The Sensitivity of Cells from Normal Mouse Bone Marrow to Gamma Radiation *in Vitro* and *in Vivo*¹

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INTRODUCTION

Normal mouse bone marrow contains a small population of cells with the capacity to give rise to macroscopic colonies containing differentiated hemic cells in the spleens of heavily irradiated animals. A radiation survival curve for these colony-forming cells after irradiation *in vitro* has been published (1). However, it remained to be shown whether this survival curve is applicable to colony-forming cells irradiated under physiological conditions in the animal. In the present paper, a survival curve for the colony-forming cells irradiated *in vivo* is presented and compared with the *in vitro* survival curve. Although these two curves are very similar, a significant difference does exist between them.

EXPERIMENTAL PROCEDURES

Measurement of the Colony-Forming Ability of Marrow Cells

The methods used to test for the colony-forming ability of cells in a marrow suspension have already been described (1). In brief, known numbers of nucleated cells from the femoral marrow of mice are injected intravenously into heavily irradiated (900 to 1000 rads) recipient animals. After 10 days, surviving animals are sacrificed, and their spleens are fixed in Bouin's solution (2). Fixation in Bouin's solution causes any macroscopic colonies that are present to stand out against the darker background of the host's splenic tissue as round or oval yellow nodules, which may readily be counted. Satisfactory counts can be made with as many as 25 colonies per spleen. However, for colony counts greater than 15 per spleen, the relationship between the number of cells injected and the number of colonies observed in the spleen ceases to be strictly linear, as illustrated by the data of Fig. 1.

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Fig. 1. The relationship between the number of nucleated cells injected and the mean number of colonies observed in the spleen after fixation in Bouin's solution. The 95% confidence limits for the points are indicated.

The curve shown in Fig. 1 is similar to that reported previously (1), but its range has been extended. The curve tends to deviate from linearity for colony numbers above 15, owing to overlap of colonies arising from crowding.

The spleens of animals receiving large numbers of colony-forming cells contain confluent colonies, which cease to be distinguishable as nodules when the normal splenic architecture is restored.

As indicated in Fig. 1, no colonies have been observed in the spleens of the few animals that have survived for 10 days after receiving 900 to 1000 rads but no injection of marrow cells. However, when lower doses of total-body radiation are used, colonies similar to those described above are observed in the spleens of animals that have not received a marrow transplant. A study of the relationship between the number of these colonies, derived from the animal's own colony-forming cells, and radiation dose, is at present under way.

The histological features of the colonies have been described (1). They consist of hemic cells in which erythroblasts, granulocytes, and megakaryocytes can be recognized. It is probable that each colony derives from a very few cells, and possibly from a single cell (1). However, as the entity in the marrow suspension that gives rise to a colony in the spleen has not yet been identified, it is referred to as a

colony-forming unit (CFU). The ratio of the number of CFU to the number of nucleated cells injected (the CFU ratio) provides a measure of the proliferative capacity of the cells in a marrow suspension. The CFU ratio usually lies between 10 and 15 colonies per 10^5 cells for normal marrow suspensions.

Donor and Recipient Mice

In most experiments C57Bl/Ha or C3Hf/Ha mice bred in the colony of the Ontario Cancer Institute, or the F1 hybrid between these strains, were used. As previously reported, colony-forming cells from these strains are similar in their response to radiation (1). In some experiments, pathogen-free Swiss mice (obtained from Darrow Laboratories, New York) served both as recipients and donors. The results obtained in these experiments were similar to those obtained with inbred mice.

The Radiation Survival Curve for Colony-Forming Cells

The dependence of the CFU ratio on radiation dose may be used to construct a radiation survival curve (1). Because the CFU ratio decreases as the dose of radiation is increased, it is necessary to increase the number of nucleated cells injected in order to obtain an adequate number of colonies in the spleen. It is impractical to use cell numbers greatly in excess of 10^7 cells per recipient mouse, and therefore, the range of cell survival that can be studied by the method is limited to approximately 2.5 powers of ten.

To satisfy the requirements of some experiments, certain modifications were made in the technique outlined above. These will be described with the presentation of results.

Irradiation Procedures

Recipient mice were irradiated in groups of 30 in a rotating circular Lucite drum 30 cm in diameter, located 65 cm from the target of 280-kvp X-ray generator, operated at 20-ma tube current, with filtration yielding a HVL of 1.12 mm Cu. The exposure dose rate was approximately 100 r/min. For some experiments 200-kvp X-Rays, HVL 1 mm Cu, were used.

Marrow cell irradiations both *in vivo* and *in vitro* were carried out with Co^{60} γ -rays. *In vivo* irradiations were performed on groups of 10 mice in a circular Lucite holder 20 cm in diameter, placed on a revolving turntable beneath the Co^{60} source. For most experiments, an absorbed dose rate of 50 to 55 rads/min was used. *In vitro* irradiations were performed on marrow cell suspensions in 12-ml stoppered polyethylene tubes, 1.7 cm in diameter, placed in a Lucite holder (wall thickness 0.5 cm) with their long axes perpendicular to the radiation beam. The tubes were agitated immediately before irradiation in order to keep the cells in suspension and to provide adequate aeration. For most experiments, an absorbed dose rate of 285

to 330 rads/min was used. In some experiments, *in vivo* and *in vitro* irradiations were carried out at the same dose rate, as will be described under Results.

Dosimetry was based on air ionization measurements made with a Philips vibrating-reed electrometer and on absorbed dose measurements utilizing the ferrous ammonium sulfate dosimeter (3). A G value of 15.5 ferric ions produced per 100 ev of energy absorbed was assumed.

RESULTS

Sensitivity of the Colony-Forming Ability of Marrow Irradiated in Vitro

A survival curve for colony-forming units (CFU) in marrow irradiated *in vitro* with γ -rays from Co^{60} has already been reported (1). The accumulated results from these and further experiments are shown in Fig. 2 (closed circles). In the figure, the 95% confidence limits for each of the experimental points are shown. The portion of the survival curve which is linear on a semilogarithmic plot has been fitted to the experimental points by the method of least squares. The curve thus obtained yielded a D_0 (the radiation dose required to reduce the surviving fraction by a factor of 0.37, measured on the exponential portion of the curve) of 105 ± 13 rads, and an extrapolation number (4) of 2.5. This D_0 is slightly less than the value of 115 rads reported previously (1). The limits quoted represent the 95% confidence limits of the D_0 .

The in Vivo Radiation Sensitivity of the Colony-Forming Ability of Marrow

Irradiation within the mouse of aliquots of a single marrow suspension was accomplished by a modification of the assay technique, as follows. The recipient mice received a part of the usual dose of 900 to 1000 rads. After this, an intravenous injection of marrow cells from the suspension under test was given. Two to three hours thereafter the balance of the required radiation dose was administered to the mice by using Co^{60} γ -rays. In this way the recipient animals received the usual heavy dose of radiation required to suppress endogenous colony-formers, but the administered cells received only the dose delivered in the second exposure. Thereafter the mice were kept for 10 days, the survivors killed, and their spleens examined for colonies. The design of a typical experiment, and the results obtained, are shown in Table I.

Data accumulated from several experiments of this design are shown in Fig. 3 (closed circles) and compared with the survival curve obtained for irradiation of cells *in vitro*. The upper curve, without points, is the *in vitro* survival curve shown in Fig. 2. The means and 95% confidence limits for the points obtained for irradiation *in vivo* are shown. These all fall below the *in vitro* survival curve. The best fit to the exponential portion of the curve yielded a D_0 of 95 ± 9 rads and an extrapolation number of 1.5. Statistical analysis indicated that the two values for D_0 (105 and 95) obtained for cells irradiated *in vitro* and *in vivo* did not differ

FIG. 2. Gamma-ray survival curve for the colony-forming ability of mouse bone marrow cells irradiated as a cell suspension *in vitro*. Closed circles: irradiated in air. Open circles: irradiated in 95% O_2 , 5% CO_2 . The extrapolation number (n) and D_0 are indicated.

significantly at the 5% level. However, the difference in the extrapolation numbers of the two curves was highly significant at the 1% level, suggesting that this difference accounts for the greater survival of cells irradiated *in vitro*, compared with cells irradiated *in vivo*.

The experimental design described above was useful because it permitted irradiation *in vivo* of aliquots of the same marrow suspension. However, before it could be accepted as a true measure of the radiation sensitivity of marrow cells functioning in their normal environment, it was necessary to do experiments in which marrow cells were irradiated *in situ* by exposing normal mice to whole-body irradiation. In these experiments, groups of 10 to 20 female donors of the same

TABLE I
SURVIVAL OF COLONY-FORMING UNITS (CFU) AFTER
IRRADIATION *in Vivo* WITH Co⁶⁰ γ -RAYS

Group	First irradiation, X-rays (rads)	Number of nucleated cells $\times 10^{-5}$	Second irradiation, γ -rays (rads)	Number of spleens	Mean CFU	CFU/ 10^5 cells	Survival (%)
1	950	1	0	22	18.9 $\pm$ 3.4 ^a	18.9	100 $\pm$ 8 ^b
2	825	3	125	19	23.8 $\pm$ 5.3	7.9	42 $\pm$ 5.4
3	700	10	250	27	23.9 $\pm$ 4.7	2.4	13 $\pm$ 1.4
4	575	30	375	27	16.9 $\pm$ 5.2	0.56	3.0 $\pm$ 0.42
5	450	100	500	19	12.1 $\pm$ 4.7	0.12	0.63 $\pm$ 0.13

^a Sample standard deviation.

^b 95% confidence limits.

TABLE II
SURVIVAL OF CFU IRRADIATED *in Situ* IN THE FEMORA OF LIVING MICE

Group	γ -ray dose (rads)	Cells per two femora $\times 10^{-7}$	Number of cells injected $\times 10^{-5}$	Number of spleens	Mean CFU	CFU per 10^5 cells	Observed survival (%)	Expected survival (%) ^a
1	0	1.3	1	5	10.1 $\pm$ 2.4 ^b	10.1	100 $\pm$ 30 ^c	100
2	185	1.5	5	7	11.5 $\pm$ 2.9	2.3	23 $\pm$ 7.4	21
3	324	1.2	23	12	17.2 $\pm$ 7.0	0.75	7.4 $\pm$ 2.5	4.6
4	438	1.5	76	20	14.5 $\pm$ 5.7	0.19	1.9 $\pm$ 0.55	1.4

^a Obtained from the survival curve of Fig. 3.

^b Sample standard deviation.

^c 95% confidence limits.

age and strain were irradiated with different doses of γ -rays. Immediately afterward the mice were killed, and their femoral marrow was pooled. The CFU ratio of the suspensions thus obtained was measured by injection of aliquots of the suspensions into heavily irradiated recipients in the usual way. The results of a typical experiment are given in Table II and, with others obtained in this way, are included in Fig. 3 as open circles.

The survivals expected from the survival curve of Fig. 3 for the radiation doses used in the experiment are included in Table II. No statistically significant difference exists between these values and those obtained with cells irradiated before extraction from the donor mice ($P = 0.25$).

These experiments support the suggestion that the *in vivo* survival curve obtained by the experimental design illustrated in Table I is valid for colony-forming cells in marrow irradiated *in vivo* under physiological conditions and is different

FIG. 3. Gamma-ray survival curve for the colony-forming ability of mouse marrow cells irradiated *in vivo*. Closed circles: irradiated after injection into the recipient mice. Open circles: irradiated *in situ* in the femora of living donor mice. The extrapolation number (n) and D_0 are indicated.

from the survival curve obtained for the same cells irradiated *in vitro*. Possible explanations for this difference will now be considered.

Effect of Oxygenation in Vitro

It seemed possible that more CFU survived irradiation in suspension than irradiation within the animal because the cells in suspension were not so well oxygenated as the cells in the animal. In order to test this possibility, an experiment was done in which oxygen (95% O_2 , 5% CO_2) was bubbled vigorously through the suspension during the irradiation. No way was found by which loss of cells during the bubbling procedure could be avoided, even though an antifoaming agent (Dow Corning Antifoam AF emulsion, Dow Corning Corp., Midland, Michi-

TABLE III
SURVIVAL OF CFU IRRADIATED *in Vitro* IN THE PRESENCE OF 95% O₂ + 5% CO₂

Group	γ -ray dose (rads)	Cell loss on bubbling (%)	Number of cells injected $\times 10^{-5}$	Number of spleens	Mean CFU	CFU 10^8 cells	Observed survival (%)	Expected survival (%) ^a
1	0	25	1.0	10	17.2 $\pm$ 3.2 ^b	17.2	100 $\pm$ 13 ^c	100
2	225	26	2.2	11	9.9 $\pm$ 3.4	4.5	26 $\pm$ 6.5	29
3	450	28	49	22	26.7 $\pm$ 3.8	0.55	3.2 $\pm$ 0.40	3.2
4	450	28	38	17	18.2 $\pm$ 5.3	0.48	2.8 $\pm$ 0.54	3.2

^a Obtained from the survival curve of Fig. 2.

^b Sample standard deviation.

^c 95% confidence limits.

gan) added to the cell suspension (dilution factor 10^{-4}) suppressed excessive foam formation. Because of this loss of cells, each aliquot of the suspension was bubbled and irradiated at maximum cell concentration, and the unirradiated control aliquot was bubbled for a similar length of time. The results of this experiment are shown in Table III, and the surviving fractions listed in the table are included in Fig 2 (open circles). Comparison of the experimental values of Table III with the percentage survivals to be expected on the basis of the survival curve of Fig. 2 indicates that no statistically significant difference exists ($P > 0.5$). This experiment thus suggests that partial anoxia cannot account for the greater survival of cells irradiated *in vitro* compared with those irradiated *in vivo*.

This result does not mean that the radiosensitivity of CFU is independent of oxygen concentration. Preliminary experiments have demonstrated that cells irradiated under anoxic conditions do show the two- to threefold decrease in radiosensitivity found in other systems (5).

Effect of Dose Rate on Survival of Cells Irradiated in Vitro

In most experiments, cells were irradiated *in vitro* at a higher dose rate than cells irradiated *in vivo*. In order to test the possibility that the observed difference between the two survival curves was due to the difference in dose rate, aliquots of the same suspension were irradiated *in vivo* and *in vitro* at the same distance from the source, i.e., at the same dose rate, and under conditions as nearly identical as possible. The results of the experiment are shown in Table IV. The results are in good agreement with the data of Figs. 2 and 3. Approximately twice as many cells survived the same dose of γ -rays *in vitro* as survived irradiation *in vivo* under nearly identical radiation conditions.

The Transition from in Vitro to in Vivo Sensitivity

The preceding experiment indicated that the observed difference between the *in vitro* and *in vivo* survival curves did not arise from differences in dose rate.

TABLE IV
SURVIVAL OF CFU IRRADIATED UNDER SIMILAR GEOMETRY
(Dose rate 55 rads/min)

<i>Irradiation in vitro</i>		<i>Irradiation in vivo</i>	
γ -ray dose (rads)	Survival ^a (%)	γ -ray dose (rads)	Survival ^a (%)
0	100 $\pm$ 16	0	100 $\pm$ 16
179	36 $\pm$ 8	179	18 $\pm$ 3.9
359	6.7 $\pm$ 1.8	359	3.7 $\pm$ 0.84
513	1.5 $\pm$ 0.4	513	0.62 $\pm$ 0.14

^a 95% confidence limits are indicated.

However, it was possible that uncertainties in dosimetry, due to differences in the absorption of radiation in the mouse as compared to the cell suspension, might be the basis for the observed difference in cell survival. Should this be the case, the survival of cells at a given dose of radiation should change abruptly when the cells are introduced into the mouse and the new conditions for absorption of energy are established. On the other hand, if the change in sensitivity is the result of changed metabolic or environmental conditions acting on the cell, it might be expected that the transition from the *in vitro* to the *in vivo* sensitivity might occupy a period of time. For this reason experiments were performed in which marrow cells were irradiated *in vivo* at different intervals after injection into irradiated hosts. The results of two experiments are given in Table V.

These results indicate that the sensitivity of the cells does not change abruptly immediately after injection into the mice; a period of time is required for the transition between the *in vitro* sensitivity and the *in vivo* sensitivity. Though the

TABLE V
THE TRANSITION FROM *in Vitro* TO *in Vivo* RADIATION SENSITIVITY
(Radiation dose, 465 rads)

<i>Irradiation</i>	<i>Experiment 1,</i> <i>survival (%)</i> ^a	<i>Experiment 2,</i> <i>survival (%)</i> ^a
<i>In vitro</i>	1.6 $\pm$ 0.23	2.6 $\pm$ 0.77
<i>Interval after</i> <i>injection of</i> <i>cells (min)</i>		
2	2.4 $\pm$ 0.63	1.4 $\pm$ 0.38
10	2.1 $\pm$ 0.59	1.4 $\pm$ 0.49
20	1.2 $\pm$ 0.30	1.1 $\pm$ 0.29
300	0.84 $\pm$ 0.18	0.68 $\pm$ 0.16

^a 95% confidence limits are indicated.

precision of the data is not sufficient to allow any more than a crude estimate to be made of the rate of change of the surviving fraction from the *in vitro* to the *in vivo* sensitivity, the results are compatible with the assumption that half the total change is accomplished in about 10 minutes.

Since the sensitivity of cells does not change abruptly on injection into the mouse, it appears that the difference in survival of cells irradiated *in vivo* compared to cells irradiated *in vitro* is probably not due to physical factors.

DISCUSSION

In previous experiments (6), using a less sensitive measure of the proliferative capacity of bone marrow, no difference between the effects of radiation *in vivo* and *in vitro* was found. Similarly, Hewitt and Wilson (7), using a transplantable murine leukemia, found that cells irradiated *in vitro* and assayed *in vivo* had a similar sensitivity to cells irradiated and assayed *in vivo*. On the other hand, Philpott, Shaeffer, and Tolmach,² using chick embryo cells, found that cells irradiated *in ovo* were slightly more resistant than cells irradiated *in vitro*. The experiments reported in this paper indicate that a small but statistically significant difference in survival does exist between marrow irradiated *in vitro* and marrow irradiated *in vivo*. This finding does not affect the observation that the D_0 for colony-forming cells from mouse bone marrow is very nearly 100 rads, since most of the difference between the *in vivo* and *in vitro* results is due to a difference in the extrapolation number.

The difference in survival for the cells irradiated *in vitro* and *in vivo* is not due to errors in absorbed dose measurements. This is demonstrated by the fact that similar survival values were obtained when cells were irradiated *in vitro* and when cells were irradiated within recipient mice a very short time after their injection. Thus, the differences in survival are probably due to biological effects resulting from the reaction of the cell with its environment.

A number of changes in the sensitivity of cells to ionizing radiation have been shown to be the result of changes in oxygen concentration (5). However, the results presented above suggest that this is not the explanation for the differences observed.

Two possible explanations for the difference suggest themselves. First, the effective radiation sensitivity of the cells might be less *in vitro* than *in vivo*, owing to mechanisms operating at the cellular level; for example, metabolically active cells in the animal might be more readily inactivated than resting cells outside the body. This sort of dependence of cell survival on the growth conditions after irradiation has been observed in the bacterium *Escherichia coli* B by Alper and Gillies (8). Alternatively, recovery processes involving changes in extrapolation number, of the type described by Elkind and Sutton (9) might be less effective *in vivo* than *in vitro*. This latter possibility is suggested by the observation that the extrapola-

² G. W. Philpott, C. W. Shaeffer, Jr., and L. J. Tolmach, personal communication (Washington University, School of Medicine, Saint Louis, Missouri).

tion number of the *in vivo* survival curve is less than the extrapolation number of the *in vitro* curve.

Second, the decreased survival of cells irradiated in the animal might result from host influences acting on the cells after irradiation *in vivo*, leading to a reduction in the number with the capacity to form colonies. For instance, nonlethal radiation effects might increase the likelihood that a cell would become more differentiated, and by differentiation lose its capacity to form a colony.

The data presented above do not permit any conclusions to be drawn concerning the basis for the difference between the survival of cells irradiated *in vitro* and *in vivo*. This difference, though small in magnitude, may provide an important clue to the mechanism of action of radiation on mammalian cells.

SUMMARY

1. A survival curve for the colony-forming ability of mouse bone marrow cells irradiated *in vivo* with Co^{60} γ -rays has been obtained. The curve may be characterized by a D_0 of 95 rads and an extrapolation number of 1.5.

2. Comparison with the survival curve obtained for cells irradiated *in vitro* indicated a statistically significant difference in extrapolation number. Possible explanations for this finding are considered.

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