Early Repair Processes in Marrow Cells Irradiated and Proliferating *in Vivo*¹

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INTRODUCTION

The radiation survival curves for the proliferative capacity of mammalian cells, both *in vitro* (1, 2) and *in vivo* (3, 4), are sigmoidal, suggesting that loss of proliferative capacity involves an accumulation of radiation damage. For mammalian cells in culture, Elkind and co-workers (5, 6) have demonstrated that accumulated sublethal damage may be rapidly repaired in surviving cells, by means of experiments in which the number of survivors was shown to increase when the radiation dose was given as two fractions separated by an interval of time. They have termed this phenomenon "recovery" (5, 7), and Elkind (7) has used the results obtained with cell cultures to predict the outcome of dose-fractionation experiments *in vivo*. Recently, it has been shown that recovery occurs in irradiated tumor cells *in vivo* (8). There has, however, been no direct experimental evidence confirming the existence of recovery in normal cells *in vivo*.

The finding (4) that normal mouse bone marrow contains cells capable of forming macroscopically visible colonies in the spleens of heavily irradiated animals provides a quantitative approach to this problem. An important advantage of the spleen-colony technique is that it may be used to study directly the effects of radiation on cells *in vivo* (9). In this paper, we report the results of dose-fractionation experiments carried out in this system. The results indicate that early repair of sublethal damage occurs in colony-forming cells irradiated *in vivo*, but suggest that the time scale of the early repair process differs from that observed for cells in culture.

MATERIALS AND METHODS

Mice

C57Bl/Ha or C3Hf/Ha mice of both sexes, bred in the colony of the Ontario Cancer Institute, or the F1 hybrid between these strains, were used for most experi-

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ments. For some experiments, Swiss mice from the same colony were used. The techniques used in the marrow transplantation procedure were similar to those described previously (4, 9). In the course of this work, it was found that anomalous results may be obtained if the mice used as donors of marrow cells and the mice used as heavily irradiated recipients of marrow transplants were not of identical strains. In particular, the use of C57Bl marrow for transplantation into C57Bl $\times$ C3H F1 hybrids yielded unusually low colony counts, as will be discussed in a subsequent publication.

Irradiation Techniques

The irradiation procedures were similar to those previously described (9). Irradiation of recipient mice was carried out with 280-kvp X-rays, HVL 1.12 mm Cu, at an exposure dose rate of approximately 150 r/min. For some experiments, 200-kvp X-rays, HVL 1 mm Cu, were used. For irradiation of marrow cells, Co⁶⁰ γ -rays were used at a dose rate of about 50 rads/min. Dosimetry was based on air ionization measurements made with a Philips vibrating-reed electrometer and on absorbed dose measurements made with the ferrous ammonium sulfate dosimeter (10).

Irradiation of Colony-Forming Cells in Vivo

The method for measuring the number of cells in a marrow suspension with the capacity to give rise to colonies in the spleen has been described (4). The modification of the technique to allow the irradiation of these colony-forming cells after their implantation within the animal has also been described (9). In brief, in the *in vivo* method, the recipient animal receives a portion of the large dose of whole-body radiation required to suppress the formation of colonies from endogenous cells (see below). After this, the cells are injected, and a period of time in excess of 1 hour is allowed, as this interval has been found to be sufficient to allow the cells to manifest the radiation sensitivity characteristic of the *in vivo* situation (9). After this interval, a second dose of whole-body radiation is given with Co⁶⁰ γ -rays. The two doses are so arranged that their sum is 950 rads. However, while the recipient animal receives the total dose of radiation, the injected cells receive only the dose delivered in the second exposure. Evidence has been presented to show that the injected cells respond to irradiation *in vivo* by this technique in a quantitatively similar way to cells irradiated *in situ* (9). In order to study the effect of dose fractionation, this design needs only to be modified by delivering the dose, after the injection of the cells, in two parts, with an interval of time between them.

An example will illustrate the method used in dose-fractionation experiments. If the cells of a transplant are to receive two doses of 200 rads with an interval between the doses, the following procedure is used: First the animals receive 550 rads of X-rays. Then, an appropriate number of marrow cells are injected intravenously in 1/2 ml of suspending fluid. After an interval of 1 hour, the animals receive 400 rads of Co⁶⁰ γ -rays in two equal doses separated by the desired interval of time.

Ten days after the second γ -ray dose, the animals are killed, their spleens are fixed in Bouins' solution, and the number of macroscopic colonies visible in the fixed spleen is determined.

Endogenous Colony Formation

In the example described above, the cells of the transplant received a total of 400 rads of γ -rays, and the recipient animals received a total dose of 950 rads of X- and γ -rays. The latter high dose is required to suppress the appearance of colonies in the spleen due to survival of the animals' own colony-forming cells, and it is sufficiently high to yield an average of less than one "endogenous" colony per spleen even under the conditions of dose fractionation used in the experiments reported in this paper. Lower doses allow the appearance of such endogenous colonies. The number of endogenous colonies visible after 11 days is related to radiation dose in the manner to be discussed under Results (see Fig. 4). Cobalt-60 γ -rays were used in all studies of the effect of radiation on endogenous colonies.

RESULTS

Cell Survival after Fractionated Doses in Vivo

The dose-response curve for colony-forming cells after a single dose of γ -rays *in vivo* has been published (9). This curve, shown in Fig. 1, is characterized by a D_0 of 95 ± 9 rads and an extrapolation number of 1.5. The effect of dose fractionation is shown in Fig. 2. Curve *b* shows the results obtained when the interval between the doses is 3 to 4 hours. Curve *a* is the single-dose curve shown in Fig. 1. For the fractionation experiments, a constant first dose of 200 rads was chosen, as the survival after this dose falls on the straight-line portion of the survival curve. Therefore, the survival curves obtained by dose fractionation are shown originating from this point. It is apparent from curve *b* that considerable restoration of the shoulder region occurred during the 3- to 4-hour interval. The results of curve *c* will be described later.

The Kinetics of the in Vivo Repair Process

Figure 3 shows the results obtained when two fixed doses of 200 rads each were given to colony-forming cells *in vivo*, separated by different intervals of time. The ordinate of Fig. 3 is the survival ratio, defined as the ratio of the percentage of surviving colony-forming cells after two doses separated by a time interval, t , to the percentage survival for the same total dose given as a single exposure. The abscissa gives the time interval, t , between the two doses.

The smooth curve drawn through the points of Fig. 3 shows an initial maximum at about 5 hours, followed by an intermediate minimum at about 11 hours. The survival ratio at the 5-hour maximum appears to be greater than would be expected

FIG. 1. Survival curve for colony-forming cells in mouse bone marrow transplants irradiated *in vivo* with a single dose of Co^{60} γ -rays (9).

from the extrapolation number of 1.5 for the single-exposure survival curve (Fig. 1). However the difference is not statistically significant ($P > 0.2$).

Validity of the Intermediate Minimum

The intermediate minimum at about 11 hours could result from a change in the slope of the survival curve rather than a change in extrapolation number. In order to test for this possibility, a survival curve was constructed for an 11-hour interval between the doses. A first dose of 200 rads was again used. The results of two experiments, shown in Fig. 2, curve *c*, indicate that the intermediate minimum is in fact due to a failure of the shoulder region of the survival curve to reappear after dose fractionation with an 11-hour interval.

FIG. 2. Survival curve for colony-forming cells from mouse marrow irradiated *in vivo* with two doses of Co^{60} γ -rays separated by an interval of time. Curve *a* (lower axes): *in vivo* survival curve, see Fig. 1. Curve *b* (lower axes): closed circles, 3-hour interval between doses; open circles, 4-hour interval between doses. Curve *c* (upper axes): 11-hour interval between doses. Results of two separate experiments. Standard errors for the individual points are shown.

Repair in Endogenous Colony-Forming Cells

The results described above show that early repair processes occur in transplanted marrow cells irradiated *in vivo*. Additional experiments were carried out to show that similar processes occur in cells irradiated *in situ*. These experiments were based on the observation that colonies are seen in the spleens of mice 11 days after irradiation with 600 to 800 rads, in the absence of a marrow transplant. The number of endogenous colonies observed is related to radiation dose in the manner illustrated in Fig. 4, in which the mean number of colonies per spleen of total-body-irradiated C57Bl mice is plotted against the indicated Co^{60} γ -ray dose. Exponential curves each having a D_0 of 95 rads have been drawn through the experimental points. It is evident that the radiation sensitivity of colony-forming cells *in situ* is

FIG. 3. Kinetics of early repair of colony-forming cells from mouse marrow irradiated *in vivo* with two 200-rad doses of Co^{60} γ -rays separated by different time intervals (t). Ordinate: survival ratio (see text). Abscissa: time interval between the doses. The different symbols indicate separate experiments. Standard errors for the individual points are shown.

similar to the radiation sensitivity of transplanted colony-forming cells (Fig. 1). It is also apparent that, in this particular experiment, a higher level of surviving endogenous colony-forming cells was found in females than in males. This difference, which preliminary experiments indicate may be due to a difference in the number of colony-forming cells in the spleens of males and females, has no detectable influence on the results of experiments in which the transplantation technique is used, since, in isologous hosts, the sex of neither donor nor recipient has been found to influence the survival of colony-forming cells.

Applying this method to a test of the effect of dose fractionation on endogenous colony-forming cells yielded the results shown in Fig. 5. The figure shows the time dependence of the survival ratio of endogenous colony-forming cells for the particular pair of doses used (first dose, 400 rads; second dose, 300 to 350 rads). Though the statistical spread of the data is quite severe, the existence of an initial maximum and an intermediate minimum in the kinetic curve, similar to that shown in Fig. 3, is apparent. The survival ratio observed in these experiments was

FIG. 4. Survival curve for endogenous spleen colony-forming cells of C57Bl/Ha mice irradiated *in situ* with total-body Co^{60} radiation. Closed circles: female mice. Open circles: male mice. Standard errors are shown.

consistently much greater than that obtained with marrow transplants. The reasons for this difference are unknown, but it is possible that they result from additional repair processes not active in marrow transplants.

DISCUSSION

The results presented above indicate that a phenomenon analogous to that studied by Elkind and co-workers in Chinese hamster (5) and HeLa (6) cells in culture also occurs *in vivo* in colony-forming cells of the mouse hematopoietic system.

Comparison of the *in vivo* results with those of Elkind and co-workers shows qualitative agreement in the forms of the kinetic curves, but the initial maximum and intermediate minimum are shifted in time (about 5 and 11 hours, respectively,

FIG. 5. Kinetics of early repair of endogenous colony-forming cells irradiated *in situ* with two doses of total-body Co^{60} radiation. First dose, 400 rads. Closed circles: second dose, 300 rads; open circles: second dose, 350 rads. Standard errors for the individual points are shown.

from cells from mouse marrow, compared with about 2 and 5 hours, respectively, for Chinese hamster cells, 5). In Ehrlich ascites tumor cells *in vivo*, Hornsey and Silini (8) found recovery to be at its maximum after 5 to 7 hours, with no significant decrease by 9½ hours, which was the longest time interval used.

In the absence of information concerning the duration of radiation-induced division delay in spleen colony-forming cells *in vivo*, it is not possible to establish with certainty that the early repair process described above for colony-forming cells is recovery in the sense defined by Elkind (7), who has applied the term only to changes in survival occurring during the period of division delay. It is still possible that the changes reported here are the result, in part, of cellular proliferation. This type of change Elkind has termed "repair." We prefer the designation "regeneration" for the effects of cellular proliferation, and in this paper we have used "early repair" as a more general term indicating processes that increase the number of surviving cells during the first day after irradiation. Experiments designed to elucidate the kinetics of proliferation of colony-forming cells *in vivo* are at present under way.

Colonies developing in the spleens of mice irradiated with doses between 600 and 800 rads have been used to study the behavior of colony-forming cells *in situ*. Although experiments designed to study endogenous colony-forming cells yield data of less precision than experiments based on cell transplantation, they have the advantage that they show the behavior of cells without any changes that might be introduced by the transplantation procedures. The experiments reported in this paper indicate that early repair processes occur after whole-body irradiation of the intact mouse. The results also indicate a difference in the number of colony-forming cells in males and females, showing that the method has general application to the study of the physiology of colony-forming cells.

SUMMARY

1. Early repair of sublethal radiation damage has been demonstrated in surviving spleen-colonizing cells *in vivo*, by means of dose-fractionation experiments.

2. A study of the kinetics of the early repair process indicated that early repair reached an initial maximum at about 5 hours after the first dose fraction, followed by an intermediate minimum at about 11 hours.

3. An assay procedure which may be used for the study of the behavior of spleen-colonizing cells *in situ* has been developed.

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