AGE RESPONSE TO X-RADIATION OF MURINE LYMPHOMA CELLS SYNCHRONIZED IN VIVO*

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Abstract.—Cells of a transplantable murine lymphoma line were partially synchronized in vivo by administration of a single dose of 0.5 mg of hydroxyurea per lymphoma-bearing mouse. At various times after administration of hydroxyurea, the femurs of the lymphomatous mice were X-irradiated, and the number of lymphoma colony-forming units per femur were then assayed by the spleen-colony technique. A well-defined age response to irradiation of the lymphoma cells in vivo, similar to that of HeLa cells in vitro, was observed.

The introduction of techniques for obtaining synchronous populations of cultured mammalian cells (see, for example, ref. 1) has permitted determination of the age responses (i.e., variations in survival through the cell division cycle) to various lethal agents, including radiation and certain cytotoxic drugs (see ref. 2 for a bibliography). The method of Puck et al.,3 in which the ability of a single cell to proliferate indefinitely and thus form a macroscopic colony is taken as a measure of its viability, has been applied in these in vitro determinations. Attempts to determine such age responses of mammalian cells in vivo have not previously been reported, despite the availability of techniques for measuring the colony-forming ability of certain cells in vivo.4-7 This omission is due to the paucity of procedures for obtaining synchronous populations of cells in vivo to which techniques for determining cell viability can also be applied. Thus, although examples of partially synchronous division in normal cell systems in vivo are well known (for example, in regenerating liver or in hair follicles after hair is plucked), it is not yet possible to measure survival of the colony-forming ability of the cells in these systems. That certain in vivo cell systems do in fact exhibit age responses to X rays has, of course, been inferred from the results of experiments in which the dose of radiation is applied in two separate fractions.4, 8, 9 In such experiments the cell survival varies with the time between the radiation doses, but the presumptive age-dependent fluctuations in survival are superimposed upon those caused by the repair of sublethal damage (i.e., the damage which is accumulated in a cell before an increment of the lethal agent can kill the cell with maximal efficiency), and it is difficult, if not impossible, to separate the two phenomena.

It seemed possible that those synchronization methods currently employed in vitro which rely upon the use of agents that selectively kill and/or block cells in a given portion of the division cycle might be applicable to cell populations in vivo. The ability of the drug hydroxyurea to kill cultured cells selectively during the period when they are synthesizing DNA (S phase of the generation cycle),10-12 and to block10-13 or at least slow down14 the progression of cells from the pre-DNA synthetic phase (G1) into the S phase, has been successfully exploited to
provide partially synchronized populations of Chinese hamster V79 cells.\textsuperscript{2, 11, 15–18} As described below, HeLa cells may also be synchronized by treatment with this agent. This communication describes the use of hydroxyurea to partially synchronize lymphoma cells \textit{in vivo}. Because the viability of these cells can readily be assayed by measuring their ability to form colonies in the spleens of recipient mice, it has been possible to determine their age response to irradiation.

\textbf{Methods.}—A murine lymphoma cell line\textsuperscript{19} (kindly supplied to us by Prof. W. R. Bruce of the Ontario Cancer Institute, Toronto) was maintained by serial transplantation in AKR/J mice. The techniques for handling these cells, which have a mean doubling time of about 11.5 hr, have been reported by others.\textsuperscript{20} When injected into the bloodstream of mice, they disseminate to a number of organs, including the spleen and bone marrow; they multiply in these various sites, overwhelming the normal body cells, and eventually cause death of the animals. For each experiment, mice received $10^6$ cells by intracaudal injection from a suspension prepared from the spleens of carrier lymphomatous mice. Four days later, groups of two to three mice were treated either with hydroxyurea alone or with hydroxyurea followed by irradiation or were left untreated to serve as controls. The survival of lymphoma cells was assayed by the spleen-colony technique.\textsuperscript{4, 5} Briefly, the femurs of the mice were removed, and the cells of the marrow were flushed out with culture medium (CMRL 1066\textsuperscript{21}). After appropriate dilutions in medium, the cells were injected intracaudally into groups of recipient mice (six to eight per group; three cell dilutions per point). One week later, spleens from these recipient mice were excised and fixed, and the number of macroscopic lymphoma colonies in the spleens were counted. In this manner, the number of lymphoma colony-forming units per femur was determined and the surviving fraction calculated. The X rays were delivered in single doses of 375 rad (220 kv constant potential; 15 ma; filtration, 0.5 mm Cu + 1 mm Al; dose rate 635 rad/min) to the femoral areas only.

\textbf{Results and Discussion.}—Preliminary experiments showed that a single intraperitoneal injection of 0.5 mg of hydroxyurea per mouse reduced the surviving fraction of the femoral lymphoma colony-forming units to a plateau level of about 20 per cent within three hours. Assuming that the duration of the \textit{S} phase is seven hours\textsuperscript{22} and that only \textit{S}-phase cells are killed, then about 35 per cent of the cells would be expected to survive. The slightly lower observed value of 20 per cent can be reconciled to the predicted value by postulating passage of some cells from \textit{G\textsubscript{1}} into \textit{S}.\textsuperscript{14} This result is therefore consistent with the expectation that \textit{S}-phase cells would be selectively killed.

When cells \textit{in vitro} are synchronized by treatment with hydroxyurea, the drug exposure is terminated by removing the culture medium containing the drug, washing with saline solution, and then adding fresh medium. Although such a washing procedure is not possible with the lymphoma cells \textit{in vivo}, it has been shown that after administration of hydroxyurea in man, the serum level of the drug declines markedly within three hours.\textsuperscript{23} Such a decline could be due to excretion or degradation of the drug. It seemed reasonable to suppose that the level of hydroxyurea in the femurs of lymphomatous mice might similarly decline, to yield essentially the same effect that is achieved by washing \textit{in vitro}. If this supposition were correct, then at about three hours after injection, the cells surviving the hydroxyurea treatment should begin to proceed through the cycle in a partially synchronized fashion. By analogy with cell lines \textit{in vitro}, such a surviving population should consist mainly of cells in \textit{G\textsubscript{1}} together with some cells in the post-DNA synthetic phase (\textit{G\textsubscript{2}}) or mitosis (\textit{M}) since, during exposure to
hydroxyurea, $S$-phase cells are killed, most $G_2$ cells progress through $M$ to $G_1$, while cells in $G_1$ are arrested in this phase or at least their progression into $S$ is considerably delayed.\textsuperscript{10-17} If the supposition were incorrect, that is, if the level of hydroxyurea in the femurs failed to decline, then the cells in $S$ would be killed and the remainder would be prevented from entering $S$; thus, no age response to subsequent irradiation would be observed.

Groups of mice were irradiated at two-hour intervals commencing three hours after a single injection of 0.5 mg of hydroxyurea per mouse. The results from four separate experiments showed excellent reproducibility, and therefore the data were pooled; they are shown plotted in Figure 1. Standard errors of the mean for each survival point are shown by the vertical bars. The time of the hydroxyurea treatment is indicated by the position of the box in the upper left part of the figure. The lower limit of the box represents the average surviving fraction after hydroxyurea treatment alone. The curve shows well-defined fluctuations in survival extending through one decade. There is an initial increase in survival with a peak at four hours (where time 0 is the predicted termination of the effect of hydroxyurea three hours after injection); this is followed by a decrease to a minimum at eight hours; subsequently, survival increases steadily up to 16 hours. In a control experiment, the femurs of mice which had not been treated with hydroxyurea were irradiated at the same times as were the treated mice. No fluctuations in surviving fraction were found. The fluctuations in survival of the lymphoma cells irradiated after administration of hydroxyurea (Fig. 1) appear to constitute an age response of these cells and hence strongly suggest that the action of hydroxyurea was essentially complete within three hours after injection.

The above interpretation is corroborated by the remarkable similarity between the curve shown in Figure 1 and the age response of HeLa cells \textit{in vitro} to X-radiation (Fig. 2). (A comparison was made with HeLa cells rather than with Chinese hamster cells, which have a similar age response to X rays with the exception that

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**Fig. 1.**—Age response of murine lymphoma cells \textit{in vivo} to X-radiation. Lymphoma-bearing mice were given a single injection of 0.5 mg hydroxyurea per mouse. Commencing 3 hr later (designated time 0 on the abscissa), groups of mice were given a single dose of 375-rad X rays to the femoral areas only. Immediately after irradiation, the number of lymphoma colony-forming units per femur was assayed by the spleen colony technique to yield the surviving fraction (ordinate). The lower limit of the box marked \textit{HOU} indicates the surviving fraction after hydroxyurea treatment only.
their \( G_1 \) phase is extremely brief,\(^{10}\) because the interpretation of an age response is facilitated when the exact position of the \( S \) phase can be determined directly by measuring the incorporation of radioactively labeled thymidine, a precursor of DNA. This can be done with cells synchronized by selective harvesting of mitotic cells, but not with cells synchronized with hydroxyurea. It is easier to synchronize HeLa cells by mitotic cell selection than it is to synchronize Chinese hamster V79 cells by this method.) HeLa cells, with a mean generation time of 22 hours, were cultivated by standard techniques,\(^{24}\) as previously described.\(^{2}\) Cell survival was determined in terms of colony-forming ability.\(^{3}\) Partially synchronized cell populations were obtained either by mitotic cell selection\(^{1}\) or by exposure of asynchronous log-phase cells to hydroxyurea.

![Figure 2](image-url)

**Fig. 2.** Age response of HeLa cells in vitro to X-radiation. Cells were synchronized either by selective harvesting of mitotic cells (\(\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cd-
of drug treatment. The continuous line (circles) shows the variation in survival of cells after irradiations carried out from 0 to 15 hours after pretreatment with hydroxyurea (upper abscissa; time 0 corresponds to the termination of the four-hour drug treatment by washing the cells with saline and placing fresh medium in the dishes). The dashed line (squares) shows the variation in survival following selective harvesting of mitotic cells (at time 0 on the lower abscissa). The abscissae have been displaced so that the troughs of the two curves coincide. The structure of the age response of HeLa cells to X rays is well known: the minimum in survival corresponds to the transition of cells from G1 to S, and as cells pass through the S phase, the survival rises steadily to a maximum.26 The peak rate of DNA synthesis, which in these experiments was observed at 13 hours after selecting mitotic cells,2 served to locate the middle of the S phase in cells synchronized by mitotic selection; the approximate distribution in the different phases of the division cycle is indicated by the box diagram at the bottom of Figure 2. That this box diagram also applies to the curve for cells synchronized with hydroxyurea is suggested by the close similarity between the two curves and was confirmed by the observation that the number of rounded (mitotic) cells peaked at 17 hours after removal of the hydroxyurea (unpublished results), while the peak in number of rounded cells after mitotic selection was observed 22 hours after collection of mitotic cells.2

Through a comparison with Figure 2, the age response of the lymphoma cells (Fig. 1) may be interpreted as follows. The minimum in survival at 8 hours may be attributed to cells at the transition between G1 and S, and the final steep increase (8–16 hours), to the passage of the cells through the S phase. Interpretation of the initial part of the age-response structure is less certain because, as described above, the population consists of a mixture of G1, M, and some G2 cells. Furthermore, by analogy with cultured cells, the G1 phase may be perturbed14–17 and the X-ray sensitivity of G1 cells may be modified.11 In addition, a residual quantity of hydroxyurea may also be present. Tentatively, however, the first peak at four hours may be attributed to G1 cells, and the initial low value at 0 hour to sensitized G1 cells and/or to cells in mitosis. The known doubling time of the lymphoma cells (11.5 hours) is compatible with this time schedule, allowing for an elongation of the G1 phase. The interpretation given above requires that the middle of the S phase should lie at about 12 hours after synchronization. It is, of course, not feasible to locate the position of the S phase directly by labeling the lymphoma cells with radioactive thymidine as in the case of HeLa cells that have been synchronized by mitotic selection, since a cell population treated with hydroxyurea consists of a mixture of doomed nonviable cells together with viable survivors. However, evidence regarding the location of the S phase was afforded by preliminary experiments in which the age responses of the lymphoma cells to the cytotoxic agents vinblastine and vincristine were determined. We have recently shown that the age response of HeLa cells to both vinblastine and vincristine exhibits a well-defined minimum in survival at the middle of the S phase.2 When lymphoma cells, synchronized in vivo as described above, were treated with vinblastine or vincristine, they were found to pass through a well-defined trough in survival at between 10 and 12 hours after synchronization. Again by analogy
with HeLa cells, this observation indicates that the middle of the S phase does indeed lie at about 12 hours on the time scale in Figure 1.

The approach described above to the problem of synchronizing tumor cells in vivo is of considerable importance because of the significance of the age response of cells in the development of more rational treatment regimens in tumor therapy. Combined hydroxyurea and X-ray treatment has already been subject to clinical trials. The protocols for such trials might profitably take into consideration the age responses of the normal and malignant cells in the patient. Further experiments to obtain more detailed X-ray age-response parameters and to explore the possibility of using other synchronizing agents in vivo are in progress.

**Summary.**—Murine lymphoma cells in vivo were synchronized by administration of a single dose of the cytotoxic drug hydroxyurea to lymphomatous mice. The age response of these synchronous cells to X-radiation was measured. It is similar to that of HeLa cells in vitro.

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