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Differing Responses to Radiation of Murine Bone Marrow Stem Cells in Relation to the Cell Cycle[1]

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SUMMARY

The response to radiation of murine bone marrow stem cells in different portions of the cell cycle has been obtained. Murine bone marrow stem cells were injected into suitable recipients and synchronized *in vivo* with hydroxyurea. At varying times thereafter, radiation was given, and surviving stem cells were assayed by the spleen colony technique. There was a 10-fold difference between the maximum survival at 9 hr and minimum survival at 10 hr. For determination of the position of the S (synthetic) phase of the cell cycle, another experiment was performed consisting of a second injection of hydroxyurea instead of radiation. A nearly inverse curve was obtained with a minimum survival at 8 hr. This allowed determination of the S phase of the cell cycle. It thus appears that murine bone marrow stem cells are most resistant in late S and are quite sensitive in the G_2-M phase.

INTRODUCTION

The introduction of techniques for synchronizing cell populations *in vitro* has led to a number of studies of the sensitivity of cells to X-radiation during different portions of the cell-generative cycle (7, 10). More recently, murine lymphoma and gastrointestinal mucosa have been shown *in vivo* to vary their radiation response as a function of positions in the cell cycle (3, 4). These studies have potential clinical significance, since, if either the limiting normal cell population or the tumor cell population could be effectively synchronized in a predictable way, treatment could be given in time to maximize tumor cell kill while minimizing normal tissue damage. This differential response to X-radiation as a function of position in cell cycle is large, perhaps as much as 10-fold (7), and hence would be very significant if it could be clinically utilized in treating cancer.

Hematopoietic stem cells[2] normally proliferate slowly, but in response to cell depletion increase their proliferative rate (2). Radiation therapy or treatment with chemical cytotoxins can thus change the proliferative activity of bone marrow stem cells. Since the bone marrow is usually the dose-limiting tissue in generalized cytotoxic therapy, it was of interest to us to determine what differences in radiation sensitivity might exist for these cells as they progress through the cell cycle.

In this study, we report that normal murine bone marrow stem cells *in vivo* do show variations in radiation response as a function of position in the cell cycle.

MATERIALS AND METHODS

A modified spleen colony method (11) was used. All mice were male C3H/HeJ mice, 8 to 10 weeks old. Radiation was delivered at 250 kVp, with the use of a 2-mm aluminum filter at 120 rads/min on a rotating turntable with maximum backscatter. The dose was measured in a mouse phantom by a Victoreen "R" meter the calibration of which was confirmed at the National Bureau of Standards. Recipient mice were given 600 rads initially and 450 rads 3 hr later. This dosage schedule suppressed spontaneous endocolony survival to less than 0.1 colony/spleen. Following radiation, these mice received syngeneic bone marrow cells i.v. We prepared bone marrow cell suspensions by flushing the medullary cavities of the tibias and femurs with cold, sterile Tyrode solution. At 18 hr after injection of bone marrow, 3 mg of hydroxyurea (Hydrea; E. R. Squibb & Sons, Inc., New York, N. Y.) were given by i.p. injection. This time period had been determined to be sufficient to allow the beginning of active proliferation, yet not long enough to allow excessive multiplicity of developing microcolonies. This was evidenced by maximum reduction in colony survival when hydroxyurea was given 18 hr following bone marrow injection. The dose given resulted in a 50% reduction in colony number, compared to untreated control groups. Hydroxyurea has been found to kill cells in the synthetic phase (6) of the cell cycle and to hold others from entering S. It has been used to attempt to synchronize cells *in vitro* (6) and, more recently, *in vivo* (4). In these experiments, after partial synchronization with hydroxyurea, 500 rads were given at varying times to different groups of mice. Nine days later, the animals were sacrificed, spleens were placed in Bouin's solution, and macroscopically visible colonies were counted. All groups contained at least 10 animals.

RESULTS AND DISCUSSION

The results of such an experiment are seen in Chart 1 (*lower curve*). As the time between synchronization and

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[2] "Stem cell," as used here, refers to a spleen colony-forming cell (11). Spleen colonies have been found to be derived from single cells (1) which have the capacity to produce 1 or more types of differentiated bone marrow forms (13), and, when secondarily transplanted, contain increased numbers of colony-forming cells (5). Thus they are capable of self-replication, as well as division into 1 or more differentiated forms, thereby fulfilling the usual criteria for stem cells.

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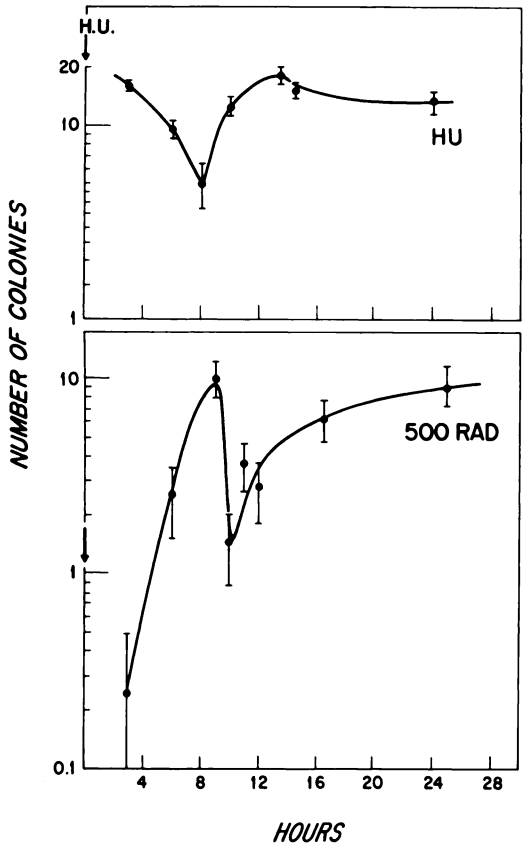

Chart 1. Lower curve, response to 500 rads delivered at varying times after administration of hydroxyurea (*HU*) at Time 0. Ordinate, number of spleen colonies; abscissa, hours following hydroxyurea administration. Each *point* is comprised of at least 10 animals, with the mean and the standard error shown. Upper curve, parallel experiment showing response to a 2nd injection of 4 mg of hydroxyurea, given at varying times after hydroxyurea at Time 0. Coordinates are the same as above.

administration of 500 rads increased, more colonies were scored. This reached a maximum at 9 hr and was followed by a precipitous fall at 10 hr, then returning to the previous value at 24 hr. There is a 10-fold difference between the 9-hr maximum and the 10-hr minimum points on the curve. Could this large difference in colony number represent in part a response to transplantation itself? McCulloch and Till (5) have noted changes in D_0 following transplantation of bone marrow. These changes might result in some difference in colony survival after treatment with 500 rads. A control experiment was therefore performed to assess the effect of 500 rads at varying times after bone marrow transplantation alone, without hydroxyurea treatment. Results of this control experiment show a gradual 2-fold increase in colony survival from 3 to 24 hr posttransplantation, with no significant fluctuations. Thus, it appears that any effect due to transplantation itself is small compared to the effect seen following hydroxyurea synchronization.

In order to determine the corresponding position in the cell cycle of each of these points, we substituted a 2nd dose of hydroxyurea for the 500 rads in a 2nd series of experiments. These results are strikingly different (Chart 1, *upper curve*). The curve obtained is nearly the reciprocal of the X-ray curve, with a minimum at 8 hr. Since hydroxyurea kills cells only in the S period of the cell cycle and holds the remainder at the G_1-S interface (6), it appears that 8 hr represents a time when most of these partially synchronized cells are in S, and therefore this can be used to locate this position in the cell cycle.

We can now consider the radiation curve and attempt to describe the radiation response as a function of position in the cell cycle. These colony-forming cells become progressively more resistant as they progress through S, reaching a peak in late S at 9 hr. This peak is followed by a sudden increase in sensitivity, probably as cells enter G_2 and/or M. In the subsequent gradual increase in survival, the most resistant level is reached at 24 hr. Since the cells pass through mitosis, one sees an increase in survival, due to the increased multiplicity of cells per colony. Presumably, this is somewhat modified by partial desynchronization, as well as by changes in sensitivity in early G_1, as compared to M. A possible criticism of the time relationships assumed from the hydroxyurea curve can be made, since hydroxyurea was given i.p. and might not be absorbed immediately. However, we have evidence that either i.p. or i.v. injection of hydroxyurea results in similar appearance time and duration of the sensitive phase, indicating little, if any, delay due to i.p. injection (unpublished data).

This study is the first demonstration of cell cycle-dependent survival differences following X-radiation in murine bone marrow colony forming cells *in vivo*. These results are similar to those of Mauro and Madoc-Jones (4), who used murine lymphoma *in vivo* and, also to those with different cell lines studied *in vitro* (8, 12). All these systems have a short G_1 period. Our interpretation of position in cell cycle differs from that of Mauro and Madoc-Jones (4), since they extrapolated from data on HeLa cells, which have a long G_1 period and are slow to enter synthesis. They did not attempt to measure the location of S by giving a 2nd dose of hydroxyurea. Our direct measurements, with such selective killing by hydroxyurea, indicate that murine bone marrow, colony-forming units can be highly synchronized by hydroxyurea, and label as the most resistant, the late S cells, with a marked increase in sensitivity as cells leave S and enter G_2 and/or M.

We have previously shown that mouse bone marrow stem cells are only slowly proliferating but are capable of rapid proliferation when subjected to daily fractionated radiation (2). Since the data here reported show as much as a 10-fold difference in radiation response, then, once significant stem cell proliferation begins, subsequent radiation will result in partial synchrony by preferentially killing those cells in sensitive phases of the cell cycle.

Tumor cells probably vary their proliferative rate little, if at all, in response to cell depletion, are known to have wide variations in duration of cell cycle (9), and might therefore remain heterogenous in spite of daily radiation. If a similar situation is found in a cancer patient, then the timing of further fractionated radiation could be important in greatly increasing the therapeutic ratio. While differing tumors in different patients may be quite heterogenous in the cell cycle times, it is likely that normal bone marrow stem cells will be more homogenous and predictable. Thus, if we can get some measure of cycle times in human normal bone marrow,

therapy could be timed to give radiation fractions at times of maximum resistance of this normal tissue. This would result in a significant increase in the therapeutic ratio, in those situations where bone marrow is the limiting normal tissue. The same reasoning is applicable to the clinical use of chemical cytotoxins. Further studies are in progress to study the cell cycle-dependent sensitivity of bone marrow stem cells to these agents.

REFERENCES

1. Becker, A. J., McCulloch, E. A., and Till, J. E. Cytological Demonstration of the Clonal Nature of Spleen Colonies Derived from Transplanted Mouse Marrow Cells. Nature, *197:* 452–454, 1963.
2. Chaffey, J. T., and Hellman, S. Radiation Fractionation as Applied to Murine Colony-forming Cells in Differing Proliferative States. Radiology, *93:* 1167–1172, 1969.
3. Gillette, E. L., Withers, H. R., and Tannock, I. F. The Age Sensitivity of Epithelial Cells of Mouse Small Intestine. Radiology, *96:* 639–643, 1970.
4. Mauro, F., and Madoc-Jones, H. Age Response to X-radiation of Murine Lymphoma Cells Synchronized *in Vivo*. Proc. Natl. Acad. Sci. U. S., *63:* 686–691, 1969.
5. McCulloch, E. A., and Till, J. E. Proliferation of Hemopoietic Colony-forming Cells Transplanted into Irradiated Mice. Radiation Res., *22:* 383–397, 1964.
6. Sinclair, W. K. Hydroxyurea Effects on Chinese Hamster Cells Grown in Culture. Cancer Res., *27:* 297–308, 1967.
7. Sinclair, W. K., and Morton, R. A. Variations in X-ray Response during the Division Cycle of Partially Synchronized Chinese Hamster Cells in Culture. Nature, *199:* 1158–1160, 1963.
8. Sinclair, W. K., and Morton, R. A. X-ray Sensitivity during the Cell Generation Cycle of Cultured Chinese Hamster Cells. Radiation Res., *29:* 450–474, 1966.
9. Steel, G. G., Adams, K., and Barrett, J. C. Analysis of the Cell Population Kinetics of Transplanted Tumors of Widely Differing Growth Rate. Brit. J. Cancer, *20:* 784–800, 1966.
10. Terasima, T., and Tolmach, L. J. Changes in X-ray Sensitivity of HeLa Cells during the Division Cycle. Nature, *190:* 1210–1211, 1961.
11. Till, J. E., and McCulloch, E. A. A Direct Measurement of the Radiation Sensitivity of Normal Mouse Bone Marrow Cells. Radiation Res., *14:* 213–222, 1961.
12. Vos, O., Schenk, H. A. E. M., and Bootsma, D. Survival of Excess Thymidine Populations *in Vitro* after X-irradiation in Various Phases of the Cell Cycle. Intern. J. Radiation Biol., *11:* 495–503, 1966.
13. Wu, A. M., Till, J. E., Siminovitch, L., and McCulloch, E. A. A Cytological Study of the Capacity for Differentiation of Normal Hemopoietic Colony-forming Cells. J. Cellular Comp. Physiol., *69:* 177–184, 1967.