Effect of the Acute Rat Leukemia L5222 on Bone Marrow Stroma Cells

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SUMMARY

The reaction of bone marrow stroma cells, labeled by the continuous thymidine-3H labeling method, was studied in rats inoculated with the acute myelomonocytic leukemia, L5222. During the 7-day course of the disease, the number of labeled endothelial cells fell to about one-half the initial level, whereas labeled reticulum cells remained constant. The decrease in endothelial cells was probably due to cell death, since degenerating labeled endothelial cells and labeled cell debris near the sinuses were observed. In addition, the constant labeling intensity of endothelial cells indicated that no proliferation occurred. At about the same time as the fall in the number of labeled endothelial cells (histologically evident as alterations in sinusoidal structure), a decrease in normal, immature hemopoietic cells in the bone marrow occurred. A decrease in bone marrow granulocytes and an increase in peripheral blood granulocytes, concomitant with the changes in sinus structure, were interpreted as a disturbance in the release mechanism of mature cells to the peripheral blood due to growth of the leukemia.

INTRODUCTION

Failure of normal hemopoiesis is an important problem in human acute leukemia with serious clinical consequences. The pathogenesis of this process is still largely unknown and may be caused by several factors such as a direct replacement of normal bone marrow cell populations by leukemic cells and/or a reduced influx from the normal stem cell pool. Since an intact bone marrow stroma is known to be an important requisite for efficient hemopoiesis, as demonstrated in recovery from irradiation injury (13, 15), one should consider whether alterations in this system are relevant for the pathogenesis of hemopoietic failure in acute leukemia.

In order to study this aspect, a transferable experimental rat leukemia, L5222 (10), has been used, in which a reduction of normal hemopoietic cells to about one-half the normal level occurs, with development of anemia and thrombocytopenia (12). Concurrently, damage to the bone marrow sinusoidal system becomes apparent and increases in its extent during the course of the leukemia, as reported from previous histological studies (11). Such alterations in the sinusoidal structure of rat bone marrow have also been found in the Shay chloroleukemia (2, 9), and their importance for the reduction in normal hemopoiesis has been emphasized by Handler et al. (8).

The aim of the present study was to establish for the leukemia L5222 the temporal relationship of alterations in the sinusoidal system of the bone marrow to the reduction in the number of normal hemopoietic cells and also to the release of mature cells to the circulation. By means of labeling with TdR-3H, changes in the number of endothelial cells and of reticulum cells have been determined and their proliferative behavior studied. Since the endothelial cells and reticulum cells have a slow turnover rate and only few can be labeled by a single injection of TdR-3H, they were labeled by the continuous application of TdR-3H during fetal life to produce complete labeling of all cell nuclei in the body (4).

MATERIALS AND METHODS

Animals. BD IX inbred rats (3) of both sexes weighing 100 to 150 g were used.

Complete TdR-3H Labeling Procedure and Transfer of Leukemia. TdR-3H was continuously administered to pregnant rats through a polythene catheter inserted into the tail vein from Day 9 of pregnancy (onset of organogenesis) until delivery (7). The dose used was 1 µCi/g body weight in 1 ml/day with a specific activity of 2 µCi/mm. Following this treatment, all cell nuclei in the newborn rats were labeled. Administration of TdR-3H to the offspring was continued s.c. every 8 hr at a dose of 1 µCi/g/day up to 5 weeks in order to maintain complete labeling of all cell nuclei during the period of rapid growth of the animal. At 5 weeks, TdR-3H administration was stopped and then 2 weeks were allowed for the rapidly proliferating cell systems to lose their label by dilution. Thus, only slowly proliferating cells in the bone marrow, i.e., reticulum cells, endothelial cells, tissue mast cells, and a proportion of the small lymphocytes, remained labeled (4). At this time (7 weeks of age) 36 of the

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2 The abbreviation used is: TdR-3H, tritiated thymidine.
offspring that had received TdR-3H and also 4 control rats of the same age without TdR-3H were inoculated with leukemia by i.v. injection of 10^7 peripheral blood leukocytes from a leukemic donor. Survival time after this cell dose was about 1 week. Six TdR-3H-labeled control animals, used to establish labeling indices without leukemic influence, received 0.9% NaCl solution only.

A comparison of daily leukocyte counts in leukemic rats that had received TdR-3H and in the 4 control leukemic rats without TdR-3H showed that the course of the leukemia was not affected by the incorporated TdR-3H.

Blood and Bone Marrow Investigation. The TdR-3H-labeled leukemic rats were killed under ether anesthesia in groups of 3 at 12-hr intervals up to 6 days after transfer of leukemia. Three nonleukemic control animals were sacrificed at the beginning and 3 at the end of the experimental period. Blood smears prepared from aorta blood taken just before death and bone marrow smears from the femur were stained with May-Grünwald-Giemsa for cytological evaluation. The tibiae were fixed in formalin for histological sections.

Autoradiographic Procedure. Bone marrow smears fixed in absolute methanol were covered with Kodak AR10 film by the stripping method and exposed for 6 weeks at 4°. Bone marrow sections (3 to 5 μm) were covered with Ilford L4 emulsion by the dipping method and exposed for 11 weeks at 4°. After exposure, all autoradiographs were developed for 5 min in D19b developer, fixed in Kodak Unifix, and washed in running tap water for 30 min. Smears were stained with dilute Giemsa at pH 5.75 and sections were stained with hematoxylin-eosin.

Evaluation. Differential counts were made on 200 nucleated cells in peripheral blood smears. The changes in absolute numbers of erythropoietic, granulopoietic, and megakaryocytopoietic cells in the bone marrow were assessed semiquantitatively in the following way. Differential counts were carried out on 1000 nucleated cells in smears. Total nucleated cells per unit area were determined from hematoxylin-eosin-stained sections of the tibia by counting nucleated cells in 50 random microscope fields to a total area of 0.125 sq mm, including capillaries and sinusoids but excluding trabecular bone. Megakaryocytes were counted at a lower magnification to a total area of 2.5 sq mm. From these counts and the bone marrow differential counts, absolute numbers of each type of cell per unit area of section were calculated.

From autoradiographs of bone marrow smears, a differential count of 200 labeled cells (erythroid cells, reticulum cells, tissue mast cells, and small lymphocytes) was made according to the morphological identification described by Fliedner et al. (4). In autoradiographs of sections, the total number of labeled cells was counted in 100 microscope fields to a total area of 1.2 sq mm. From this number and the differential count of labeled cells in smears, the absolute number of labeled reticulum cells per unit area of section was calculated. The absolute number of labeled endothelial cells per unit area and the percentage of labeled endothelial cells were determined directly on sections, counting only the endothelial cells lining the walls of sinusoids and, thus, identifiable with certainty (Figs. 1 and 2).

Mean grain counts for the endothelial and reticulum cells were made on smears of 100 cells for each type. For this determination, only those labeled endothelial cells, which could be well identified by their typical arrangement in rows in the connective tissue, were included (Figs. 3 and 4). All slides were coded before evaluation.

RESULTS

The mean labeling index of the endothelial cells, determined from the TdR-3H-labeled nonleukemic control rats sacrificed at the beginning and end of the experiment, was 65%. Thus they represent the majority of the sinus endothelial cell population. During the course of the leukemia, the number of labeled endothelial cells in the bone marrow decreased to about one-half their initial level (Chart 1A); this decrease becomes obvious after the 2nd day following transfer of leukemia. In the later stages of the leukemia, degenerating labeled endothelial cells and labeled debris in the area of the sinusoids appeared. No increase in number of labeled cells of any type was observed. The mean grain count of the remaining labeled endothelial cells did not change over the whole period (Chart 1B).

That labeled endothelial cells of the bone marrow react in a specific way to the growth of the leukemia is borne out by the behavior of the reticulum cells. In contrast to the endothelial cells, labeled bone marrow reticulum cells (Chart 2A) did not show an appreciable change in number during the entire period, nor did they show any diminution in labeling intensity (Chart 2B).

Chart 3 illustrates the behavior of the immature cells of erythropoiesis, granulopoiesis, and megakaryocytopoiesis in the bone marrow after transfer of the leukemia. There is a reduction in all these cells, varying in degree and time of onset for the 3-cell series. Erythropoietic and megakaryocytopoietic cells began to decline in number after about the

<chart1>

Chart 1. Behavior of labeled endothelial cells in the bone marrow during development of leukemia. A, decrease in absolute number of labeled endothelial cells; B, mean grain count per labeled endothelial cell; O, control animals without leukemia.
Proliferation as a cause for this decrease, which would be demonstrable by a decrease in the labeling intensity, could be largely excluded, since the mean grain count per cell remained constant throughout the observation period. Cell death was considered the most probable explanation for the decrease, because degenerating labeling cells and labeled cell debris in the region of the sinusoids were observed. As a result, one would also expect histological evidence for damage in the sinusoidal system. This is in agreement with earlier histological observations (11), which revealed that as soon as the

2nd and 3rd day to approximately 10 and 25% of control values, respectively, and granulopoiesis, after more than 4 days, declined to one-half the control value.

The changes in the numbers of mature granulocytes and leukemic blast cells in the bone marrow and peripheral blood, as a function of time after transfer of leukemia, are shown in Charts 4 and 5, respectively. The band and segmented granulocytes in the bone marrow decreased markedly between 2.5 days and 3.5 days, and thereafter more slowly, to about one-third of control values. There was a corresponding rise of granulocytes in the peripheral blood after 2.5 days, to 7 to 8 times that of control values. The number of leukemic blast cells rose continually from the beginning in the bone marrow; in the blood, the 1st leukemic cells appeared between Days 2 and 3.

**DISCUSSION**

In the experiment described here, there was a clear decrease in the number of TdR-3H labeled endothelial cells during the course of leukemia development. Proliferation as a cause for this decrease, which would be demonstrable by a decrease in the labeling intensity, could be largely excluded, since the mean grain count per cell remained constant throughout the observation period. Cell death was considered the most probable explanation for the decrease, because degenerating labeling cells and labeled cell debris in the region of the sinusoids were observed. As a result, one would also expect histological evidence for damage in the sinusoidal system. This is in agreement with earlier histological observations (11), which revealed that as soon as the
2nd day after transfer of the leukemia the sinuses in some parts of the bone marrow were widely dilated and in others the sinuses were constricted or collapsed to a much greater extent than is seen in normal rat bone marrow (5). These changes became progressively worse so that in the terminal stages of the leukemia the sinusoidal system appeared to be almost completely collapsed and unable to fulfill its normal function. That not all the bone marrow stroma cells are similarly affected by the leukemia is apparent from the behavior of the TdR-3H-labeled reticulum cells, which were not altered throughout in their number or labeling intensity.

The slowly proliferating cells of the bone marrow labeled by continuous application of thymidine-3H in the manner described (in particular the endothelial cells) are not damaged by a radiotoxic effect of the contained tritium, since they are still able to react to appropriate stimuli. Thus, it was shown by Meyer-Hamme et al. (14) that, following partial depletion of the femoral bone marrow by mechanical means, the endothelial cells in the undisturbed part of the femur showed a high compensatory proliferative activity, as indicated by a fall in their labeling intensity accompanied by a marked decrease in their labeling index.

The reaction of TdR-3H-labeled endothelial cells during the leukemia is in contrast to their nonreactive behavior during bone marrow regeneration following treatment with such agents as X-irradiation (6), nitrogen mustard (7), or hydroxyurea (1), after which they show no change in number or in their labeling intensity. The resistance of the endothelial cells to these noxious agents suggests that their disappearance during the development of leukemia must be specific for the leukemic process. The influence of the leukemic cell population, either direct by cell-to-cell interaction or indirect through some humoral agent or other unknown mechanism, is such that endothelial cells are destroyed and those remaining are apparently unable to perform compensatory proliferation, at least within the period of observation.

The resulting collapse of the bone marrow sinusoidal system might have consequences for normal hemopoiesis in several ways. First, the microcirculation is altered, which could mean that the supply of nutrients for normal cells is inadequate. Second, the microenvironment envisaged by Trentin (16) would be changed, assuming that endothelial cells form a part of this entity. Since Wolf and Trentin (17) have indicated that a functioning microenvironment is important for the differentiation of the normal hemopoietic stem cell, this differentiation may be disturbed in the leukemic bone marrow. Third, the controlled release of mature cells to the circulation through the sinusoidal walls may no longer function normally.

Regarding the decrease in the normal immature cells of the bone marrow shown in Chart 3, the different times at which the fall in erythropoiesis, megakaryocytopoiesis, and granulopoiesis occurs could be explained by: a reduced influx of cells from the stem cell pool and the known varying transit times of cells in the 3 series; or a direct influence of the leukemic cell population and different sensitivities of the 3-cell series to this influence (12). As seen in Chart 1, the decrease in labeled endothelial cells begins at about the same time as in the immature hemopoietic cells and could thus be subject to the same process which acts on the proliferating cells. Our data do not clarify whether these are 2 simultaneous reactions to the same influence or whether the damage is primarily to the endothelial cells, which could affect the proliferating cells later. However, the coincidence of the 2 events is noteworthy, and perhaps destruction of bone marrow sinusoids has consequences for normal hemopoiesis, in particular, for possible regeneration during remission that is induced by therapy.

The effect of an altered sinusoidal system on the normal release mechanism of mature cells to the circulation may also have consequences for normal hemopoiesis. From the results presented in Charts 4 and 5, there are indications of a relationship between the degenerating sinusoidal system and an altered release of cells from the bone marrow to the blood. The decrease in bone marrow granulocytes becomes more pronounced between 2 and 3.5 days after transfer of leukemia and is accompanied by an increase of these cells in the peripheral blood. Here, both events occur when changes in the sinusoidal system commence, as indicated by the decline in endothelial cells. An increase in peripheral blood granulocytes was also found by Handler and Handler (8) in the Shby chloroleukemia. They concluded from their investigations that it must be due to extramedullary hemopoiesis (8). But this cannot be the case in the L5222 leukemia, since in earlier investigations (11) no appreciable extramedullary hemopoiesis was found. A further explanation could be that the granulocytosis reflects a subacute inflammation accompanying the leukemia, but only weak signs of inflammation were noted in previous histological observations of this leukemia (11). Thus, some evidence exists that augmented release of granulocytes from the bone marrow in this leukemia may be associated with alteration in sinusoidal structure. It should be mentioned, however, that the release mechanism must still be at least partially intact, since only a few immature cells are seen in the peripheral blood. A further explanation for the granulocytosis might be abnormally high levels of humoral "releasing factors," although evidence from the reduced response to endotoxin of rats with advanced chloroleukemia (8) contradicts this theory.

ACKNOWLEDGMENTS

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Figs. 1 and 2. TdR-3H-labeled endothelial cells in autoradiographs of histological sections of tibial bone marrow. Ilford L4 emulsion exposed for 11 weeks. × 1250.

Figs. 3 and 4. TdR-3H-labeled endothelial cells in autoradiographs of femoral bone marrow smears. Kodak AR10 emulsion exposed for 6 weeks. × 1250.
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