PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 145, 1181–1186 (1974)

Impaired Elkind Recovery in Hematopoietic Colony-Forming Cells of Aged Mice[1,2] (37977)

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For several years we have been investigating changes with senescence in the number and activity of progenitor cells in the immunohematopoietic system (1–3). The changes fall into two general categories: those intrinsic to the cell populations (i.e., numbers, proliferating capacity) and those of an extrinsic nature (i.e., microenvironmental factors, humoral factors).

Variations in radiosensitivity as a function of the age of hematopoietic stem cells —the colony-forming units (CFU) of Till and McCulloch (4)—have been reported by Proukakis and coworkers (5, 6), who consider that there is some agreement between this intrinsic property and sensitivity to an acute midlethal dose ($LD_{50/30}$) of whole-body 15-MeV electron beam radiation (7). The most interesting finding in their published data on the radiosensitivity of the CFU from mice of different ages is the apparent absence of a significant shoulder on the dose–survival curve in the oldest (100 weeks) mice tested. This finding suggested to us an impaired ability to repair sublethal radiation damage of the type described by Elkind (8).

In the present work we investigated possible age-dependent changes in Elkind recovery of CFU from the bone marrow of a long-lived mouse hybrid, using radiation dose fractionation *in vivo* (9). A difference in the survival ratio for the 5-hr split-dose response was found between young and old cells, which could be attributed in part to a decreased radiosensitivity following the first dose in the young stem cells but not in the old.

Materials and Methods. Male (C57BL/Cum ♀ × C3H/Anf Cum ♂)F_1 mice (hereafter $BC3F_1$) ranging in age from 3 to 5 months were used as recipients of bone marrow. Young male $BC3F_1$ bone marrow donors were 3 months of age; old $BC3F_1$ donors (mostly male) were 25–27 and 33 months of age. The technique used for *in vivo* irradiation of the spleen CFU was that of McCulloch and Till (10); the method of dose fractionation and the expression of degree of repair as the survival ratio were from Till and McCulloch (9).

Using the latter method, Elkind recovery in CFU from bone marrow of young and old donors was assessed in young recipients. The recipient mice first received 500 R whole-body irradiation from a G.E. Maxitron X-ray machine under the following irradiation conditions: 300 kVp at 20 mA; ~168 R/min at a target–object distance of 70 cm; inherent filtration, 4.75 mm Be; added filtration, 3 mm Al; and a half-value layer of 0.470 mm Cu. Soon after this, bone marrow cells from the young or old donors were injected intravenously, and after an interval of 1 hr the animals received an additional 400 R of X-rays in two equal exposures separated by the desired interval of time. Nine days after the second X-ray dose, the mice were killed and their spleens were removed and fixed in Bouin's solution.

[1] Presented in part at the Symposium on Cellular and Macromolecular Aspects of Aging, 23rd Annual Biology Division Research Conference, Gatlinburg, Tennessee, April 6–9, 1970.

[2] Research sponsored by the U. S. Atomic Energy Commission under contract with Union Carbide Corporation.

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Macroscopically visible colonies on the surface of the spleen were counted under 10× magnification. The survival ratio obtained by this dose–fractionation procedure is the ratio of the percentage of CFU surviving after the fractionated dose to the percentage that survive the same total dose given in a single exposure (9).

In order to determine whether the survival ratio was influenced by a change in radiosensitivity at the time the second radiation fraction was administered, survival curves were determined at 5 and 10 hr after the first fraction. A first fraction of 200 R was again used, with 100, 200, or 350 R given as the second fraction. The initial exposures of whole-body radiation to the recipients (before transplantation) were 600, 500, and 350 R, respectively, making a total of 900 R to each recipient. Larger numbers of bone marrow cells were infused in the mice that received the higher initial exposures, to give sufficient numbers of splenic nodules for counting.

In addition to the above studies, more detailed data for survival after a single dose of radiation were obtained for mice from three different age groups (3, 25–27, and 33 months of age) to provide an estimate of the extrapolation number (n) and radiosensitivity (D_0) within reasonable confidence limits. The *in vivo* radiation technique of McCulloch and Till (10) was used, i.e., various doses of radiation were given to groups of 12 young recipient mice, which then were injected within 2 hr, with various numbers of cells, and 2 hr later a single dose of radiation was given to the recipients. Total exposure to the recipients was 900 R, which preliminary studies had shown to be sufficient to reduce endogenous CFU levels to less than one colony per spleen.

Dose–survival curves were analyzed by computer program (Metro Com Time Sharing, Charlotte, NC); exposures of 150 R and greater were used for determinations of slope and extrapolation number. Curves in the figures below represent computer-calculated least-squares fits to the data.

Results. Figure 1 shows the survival ratios obtained for CFU from old and young donors irradiated *in vivo* in young recipients with two equal fractions of 200 R separated by various intervals of time. Data are from five separate experiments, in two of which old and young CFU were run concurrently. The upper curve (young CFU) shows a maximum survival ratio at 5 hr of 3.1 ± 0.2, a value somewhat higher than the 1.9 reported by Till and McCulloch (9). The lower curve shows a slight maximum at 4–7 hr of ~1.5 ± 0.1, significantly different from the maximum value for young CFU.

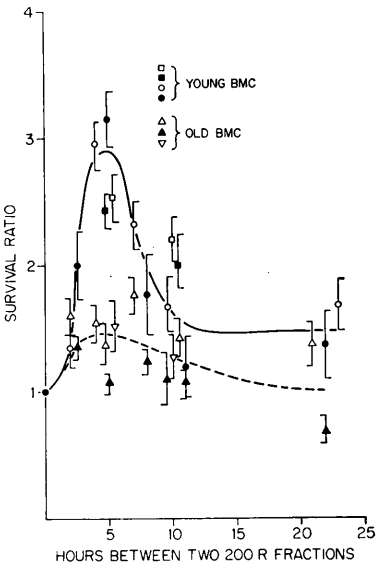

FIG. 1. Comparison of repair of sublethal radiation damage (Elkind recovery) in spleen CFU from bone marrow of old (25–27 months) and young (3 months) $BC3F_1$ mice, showing survival ratios as a function of the time interval between two 200-R doses of X-rays *in vivo*. Different symbols indicate separate experiments. Vertical bars represent ± one standard error of the mean.

Fractionation survival curves (11) for young and old CFU after various fractionated exposures are depicted in Figs. 2 and 3, respectively, and provide an *estimate* of D_0 and n for the CFU surviving the first dose. These data are summarized in Table I. There are significant changes in D_0 and n at 5 hr for the young CFU, similar to that reported by Frindel *et al.* (12) and by Hendry and Howard (13), and in this experiment the survival ratio for young

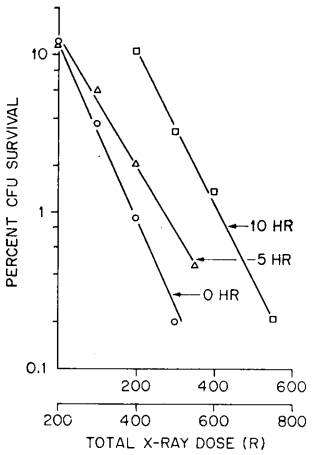

FIG. 2. Survival curves for spleen CFU from young (3 months) bone marrow cells irradiated *in vivo* with two doses of X-rays separated by various intervals of time. Curves for 0 (no fractionation) and 5 hr between doses, lower axes; for 10 hr between doses, upper axes.

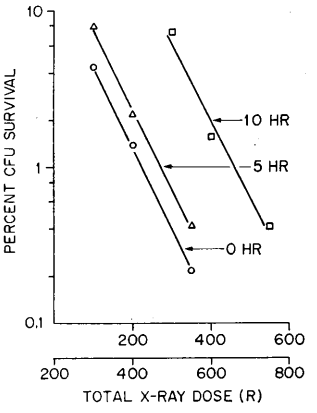

FIG. 3. Survival curves for spleen CFU from old (27 months) bone marrow cells irradiated *in vivo* with two doses of X-rays separated by various intervals of time. Curves for 0 (no fractionation) and 5 hr between doses, lower axes; for 10 hr between doses, upper axes.

CFU after two 200-R doses separated by 5 hr was smaller than that found previously (Fig. 1). In contrast, the D_0 at 5 hr was not significantly different for the old CFU, and n increased. On the basis of these curves, the survival ratio appears to be most influenced by the change in slope of the survival curves for fractionated doses in relation to those for single doses.

Dose–survival curves for unfractionated doses to CFU from donors 3, 25–27, and 33 months old are depicted in Fig. 4, and the radiosensitivities and extrapolation numbers (with 95% confidence limits) are given in Table II. In this hybrid mouse, there is no significant change in D_0 or n between 3 months and 2 years (15% cumulative mortality). However, there is an appreciable increase in D_0 for the 33-month-old mice. It should be pointed out that considerable selection by mortality is represented by these old mice, 80% of whose littermates had already died. Of greatest interest to the present studies was the absence of appreciable changes in the shoulders of any of the survival curves, as reflected in the extrapolation numbers. This is in contrast to the report of Proukakis *et al.* (6). All the ns were close to the 1.5

TABLE I. Split-Dose X-Ray Survival Curve Parameters for Old (25–27 Months) and Young (3 Months) Spleen CFU.

CFU	Dose	D_0 (rads)	n
Young	Single	73.0 ± 3.8[a]	1.5 (1.3–1.9)[b]
	Split (1st dose 200 R)		
	5 hr	99.5 ± 11.5	0.9 (0.7–1.2)
	10 hr	84.3 ± 7.3	1.0 (0.8–1.3)
Old	Single	77.4 ± 1.9	1.7 (1.4–2.2)
	Split (1st dose 200 R)		
	5 hr	80.1 ± 4.1	2.5 (1.6–4.0)
	10 hr	83.7 ± 13.9	1.9 (0.5–7.0)

[a] Standard error of the estimate.

[b] 95% confidence interval.

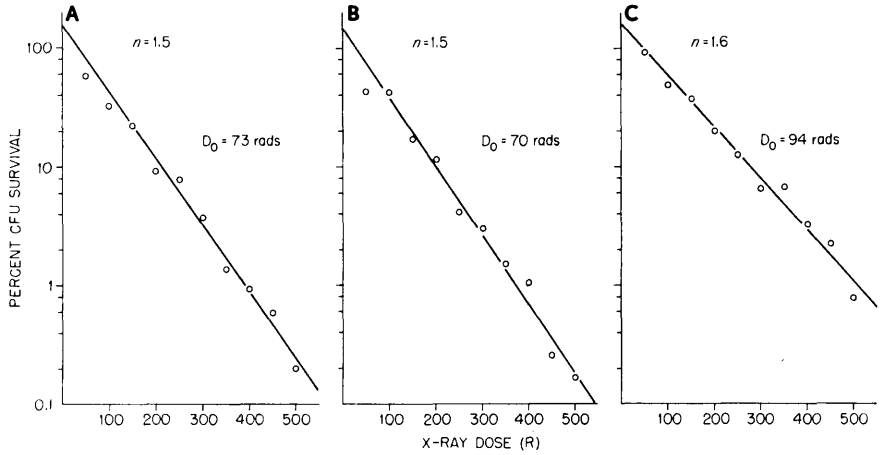

FIG. 4. Survival curves for spleen CFU in bone marrow transplants from mice of three different ages irradiated *in vivo* with a single dose of X-rays. (A) 3 months, (B) 25–27 months, (C) 33 months.

value originally reported by McCulloch and Till (10).

Discussion. Comfort (14) has defined senescence as a deteriorative process in which there is a decrease in viability and an increase in vulnerability. Aside from producing morphological changes which in some ways resemble senescence, ionizing radiation provides a quantitative tool with which to study the vulnerability of the organism to injurious agents. An index of this vulnerability is the ability of the stem cells of a cell renewal system with rapid turnover to recover from sublethal radiation damage.

The present studies provide evidence for an age-related decrease in the ability of hematopoietic stem cells to recover from sublethal radiation damage. With the *in vivo* dose–fractionation method of Till and McCulloch (9), a significant difference in the survival ratios at the time of maximum early repair (about 5 hr between fractions) was found between young and old CFU irradiated in young recipients. The fractionation survival curve for the young CFU at 5 hr (Fig. 2) shows a significant increase in D_0 (with no increase in n), which apparently contributes strongly to the enhanced survival ratio at this maximum. Both values, of course, are extremely susceptible to the arbitrary nature of curve-fitting over a mere 100 fold kill range. This shift in D_0 and decrease in n at 5 hr agree with data of Hendry and Howard (13), who used a method involving dose fractionation to the donor mice followed by assay in lethally irradiated recipients, but disagrees with the original work of Till and McCulloch (9), in which an increase in n with little change in D_0 was implied to be the contributing factor in the enhanced survival.

TABLE II. Single-Dose X-Ray Survival Curve Parameters for Spleen CFU from $BC3F_1$ Mice 3, 25, and 33 Months Old.

Age of mice (months)	D_0 (rads)	n
3	73.0 ± 3.8[a]	1.5 (1.3–1.9)[b]
25	70.4 ± 4.2	1.5 (1.2–1.8)
33	94.2 ± 6.8	1.6 (1.3–2.0)

[a] Standard error of the estimate.
[b] 95% confidence interval.

On the other hand, the data from the 5-hr fractionation survival curve for the old CFU (Fig. 3) support the concept of the restoration of a shoulder, although this is not apparent from the low survival ratio. The reproducibility of the lower survival ratio in the old as compared to the young CFU in several experiments is convincing evidence to us that there is an age-related change. Comparison of the fractionation survival curves for young and old CFU suggests that the old CFU surviving the

first 200 R may not be as radioresistant as the young survivors.

A second surprising finding is the similarity of the single-dose (0 hr) curves of young and old stem cells with respect to slope (i.e., D_0) and extrapolation number, which contradicts the report of Proukakis *et al.* (6). This finding prompted the more detailed single-dose studies (Fig. 4), which substantiate the earlier observations; i.e., comparison between CFU from donors 3 and 25 months old (the ages used in the dose-fractionation experiments) shows no significant differences in the parameters of the survival curves. Both D_0 values are in close agreement to those reported by McCulloch and Till (10), allowing correction for the difference in the relative biological effect of γ-rays and 300-kVp X-rays. In all three age groups, the *n*s are virtually the same. RBE values for electrons in the energy range and at the dose rate used by Proukakis and coworkers (5) are similar, and we are not aware of studies showing a unique radiobiological effect for that modality (R. F. Kallman, personal communication).

The apparent similarity in the dose–survival curves for unfractionated exposures, however, does not preclude considerable heterogeneity with age with respect to the radiosensitivities of the populations making up each tissue (15, 16). Perhaps perturbation by a first fraction of radiation brings out these differences. An attractive hypothesis, along lines outlined by Kallman (17), is that there may be age-related differences in the distribution of CFU in the various phases of the cell cycle and/or in the radiosensitivities of the phases of the cell cycle themselves. Duplan and Feinendegen (18) and Chaffey and Hellman (19) have recently reported different radiosensitivities for CFU in different phases of the cell cycle, which parallel findings for cultured cells (20, 21).

Although age-related differences in sensitivities and numbers of CFU in various phases of the cell cycle are attractive concepts, it must also be remembered that in the steady state most CFU are in G_0 (i.e., not in the cell cycle) or in a long G_1 phase (22). Lindop and coworkers [as reported in Lajtha and Schofield (23)] have reported a slight increase with age in the number of CFU in cell cycle, as determined by the percentage of cells that are killed by high-specific-activity tritiated thymidine (8% at 30 weeks, 13% at 3 years); but we have (Chen and May, unpublished data) found no significant difference between the number of CFU in the cell cycle in 3- and 33-month-old mice. An increase in G_0 cells with senescence might find some analogy to certain plateau-phase mammalian cells *in vitro* which apparently lose their capacity for Elkind recovery (24). Finally, senescence might contribute to a defective or retarded recruitment of stem cells from the resting state (G_0) into the cell cycle, so that cells would not progress into relatively radioresistant phases after the first sublethal radiation challenge.

The best evidence at present for age-related changes in the cell cycle of progenitor cells of a cell-renewal system comes from studies of cell kinetics with labeled precursors in the gastrointestinal tract. Lesher *et al.* (25, 26), Thrasher (27), and Thrasher and Greulich (28, 29) have shown increases with age in the generation time of the progenitor cells in the crypts of the small intestine and colon of the mouse, which can be attributed largely to increases in the G_1 phase. The larger shoulders on the radiation dose–survival curves for the gastrointestinal tract might provide a better system in which to demonstrate senescent changes in the parameters of Elkind recovery.

Summary. Spleen colony-forming cells (CFU) in the bone marrow of old mice (25–27 months) are deficient in their ability to recover from fractionated sublethal doses of X-rays *in vivo*. This lack of repair is not evident from single-dose radiation survival data. Fractionation survival curves suggest that old CFU which survive the first dose of fractionated radiation may not be as radioresistant as comparable young (3 months) survivors.

We are grateful to Drs. T. Makinodan and C. F. Gottlieb for helpful advice, to Dr. J. M.

Yuhas for generous help in computing radiosensitivity parameters, and to Mrs. Lynda F. May and Mr. J. M. Ellis for expert technical assistance.

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Received Sept. 1, 1972. P.S.E.B.M., 1974, Vol. 145.