of the primitiveness of the HSC subsets.

In BMT protocols that are aimed at eradicating leukaemic cell populations, high-dose total-body irradiation is often a major part of host preparation. The modification of TBI in fractionated and low-dose-rate protocols have reduced side-effects in intestine and lung, but also increased the proportion of patients failing to engraft donor marrow (Patterson *et al.* 1986, Guyotat *et al.* 1987, Iriondo *et al.* 1987). The events leading to graft failure are complex and the parameters involved include the presence of donor T-cells (Patterson *et al.* 1986, Martin *et al.* 1985, Mitsuyasu *et al.* 1986), the radiosensitivity of the host immune system and that of the normal and leukaemic cell populations. The reduced sublethal damage repair of CFU-S implied from fractionated or low-dose rate irradiation studies (Fu *et al.* 1975, Glasgow *et al.* 1983, Peacock *et al.* 1986, Tarbell *et al.* 1987) has been contrasted with the large repair capacity of dose-limiting late complications in the lungs and provided the basis for using fractionated and/or protracted TBI to prevent normal tissue

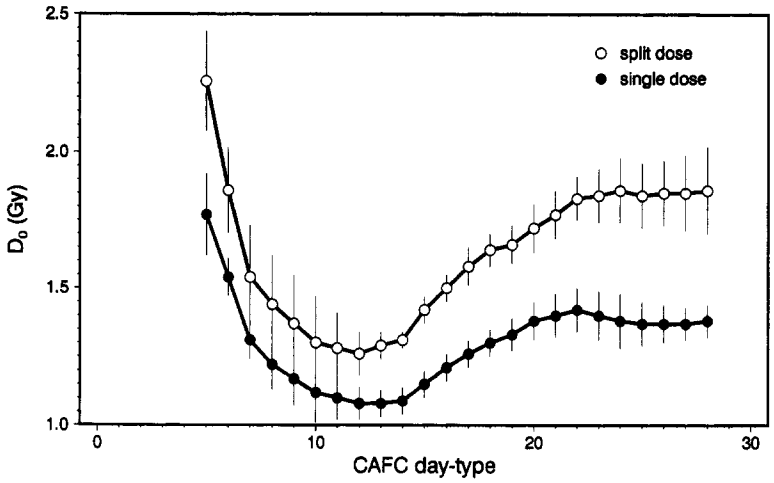

Figure 4. Summary of radiation sensitivities of various CAFC subsets showing clonal amplification between 5 and 28 days of culture. Data represent arithmetic means (1 SEM) of five separate experiments. Two-sample analysis using Student's *t*-test gave significance levels for the difference between split and single doses between 0·97 and 0·91 for day 13 and later time points. Single radiation dose, ●, split dose, ○.

toxicity. Since the CFU-S-12 assay does not measure the presence or frequency of primitive HSC with MRA and LTRA in a cell suspension (Jones *et al.* 1990, Ploemacher and Brons 1988c, Van der Sluijs *et al.* 1990), there is consequently little information regarding the radiosensitivity and repair capacity of cells in the pre-CFU-S stem cell compartment.

In the present comparative study on the radiation sensitivity of subsets from the HSC hierarchy we have used the recently characterized CAFC assay in addition to a complete series of *in vivo* stem cell assays. The obvious advantages of the CAFC assay includes the fact that culture conditions for the different HSC subsets are similar, and that data interpretation is not complicated by shifts in homing patterns or non-specific loss of cells in the microcirculation as is the case when applying *in vivo* stem cell assays. Recent studies carried out in our laboratory clearly demonstrate that the CAFC assay, when scored at 6–8 weeks, closely correlates with both the enrichments and actual frequencies of stem cells, that are responsible for long-term *in vivo* engraftment of both lymphoid and myeloid lineages in a sex-mismatched chimeric mouse model (unpublished observations). Finally, the CAFC assay may possibly also be made applicable to other species, including man.

The committed progenitor cells assayed after only

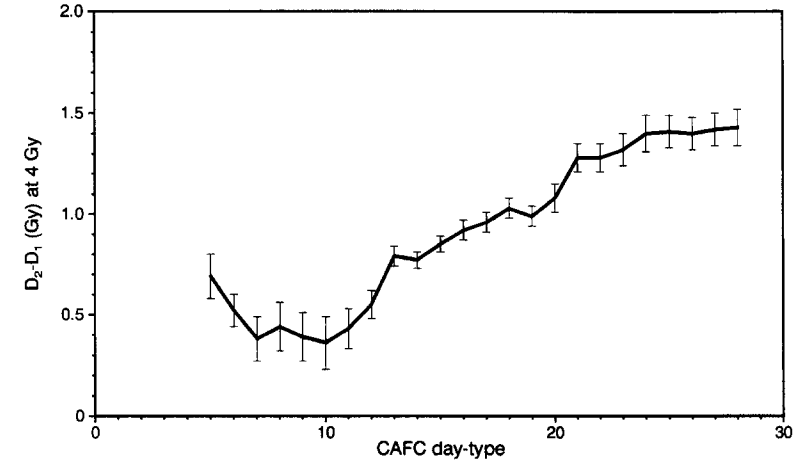

Figure 5. Dose-sparing (D_2–D_1) of split-dose irradiation for various CAFC subsets. Comparison of all doses was done at the same isoeffect survival level given by a single dose of 4 Gy. Bars denote 1 SEM for the difference D_2–D_1, and includes the SEM for D_1 and D_2.

Table 1. Radiosensitivity of stem cell subsets as determined by a variety of clonal and repopulation assays

Precursor subset	D_0 single dose	D_0 split dose	D_2-D_1 at 4 Gy
CFU-C	1·33 (0·10)	1·63 (0·20)	0·65 (0·29)
CFU-S day-7	0·79 (0·01)	0·82 (0·02)	0·14 (0·05)
ERA	0·97 (0·11)	1·13 (0·09)	0·66 (0·22)
CFU-S day-12	0·91 (0·01)	0·99 (0·11)	0·33 (0·13)
SRA	0·82 (0·03)	1·15 (0·15)	1·42 (0·36)
MRA	1·25 (0·12)	1·63 (0·14)	1·27 (0·31)

Data represent arithmetic means (1 SD) of three separate experiments in which three radiation doses (1, 3, 5 Gy, or 2, 4, 6 Gy, delivered either as single or fractionated dose) were included per experiment. CFU-C, colony-forming units in culture; CFU-S, spleen colony-forming cells; ERA, erythroid repopulating ability; SRA (spleen) and MRA (bone marrow) repopulating ability.

a few culture days have remarkable resistance to γ-radiation. This compares well with the relatively high D_0 of the CFU-C population following exposure to ionizing radiation as measured in this study and reported elsewhere (Hendry and Lord 1983, Imai and Nakao 1987, Baird *et al.* 1990, Meijne *et al.* 1991). With increasing probability for self-renewal of HSC their radiosensitivity sharply increases; however, then it gradually decreases again with CAFC-28 being relatively radioresistant. Clearly, the radiosensitivity as determined here for the various CAFC subsets does not merely reflect their cycle status as implied from their sensitivity to the cytostatic agent 5-fluorouracil (Hodgson and Bradley 1979, Ploemacher *et al.* 1989). In addition, the data strongly support the conclusions from our recent study on the different sensitivity of CFU-C, CFU-S-7 and CFU-S-12 and MRA cells for X-irradiation (Meijne *et al.* 1991).

The D_0 value of 0·79 Gy obtained for CFU-S-7 is in agreement with reported values in the range of 0·60–1·20 Gy for similar cell populations using γ-radiation or 200–300 kVp X-rays (Hendry and Lord 1983, Hendry *et al.* 1986, Meijne *et al.* 1991). Our data extend previous work showing that CFU-S-7 are more sensitive to X-irradiation than are CFU-S-12 (Meijne *et al.* 1991), and indicate that CFU-S-7 represent a highly radiosensitive HSC subset. In the CAFC assay the best fit with CFU-S-7 has been previously found around day 6, but the relatively low correlation coefficients prohibit the unequivocal use of the CAFC-6 assay for enumeration of CFU-S-7 (Ploemacher *et al.* 1991). The highest radiosensitivity values for CAFC are found between day 10 and 14. It is therefore possible that a highly radiosensitive subpopulation of CFU-S-7 is not expressed in the CAFC assay. However, the large differences in D_0 of the various CAFC subsets varying in primitiveness (CAFC-5, CAFC-10, CAFC-28) clearly reflect those of comparable subsets assayed using the conservative assays (CFU-C, CFU-S-12, MRA), both using γ-rays (present data) and X-rays (Meijne *et al.* 1991).

Not only are CAFC-28 and MRA cells less sensitive to the effect of a single exposure of γ-rays, they are also more spared by split-dose irradiation than are CFU-S-7 and CFU-S-12 and CAFC-10. These data show that the survival of the primitive MRA and LTRA cells, and accordingly recipient type repopulation of the haemopoietic system, following high-dose single or fractionated irradiation is importantly larger than previously estimated on the basis of radiation data for CFU-S. Thus, it can be calculated that the fractional survival of MRA is 16 times

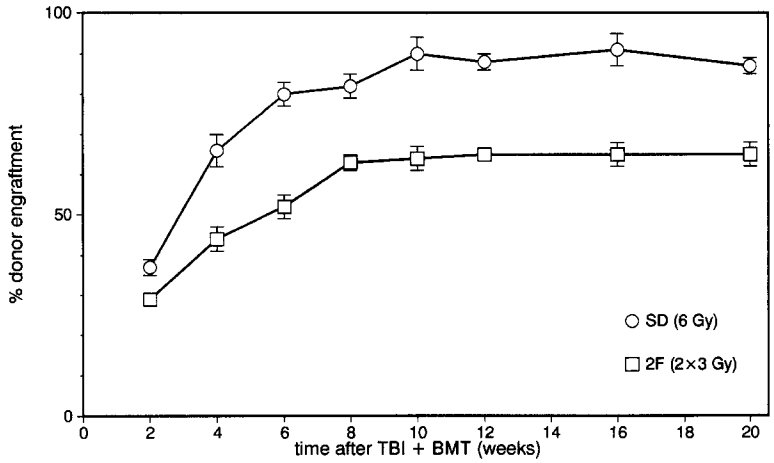

Figure 6. Level of erythroid donor (B6-Gpi-1^a) engraftment in B6-Gpi-1^b hosts as a function of time after 6 Gy TBI and syngeneic (10^7 cells) BMT. TBI was given either as a single acute dose (SD) of 6 Gy or as two fractions of 3 Gy with a 6 h interval (2F).

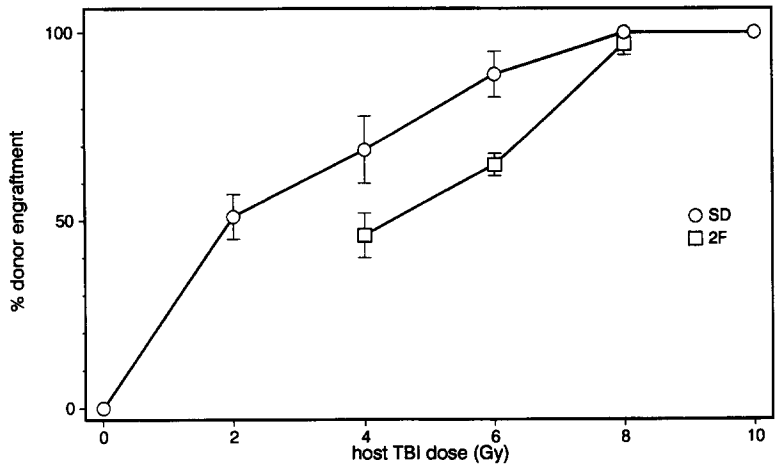

Figure 7. Donor marrow engraftment as a function of total TBI dose (radiation dose-responses) at 3 months after BMT to show dose-sparing effect of two fractions with a 6 h interval (2F) or a single radiation dose (SD).

higher than that of CFU-S-7 following an acute single dose of 6 Gy γ-irradiation, while it is even 37 times higher when the same dose is split. Our data suggest that the dose sparing with fractionated irradiation, as well as the radiosensitivity, is a function of the primitiveness of the HSC subsets.

The restored survival of primitive stem cells on introduction of a 6 h rest period between radiation treatments is consistent with an early intracellular repair process. This appeared to be more pronounced in cells that were already of higher resistance to acute radiation doses and it may therefore underlie the intrinsic variation in radiosensitivity among the HSC subsets. Repopulation on behalf of *proliferating* CFU-S has also been implicated in recovery during radiation treatment but it necessitates treatment times in excess of 1 day (McCulloch and Till 1964, Hendry and Howard 1971). Furthermore, it would have had the effect of increasing the radiation dose-survival of cycling CFU-S towards that of non-cycling pre-CFU-S. Within the interfraction time interval as used in our studies the quiescent proliferative state of the primitive HSC compartment may provide the conditions favourable to repair.

Our assessment of the corresponding modifying role of fractionated TBI on the development of long-term bone marrow chimerism has allowed us to ascertain the functional significance of HSC subsets after irradiation in the transplant setting. The appearance of the donor Gpi-1^a marker in the blood is dependent on the emergence of mature circulating erythrocytes following the engraftment, growth and differentiation from a particular HSC subset at the expense of radiation-sterilized host cells. The earliest donor erythrocytes will have originated from committed progenitor cells followed by a cohort of HSC subsets of increasing order of primitiveness with time until stable engraftment is achieved by continuous output from the most primitive stem cells of high self-renewal. Hence the pattern of blood chimerism after TBI should theoretically reflect the radiosensitivity of cells within the haemopoietic hierarchy. Splitting the radiation dose had the effect of encouraging host marrow repopulation and suppressing donor chimerism. The split-dose recovery was minimal at the early time points and progressively increased with time within the first 2 months. While the precise relationship between engraftment of specified HSC subsets and the development of erythroid chimerism has yet to be defined, these results are consistent with a higher repair capacity in pre-CFU-S with MRA and LTRA qualities as compared to transient CFU-S cells. The construction of radiation dose-response curves for long-term marrow engraftment allowed us to directly compare the extent of dose recovery, i.e. the extra dose required to produce the same effect (D_2-D_1), with that obtained from cell survival measurements using the *in vitro* or *in vivo* clonogenic assays. At the same isoeffect level given by a single dose of 4 Gy, the D_2-D_1 values of around 2 Gy are indeed close to those of MRA and CAFC-28. These similarities add special emphasis to the role of pre-CFU-S survival in long-term marrow engraftment.

It is interesting that analysis of data on dose-incidence relationships for marrow failure in mice has characterized the D_0 for tissue-rescuing units that prevent marrow failure to be about 1·0 Gy (Hendry and Roberts 1990). These data support and extend our suggestion that CFU-S-12 are responsible for

early survival of mice following acute radiation, while the more radioresistant primitive MRA and LTRA cells are unable to do so, but ensure long-term engraftment (Ploemacher and Brons 1989).

Increased radiation effects due to high doses in bone and neighbouring marrow cells, as suggested for orthovoltage X-rays (Hendry and Lord 1983), would be negligible in our use of higher-photon-energy γ-rays. Furthermore, the higher radioresistance of MRA cells as compared to CFU-S-7 has recently been shown to be retained in isolated bone marrow cell suspensions irradiated *in vitro* (unpublished results). Hence regional factors such as the level of oxygenation (Alalunis *et al.* 1983) and spatial relationships with bone surfaces (Hendry and Lord 1983) are unlikely to account for the disparate radiosensitivities observed in the present study.

In the light of the toxic effects of conditioning regimens antecedent to BMT, the present observations necessitate a reconsideration of established opinions concerning the high radiosensitivity of haemopoietic stem cells as inferred from *in vivo* spleen colony assays. The data also pertain to the modifying effects of fractionated irradiation and the role of radiation repair in primitive stem host cells responsible for long-term haemopoietic support. Hence host marrow repopulation, and possibly leukaemia regrowth, may not be adequately suppressed by extended irradiation treatments in the bone marrow transplant setting. These problems are of particular interest to recent clinical evidence for poorer donor engraftment (Patterson *et al.* 1986, Guyotat *et al.* 1987, Iriondo *et al.* 1987) and higher Leukaemic relapse (Scarpati *et al.* 1989, Socie *et al.* 1991) with fractionted or protracted TBI regimens.

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