Studies on the Kinetics of Marrow Regeneration after Local Irradiation of the Femur in Rabbits

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SUMMARY

The development of 2 laboratory techniques for more accurate quantification of hematopoietic activity, (a) radioactive iron (59Fe) uptake and (b) DNA content of marrow cells, are described, utilizing a regenerating marrow system in rabbits after local irradiation of 1 femur while shielding the contralateral leg. The sensitivity and reproducibility of the 2 methods were determined; there was good correlation of the results obtained with these 2 techniques. The significance of these tests of bone marrow activity as useful indicators in the experimental evaluation of new myelosuppressive agents is discussed.

INTRODUCTION

A major problem in cancer chemotherapy is the achievement of selective effect, since most of the chemotherapeutic agents currently available can be expected to inhibit rapidly proliferating tissues, but lack discrimination between normal and malignant cells (4, 5). Many clinical experiences in the past have resulted in relatively severe manifestations of toxicity to normal tissues, particularly with intensive use of chemotherapy (10, 11, 12, 28). Bone marrow depression is usually the limiting factor with respect to the amount of drug that can be administered and the margin of safety may be precariously thin. Attempts to protect the hematopoietic system during administration of these drugs have included regional perfusion (7), autologous marrow transplantation (23), circulatory occlusion to bone marrow areas either by arterial tourniquets (6) or by balloon catheters (21), general body hypothermia (22), and the systemic or regional administration of specific antidotes during chemotherapy with the corresponding drugs (3, 16, 25).

Although these techniques have produced varying degrees of success, none has enjoyed practical clinical usefulness and there has been a relative paucity of experimental models designed to measure quantitatively the effectiveness of hematopoietic protection. There is a need in clinical pharmacology, and specifically in cancer chemotherapy, for the development of accurate methods for predicting dosage, specificity and toxicity of drugs.

The present study describes the development of 2 simple laboratory techniques for more accurate quantification of bone marrow activity and their possible application in the experimental evaluation of chemical or physical myelosuppressive agents. In this report, the 2 methods are compared with respect to their ability to measure daily changes in the hematopoietic function after local irradiation to the femur of rabbits.

MATERIALS AND METHODS

Seventy-six New Zealand albino rabbits weighing 1.4-2.0 kg were used in these experiments. Male and female animals were used since previous experiments had shown that no measurable difference in response of the bone marrow to irradiation could be correlated with sex (26). The source of X-ray was a 250 KVP Westinghouse Quadroconex Model therapy unit operated at 15 ma with filters of 0.5 mm copper + 1 mm aluminum, a TSD of 50 cm and an HVL of 1.65 Cu. The beam was calibrated with a Phillips ionization chamber giving an output of 116.7 R/min. The tissue dose was measured using a Phillips dosimeter inside the femur of a rabbit, embedded in paraffin in the shape of a rabbit thigh (Figs. 1, 2). A cone 6 × 8 cm delivered 2250 roentgens to the bone marrow cavity. This particular dose resulted in maximum depression of marrow activity, but permitted recovery of marrow function over the succeeding 11 days of observation.

All rabbits were anesthetized with pentobarbital sodium, 107 mg/kg, administered in a single injection into the marginal vein of the ear. The animals were secured to the table with their hind legs spread and flat. The cone was placed against the medial aspect of the right femur and angled away from the left. The left femur was completely shielded with 4 mm of lead. A tracer dose of 59Fe (5 μC of Squibb radioferrous citrate) was given intravenously 8 hr before sacrifice. Previous studies in our laboratory had shown that maximum uptake of 59Fe by the bone marrow in unirradiated rabbits occurred in 6 to 8 hr and remained constant until 10 to 12 hr after injection. The rabbits were sacrificed by an overdose of pentobarbital sodium (180-200 mg), at daily intervals from Day 1 through Day 11 after irradiation. Both femurs were severed immediately and a 3 cm segment of the shaft was sawed close to the junction of the distal epiphysis. The entire volume of marrow from this segment was removed from the cavity, weighed, and...
counted for $^{59}$Fe radioactivity in a well-type scintillation counter (Fig. 3). The specimen was then frozen immediately and stored for subsequent measurement of DNA content. Uptake of $^{59}$Fe was calculated as cpm/gm of marrow.

A modification of the Dische-Burton diphenylamine reaction (2) was used to assay the DNA content of bone marrow. The marrow was placed in graduated test tubes and the volume was brought up to 3 ml with 0.5 N PCA. The test tubes were incubated in a constant temperature water bath at 85°C for 30 min. They were then cooled to room temperature and filtered. Aliquots of 0.02, 0.05, 0.08, and 0.10 ml were taken of the filtrate and PCA was used to bring all the aliquots to an equal volume. Standards were tested using deoxyadenosine (Calbiochem) at 1 μmole/ml; a control with PCA was also used. The diphenylamine reagent, which must be prepared for each series of determinations, consisted of 1.5 gm diphenylamine (Fisher Scientific), 1.5 ml concentrated H$_2$SO$_4$, and 100 ml glacial acetic acid. Acetaldehyde (20 mg/ml) was added immediately before use (0.1 ml/20 ml reagent). Two volumes were added to all test tubes, standards, and control. The tubes were then covered and kept in the dark at 26-30°C for 17 hr before they were read on a Zeiss spectrophotometer at 600 nm.

The Dische-Burton diphenylamine reaction offers a more sensitive determination, which is less susceptible to interference by other compounds, than does the original Dische procedure (8). The Dische-Burton method had to be modified for use in a rabbit bone marrow system in which the total nucleic acid content was less than 5 μmoles. The DNA was calculated as μmoles/gm of marrow.

RESULTS

Seventy-two rabbits received local irradiation to 1 femur, while the contralateral extremity was shielded. Six rabbits were sacrificed 8 hr postirradiation and 6 each day thereafter. The average values of marrow DNA content were plotted as shown in Chart 1; the results of the $^{59}$Fe uptake studies are illustrated in Chart 2. The magnitude of the difference in $^{59}$Fe uptake and total DNA content of the marrow in the shielded leg and the irradiated leg is readily apparent and the close correlation obtained with the 2 methods is also demonstrated. Both curves of hematopoietic function in the shielded leg show a similar depression, which reaches its nadir after the marrow of the irradiated leg is already in a phase of rapid recovery. Maximal depression occurred 3 to 4 days after irradiation when the percent of residual marrow activity (irradiated femur/shielded femur) was 7% $^{59}$Fe and 17% DNA content. Recovery began on Day 4 and, in these experiments, marrow samples were evaluated daily until 11 days after irradiation.

A control group of 4 unirradiated rabbits was sacrificed and $^{59}$Fe uptake and DNA content were determined for both femurs. The standard error of the mean was calculated for the 8 samples of normal bone marrow. The data listed in Table 1 demonstrate no significant variation in hematopoietic function between the left and right femurs in this control group.

DISCUSSION

Before exploring the possibilities of protecting hematopoietic function from the damaging effects of various forms of antineoplastic therapy, quantitative technics must be developed to determine the extent and duration of myelosuppressive action for an appropriate system. The technics described in this report were devised in rabbits because of the inherent advantages in using an animal with vessels large enough to permit the use of continuous intraarterial infusions of drugs in future studies.
The use of chemotherapeutic agents by this method in rabbits bearing VX2 carcinoma in each limb has been described (24).

By using this transplantable epidermoid tumor in conjunction with our technics for studying normal marrow in the same animal it is feasible to reproduce an experimental situation that resembles the clinical problem of administering regional therapy to an unresectable neoplasm with concomitant regional protection of the bone marrow.

Although several antimetabolites are currently being evaluated in this system (15), the present study was designed to assess the effects of local irradiation upon hematopoietic function. The expected myelosuppressive effect and recovery phase were observed with excellent correlation of the 2 quantitative methods employed. An interesting finding was a consistently reproducible depression of DNA content and $^{59}$Fe uptake by the marrow of the contralateral (shielded) limb. The amount of radiation delivered to the shielded femur was measured and found to be below 7.5 R consistently on repeated determinations. Subsequent data by our group have shown that a minimum of 15 R delivered to 1 femur is needed to produce a 3% depression in the shielded limb, as determined by these same 2 methods. Since the above observation appears compatible with an abscopal effect from irradiation, our system may prove useful for studying this unexplained phenomenon (9, 18).

After destruction of hematopoietic tissue by irradiation, repopulation depends upon the restoration of marrow cells capable of regeneration and proliferation (1, 14). These have been called clone-forming cells and are thought to represent the stem cells of hematopoietic tissue (17, 27). The regenerating phase of hematopoietic function following radiation injury provides a valuable opportunity for studying the myelosuppressive properties of various cytotoxic drugs upon rapidly proliferating marrow cells. In lethally irradiated mice treated with aliquots of marrow cells from syngenic donors, the regenerating marrow demonstrates increased sensitivity to chemotherapeutic agents, with respect to normal marrow (13). The use of normal metabolites to reverse the inhibitory effects of specific antimetabolites, or the combination of several anti-neoplastic agents to detect possible enhancement of action, have been reported with this sensitive technic (19). In addition to the opportunity of administering drugs or antidotes by continuous intraarterial infusion, the rabbit method described in this report offers other advantages with respect to the regenerating marrow system in the mouse. These include the absence of need for pooling aliquots of marrow for quantitative determinations (13), the fact that 2 comparable areas of marrow can be evaluated in the same animal, and the avoidance of external manipulation of cells as necessitated by the usual grafting procedures (20).

The relatively high degree of sensitivity, reproducibility, and correlation of the methods described and the absence of major technical complications suggest that this may be a useful system for determining the myelosuppressive effects of antineoplastic agents alone or in combination.

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**REFERENCES**


Fig. 1. The tissue dose of irradiation was measured using the Phillips dosimeter inserted into a paraffin and bone phantom.

Fig. 2. Roentgenogram demonstrating the dosimeter probe in place in the marrow cavity of the phantom.

Fig. 3. Stages in the removal of bone marrow for 59Fe uptake and DNA content. Both femurs have been amputated and separated from the surrounding structures; the right femur has been severed to demonstrate the 3-cm portion of the shaft that was used for these experiments. On the far right, the total volume of marrow obtained from the 3-cm segment has been removed, weighed, and placed in a planchette which was then inserted into a deep-well scintillation counter to determine the 59Fe uptake.
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