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COMPARISON OF CELL PROLIFERATION IN THE REGENERATING BONE MARROW AND SPLEEN OF IRRADIATED MICE WITH THE USE OF ^{125}I-IODODEOXYURIDINE

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Recovery of hemopoietic injury in a sublethally irradiated organism is an expression of the activity of homeostatic mechanisms which tend to establish new steady states. The surviving hemopoietic cell populations are under the action of a proliferation stimulus by which the organism accelerates the regeneration of the hemopoietic system. Evidence that the proliferation control is rather local than circulating in nature (GIDALI & LAJTHA 1972, PATT & MALONEY 1972) implies the possibility of differences in repopulation between the various hemopoietic localities or organs. An analysis of these differences may contribute to better understanding of the mechanisms of effective postirradiation recovery.

The purpose of the experiments now presented was to analyse some characteristics of the repopulation activity in the bone marrow and spleen of sublethally, whole-body irradiated mice with the use of ^{125}I-iododeoxyuridine (^{125}IUdR). ^{125}IUdR is incorporated by DNA synthesizing cells as an analogue of thymidine and offers several methodologic advantages for analysis of cell population kinetics in vivo, i.e. specificity for DNA synthesis, stability, inefficient re-utilization and easy counting of its gamma emission (HUGHES et coll. 1964, COMMERFORD 1965). It may be used either as an indicator of the proliferative activity of cells (CUDKOWICZ et coll. 1964, DEGOWIN 1967, SIEGERS et coll. 1979), or as a cell tracer which makes it possible to obtain some evidence about the organ cell losses (including cell deaths) or immigration of labelled cells (JOEL et coll. 1977, WEBB et coll. 1980). Both these methodical possibilities were utilized in the present experiments.

Material and Methods

Inbred C 57Bl/10 male mice, aged 12 to 14 weeks and weighing 25 to 30 g, were used. Standard stock diet and drinking water were given ad libitum. The mice were irradiated with single sublethal whole-body doses of 5.5 Gy from a ^{60}Co gamma ray source at a dose rate of 0.47 Gy/min. Irradiation was performed in the morning hours.

^{125}IUdR labelling and measurement of incorporated activity. Mice were given intraperitoneally 3.7×10^4 Bq (1 μCi) ^{125}IUdR (The Radiochemical Centre, Amersham, England) in 0.4 ml saline. In order to inhibit endogenous thymidylate synthesis, fluorodeoxyuridine (10^{-7} mol) was injected intraperitoneally 30 min before ^{125}IUdR (HUGHES et coll., TAKADA et coll. 1971). At various intervals after the isotope injection the mice were killed by cervical dislocation, the spleen and left femur were excised and placed in 10% buffered formalin for 48 h to remove radioactive iodine not incorporated into DNA (BÜRKI et coll. 1971). The activity of each organ was measured using the Nuclear Chicago Automatic Gamma Well Counting System and ex-

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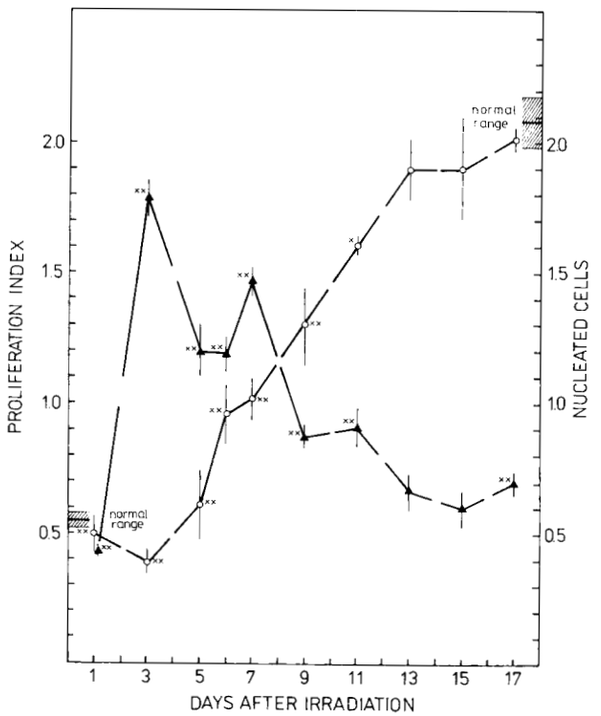

Fig. 1. Number of nucleated cells in the femur $\times 10^7$ (○) and the proliferation index (▲), calculated as per cent of incorporated ^{125}IUdR activity/10^8 nucleated cells on different days after irradiation with 5.5 Gy. Difference from controls: × $p<0.05$, ×× $p<0.01$.

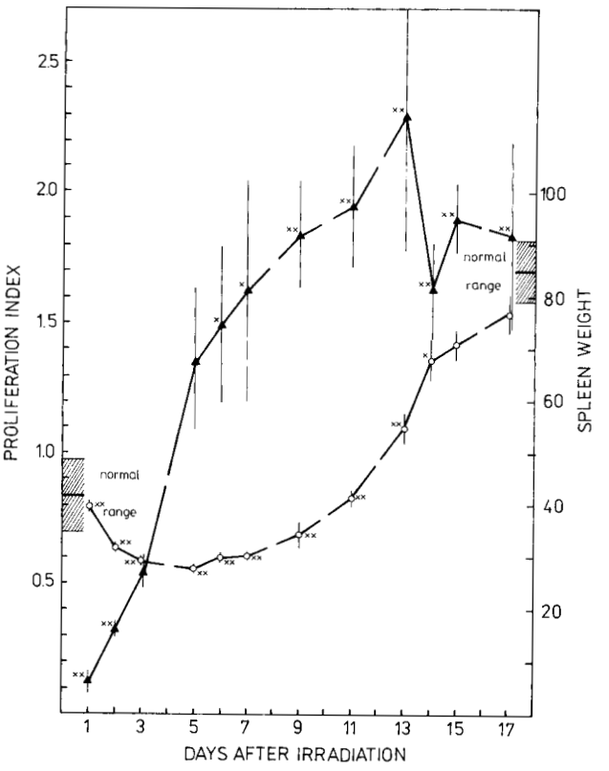

Fig. 2. Spleen weight in mg (O) and proliferation index (▲), calculated as per cent of incorporated ^{125}IUdR activity per 100 mg of spleen weight (before calculation 25 mg were subtracted from the real spleen weight) on different days after irradiation with 5.5 Gy. Difference from controls: × $p<0.05$, ×× $p<0.01$.

pressed as per cent of the total radioactivity administered.

Spleen weight and femoral cellularity. Before fixation the wet spleen weight was estimated. After washing off the marrow into Hanks' solution and removing erythrocytes by saponin, counts of nucleated cells in the femur were determined by a Coulter Counter. In separate experiments it was confirmed that the cellularity of the spleen in the observed intervals of postirradiation regeneration is a linear function of wet spleen weight from which 25 mg (capsule weight) were subtracted (WANGENHEIM et coll. 1980).

The proliferation index of the organs was estimated in animals killed 6 h after ^{125}IUdR injection. The proliferation index of the femoral bone marrow was calculated as per cent of incorporated activity per 10^8 nucleated cells. The mean values of bone marrow cellularity which were used for calculation of the proliferation index were obtained from different groups of animals killed at the time of day corresponding to the time of isotope injection. The proliferation index of the spleen was estimated as per cent of incorporated activity per 100 mg of spleen weight (minus 25 mg allowance of capsule weight). In this calculation the corresponding individual spleen weights of isotope injected animals were used. Because the half-time of incorporation of ^{125}IUdR into DNA is only a few minutes (HUGHES et coll.), the calculation of proliferation index for the spleen weight 6 h after isotope injection may be slightly erroneous. In the period of increased splenic weight after irradiation the values of spleen proliferation index are probably underestimated. However, in view of the results emphasized, i.e. high values of the proliferation index, the calculations used will err on the conservative side.

Estimation of radioactivity retention in organs with time. In these experiments animals were killed 1, 2, 3, 4 and 5 days following ^{125}IUdR injection. The activities of spleen and femur were determined as mentioned and related to the whole organ. Day 1 was chosen as the initial reference point and the retention of activity in femur and spleen in the next days expressed as per cent of this mean reference value. Retention of ^{125}IUdR in the organs of

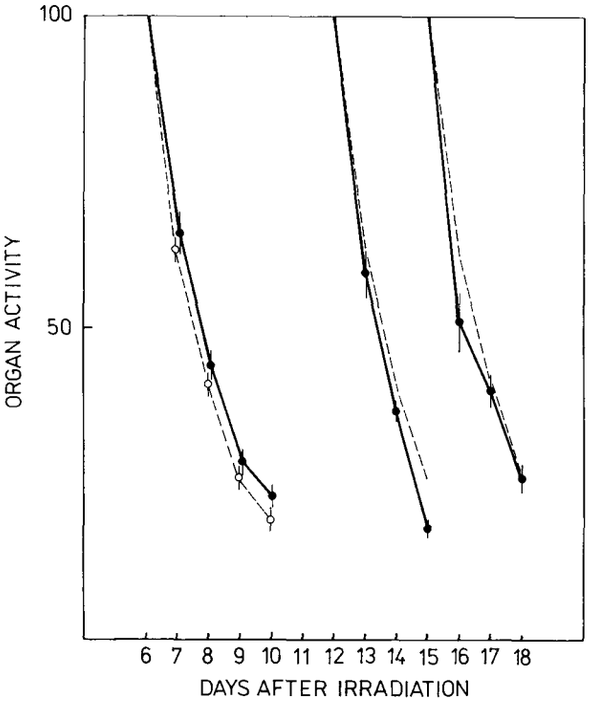

Fig. 3. Activity of the femur expressed as per cent of activity estimated 24 h after ^{125}IUdR injection (100%) 5, 11 and 14 days after irradiation with 5.5 Gy (—). Activities in unirradiated animals (- - -).

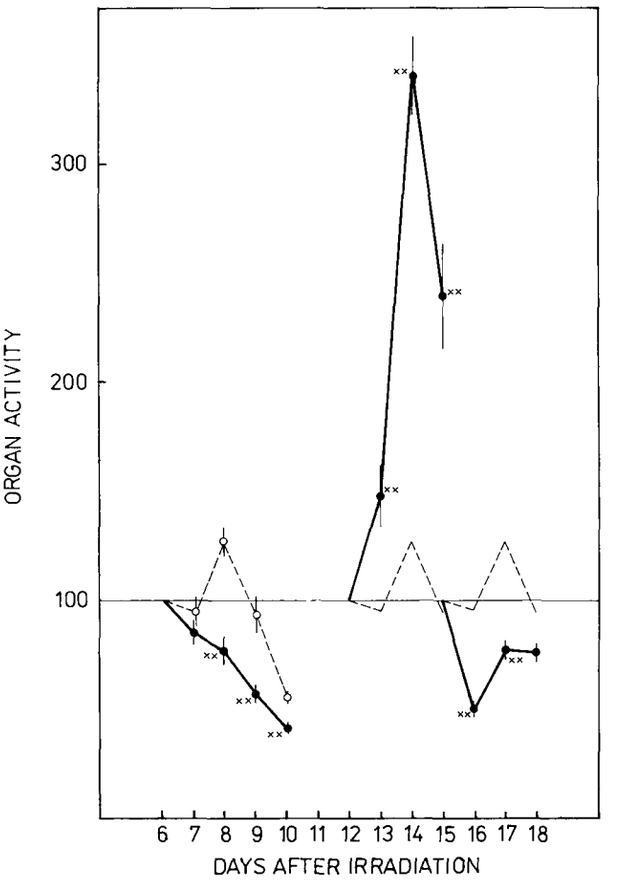

Fig. 4. Activity of the spleen expressed as per cent of activity estimated 24 h after ^{125}IUdR injection (100%) 5, 11 and 14 days after irradiation with 5.5 Gy (—). Activities in unirradiated animals (- - -). Difference from controls: ×× $p<0.01$.

irradiated animals was estimated in three periods, i.e. after its injection on day 5, 11 and 14 after irradiation. Parallelly unirradiated control animals were subjected to the same procedure and the results pooled.

Statistics. The values given in the data represent the mean ± SE. For every point 8 to 15 animals were used. Statistical significance of the results was evaluated using the distribution-free sequential test.

Results

The postirradiation changes of the cellularity and of the proliferation index in the bone marrow of the femur appear in Fig. 1. The values of bone marrow cellularity exhibited the lowest level on day 3 after irradiation, then rose and reached approximately the control range on day 13. The proliferation index was slightly under the control range on day 1 after irradiation, then increased rapidly to its highest level on day 3, and from this time on decreased to reach the control level on about day 15.

Fig. 2 gives the results of experiments evaluating the proliferation dynamics in the spleen of irradiated mice. The spleen weight was mostly decreased on day 5 after irradiation; then it increased and reached the control level at the end point of the experiment, i.e. on day 17 after irradiation. The proliferation index of the spleen was more severly suppressed than that of the bone marrow. It rose from day 2 after irradiation, surpassed the control range on day 5, reached the highest level on day 13, and remained above the values of steady state controls up to day 17 after irradiation.

The retention of labelled ^{125}I in the femoral bone marrow of control as well as irradiated animals as a function of time after the injection of ^{125}IUdR is shown in Fig. 3. The decline of organ activities calculated as per cent of activity estimated 24 h after ^{125}IUdR injection was fast and reached approximately 25 per cent of the reference value 3 days later. The rates of activity loss were similar in all the measured postirradiation intervals and were not different from controls.

The retention of activity in the spleen of control and irradiated animals is compared in Fig. 4. In the control spleen the decline of activity did not start so early as in the femur, and 3 days after isotope injection the retention was higher. The increased organ activity 3 days after isotope injection as compared with the values of 24 h incorporation was repeatedly observed and cannot be regarded as a random phenomenon. In animals injected with ^{125}IUdR at various intervals after irradiation the fate of splenic activity was markedly different from controls. Animals injected 5 and 14 days after irradiation exhibited significantly higher losses of organ activity. In animals injected on day 11 after irradiation a marked and highly significant increase in splenic activity on days 13 to 15 after irradiation was recorded.

Discussion

Already previous experiments on sublethally irradiated mice had demonstrated marked differences in hemopoietic postirradiation recovery in the spleen as compared with the femoral bone marrow. After an initial postirradiation drop in total nucleated cells of both organs, the repopulation of the bone marrow starts earlier (GUZMAN & LAJTHA 1970, BERAN & TRIBUKAIT 1972, OKUNEWICK et coll. 1972). Similarly VÁCHA et coll. (1974) described in various strains of mice a faster normalization to the preirradiation level of erythropoiesis in the femoral bone marrow as measured by the ^{59}Fe uptake method. The present findings are compatible with these results and indicate an earlier onset of effective cellular repopulation in the marrow as opposed to that of the spleen.

The initial postirradiation dip of the proliferation index observed in both organs may be caused by a complex of events including the radiation injury of cells, the relative preponderance of surviving (radiation resistant) nonproliferating functioning cells, as well as the increase in thymidine pool due to degradation of DNA, which may lower the IUdR uptake via the salvage pathway. The suppression of the proliferation index on day 1 after irradiation is more evident in the spleen and indicates a higher radiation injury of this organ. Similarly, GUZMAN & LAJTHA described in the spleen of sublethally irradiated mice a sharper and longer lasting dip in the number of colony-forming units as compared with the femur. The initial lowering of ^{125}IUdR incorporation seems to be rapidly overcome, and the proliferation index starts increasing from day 2 or 3 after irradiation.

In a repopulating femur the cellularity and proliferation index are inversely related in time. The proliferation index reaches its highest level concomitantly with the lowest counts of nucleated cells. Afterwards, with the increase of cell content in the femur the proliferation index decreases with a damped oscillation approximately to the control level at a time at which cellularity approaches its normal range. PATT & MALONEY observed in the locally disturbed femoral bone marrow of rabbits a similar negative correlation between cellularity and changing fraction of proliferating cells as measured by ^{3}H-TdR uptake. These authors showed that bone marrow production is geared to cell population density and their findings indicated a self-regulatory local homeostatic mechanism. The present findings may be evaluated in a similar way and agree with a widely accepted conception that the regulation of mitotic activity of tissues generally depends upon the number of actually present cells. Updated evidence indicates that the mechanisms of local proliferative regulation in the hemopoietic tissue involve certain inhibitory materials (chalones), particulary in the recognizable maturing end cells exhibiting a specific feedback control effect on the proliferation of the respective cell lines (BULLOUGH 1975). Moreover, humoral factors released from yet unidentified cells of the heterogeneous macrophage populations and acting as inhibitors or stimulators of hemopoietic stem cell proliferation were described (LORD & WRIGHT 1980).

The relationship between the repopulation of spleen (as demonstrated by organ weight) and the proliferative activity of this organ indicates a different regulatory situation. The proliferation index of the spleen surpasses the normal range on day 5 after irradiation, continues increasing parallelly with the organ weight, and attains the high level even at the time of normalization of the spleen weight. Assuming that spleen weight represents cellularity (cf. methods), these results may be discussed as follows. As compared with the bone marrow, the observed proliferation characteristics of the irradiated and regenerating spleen may be either an expression of physiologic peculiarities of the local control of splenic cellularity, or a result of the ineffectiveness of control mechanisms due to radiation injury of cells. LEWIS et coll. (1977) compared the mode of exogenous and endogenous hemopoietic repopula-

tions following radiation injury in mice. They deduced that splenic hemopoietic stem cells were not capable of undergoing radiation repair as easily as were the stem cells of the bone marrow. If so, a higher proportion of genetically injured cells may persist in the irradiated spleen and pass through a few mitotic cycles before death (BOND et coll. 1965). It even appears probable that a high proliferation stimulus enables the injured cells to pass through more mitotic cycles than is normally the case (WANGENHEIM et coll.). The injured cells may thus temporarily contribute to the cellularity of the organ, but the cell-mediated control feedback may be absent or weakened due to functional injury, premature elimination, or death of the cells participating in the proliferative control. These mechanisms may operate for several days after irradiation. WANGENHEIM et coll. observed mitotic activity in the spleen of mice for at least 12 days after irradiation with 8 Gy. They emphasized that following this fatal dose most of the mitotically active cells must be expected to lose acentric chromosome fragments. Probably for these reasons and despite the persistently increased proliferation index, the repopulation effectiveness of the spleen at early intervals after irradiation is lower than that of the bone marrow.

Experiments with measuring the retention of ^{125}IUdR in hemopoietic organs in time give a further evidence of different cell population dynamics in the regenerating bone marrow and spleen. The rate of decline of femoral activity in unirradiated animals is comparable with the results of other authors (JOEL et coll., WEBB et coll.). No evident differences existed between control and irradiated animals measured in three distinct intervals after irradiation. These results suggest that the migration or disintegration of recently produced cells in the regenerating bone marrow is not modified. The fate of activity in the spleen indicates a more complicated picture. The transient elevation of activity in the spleen of unirradiated animals 3 days after isotope injection suggests that the retention of ^{125}IUdR in this organ is the result of a balance between cell losses and the immigration of labelled cells. A similar phenomenon has not yet been observed and may be due to the strain of mice used. As compared with the control curves the retention of labelled cells in the spleen of irradiated mice exhibits marked deviations. Experiments with measuring the fate of spleen activity between days 6 and 10 as well as between days 15 and 18 after irradiation indicate higher losses of labelled cells. A probable contribution of cell lysis or a fast traffic of cells through the organ cannot be ascertained. However, at least in the interval between days 8 and 10 after irradiation, spleen activity losses could be mediated by a suggested disintegration of injured and proliferating cells as discussed. The peculiar situation in the spleen is further characterized by increased organ activity in the interval between days 13 and 15 after irradiation. This result may be considered as evidence of an immigration of labelled cells into the spleen. The nature and source(s) of the cells participating in this immigration are not known and deserve further experiments. However, in this context the phenomenon of lymphocyte migration and recirculation (YOFFEY 1964) as well as the auxiliary role of some lymphocyte populations in the proliferation of hemopoietic tissue (GOODMAN et coll. 1978) should be mentioned. It is quite possible that the relative 'opening' of the spleen to immigration of cells from other sources, together with the ability of this organ to increase its volume (in contrast to the bone marrow), may enable the shifting (elevation) of a set point of local cellularity control and thus contribute to the persistance of a high proliferation index. Due to these properties the spleen of mice may function in a booster capacity to marrow hemopoiesis and exhibit the known overshoot in cellularity which follows the recovery after sublethal irradiation (BOZZINI et coll. 1970, TAKADA et coll., VÁCHA et coll., BRADY et coll. 1976, PAVELIĆ et coll. 1979).

SUMMARY

The method of ^{125}IUdR labelling of hemopoietic cell populations was used to estimate differences of repopulation kinetics in the femoral bone marrow and spleen of sublethally irradiated mice. In regenerating bone marrow the cellularity and proliferation index, as measured by 6 h ^{125}IUdR incorporation, were found to be inversely related. In the spleen the proliferation index increased parallelly with the increase in cellularity and indicates an ineffectiveness or a change in the set point of local cellularity control. Experiments with measuring the retention of ^{125}IUdR in hemopoietic organs revealed, in the spleen of irradiated animals, phases of increased cell losses as well as immigration of labelled cells into this organ.

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