Sequestration of Heat-injured Erythrocytes in Perfused Spleens of Acute Myelogenous Leukemic Rats as a Measure of Reticuloendothelial System Function

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SUMMARY

This study was undertaken to determine whether splenic reticuloendothelial system function is altered in the spleens of rats with acute myelogenous leukemia. For this purpose, the isolated rat spleen perfusion technique was used to assess the sequestering capacity of normal and leukemic rat spleens for $^{51}$Cr-labeled rat erythrocytes that were damaged by heating in a 49.5°C water bath for 20 min. Leukemia was induced in rats by i.v. injection of 10 $\times$ 10$^6$ tumor cells obtained from Shay chloroleukemic donor rats. On the 10th postinjection day the recipient animal spleens were perfused. The results indicate that, as the leukemia progresses within the rat, splenic sequestration of damaged red blood cells is profoundly decreased. Reduced sequestering capacity was consistently evident in spleens obtained from leukemic animals in the late stages of the pathogenesis, as indicated by the percentage of leukemic myeloblasts in the bone marrow, and by splenic weight and histology. Reduced or unaltered sequestering capacity was found in spleens obtained from leukemic rats in which the pathogenesis was less advanced. In none of the leukemic spleens studied was cell sequestration enhanced. The findings suggest some form of mechanical impedance as one possible mechanism for the altered sequestration pattern of heat-injured erythrocytes in the spleens of leukemic rats.

INTRODUCTION

A relationship between neoplasia and RES$^2$ function has been demonstrated in recent years in studies performed with both animal (1, 17, 24, 29, 39, 40, 42) and human subjects (4, 7, 16, 34, 37). The findings of such investigations have not always been in agreement; RES phagocytic function has variously been reported to be depressed, stimulated, and in some circumstances unaltered by the neoplastic processes. Of the studies performed only a few have been directed toward the assessment of RES function in leukemia (4, 16, 29, 34). These have been limited, with minor exception (29), to human subjects where interpretation of the results may be complicated by direct damage to RE cells due to the administration of chemotherapeutic agents. Moreover, further difficulties arise from dissimilarities in the overall state of health, age, sex, and genetic constitution of the individuals examined.

The purpose of this investigation was to evaluate the effect of leukemia on splenic RES function using rats with an acute myelogenous leukemia (Shay chloroleukemia) (36, 45). The technique developed for perfusion of the isolated rat spleen (11) was used to assess the removal of $^{51}$Cr-labeled heat-injured rat erythrocytes from whole blood perfused through spleens obtained from leukemic rats. The important role of the RES of the spleen in the removal and destruction of aged erythrocytes and erythrocytes altered by chemical, physical, or immune injury is well documented (5, 6, 21, 44). The sensitivity of the isolated perfused rat spleen preparation for selectively sequestering heat-injured rat erythrocytes (10) makes it a useful tool affording several advantages. Of major importance is the fact that the technique allows for the accurate quantitative assessment of the clearance of altered erythrocytes by the spleen. This is due in part to the absence of other RES functioning organs, such as the bone marrow, lymph nodes, and particularly the liver, which are present in the intact animal and can complicate interpretation of splenic RES function (3). Evaluation of RES function was further facilitated because the leukemic spleens were perfused with blood obtained from nonleukemic rats, which eliminated the possibility that any altered RES function in the leukemic rat could be wholly or in part attributable to serum opsonin (9, 13, 22, 23, 25, 32, 33) or hemoantibody (26, 41, 46) deficiencies in the blood of the leukemic animal. In addition, use of the perfusion technique precludes the possibility that any alteration in RES function in the leukemic animal could be solely a reflection of some change in the rate of blood flow through the organ (2), since total blood flow through the perfusion leukemic spleen was precisely measured and regulated to correspond to the blood flow through perfused spleens obtained from normal rats. Finally, the use of heat-injured erythrocytes provided a more physiological test clearance material than the colloidal type particles used in many earlier studies.

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2 The abbreviations used are: RES, reticuloendothelial system; RE, reticuloendothelial; RBC, red blood cells.

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MATERIALS AND METHODS

Male Long-Evans rats were used for all phases of this study. The Shay chloroleukemia (36) was transferred to rats as previously described (45). In essence, animals weighing 240 to 270 g used as leukemic spleen donors each received 10 x 10⁶ tumor cells via jugular inoculation. Administration of tumor cells i.v. induces an acute myelogenous leukemia. On Day 10 postinjection, the animals were operated on and the spleens prepared for perfusion. At the same time, brush preparations of femoral bone marrow were prepared and stained with May-Grunwald, and the percentage of marrow myeloblast content determined by enumerating the number of myeloblasts in 1000 nucleated cells. Normal spleens used for perfusion were obtained from normal untreated rats. The technique for perfusion of the isolated rat spleen, including the cannulation procedure and the method used for the collection and preparation of the whole blood used for perfusate, has also been described in detail (10-12). However, it should be restated that the spleens were completely isolated from the donor rats and perfused in a constant 37° temperature chamber. The blood used as perfusate was collected from donor rats no more than 1 hr before the start of the spleen perfusion and kept in a siliconized flask immersed in an ice bath until the time of introduction into the perfusion apparatus. This procedure was necessary to minimize damage to the erythrocytes in the blood perfusate, which would otherwise then become sequestered in the spleen (10). The spleens were perfused so that the rate of outflow of blood from the splenic vein was maintained at about 0.3 ml/min, which is a rate approximating the physiological blood flow through the spleen of the normal rat (11, 15). Arterial and venous samples of blood were collected at 5-min intervals during the first 30 min of perfusion, and at 15-min intervals thereafter until the conclusion of the experiment. Erythrocyte counts were made on the samples with a Coulter electronic cell counter, and hematocrit determinations were performed by the microhematocrit method. A Beckman physiological gas analyzer was used to measure the arterial and venous blood PO₂ and P CO₂ values. Spleens were perfused for a period of 1 hr and blood perfusate once passed through the spleen was not recirculated.

Erythrocytes were labeled with ⁵¹Cr by incubating 2 ml of an erythrocyte suspension that had been washed free of plasma with sterile 0.9% NaCl solution with 60 µCi sodium ⁵¹chromate (200 to 316 mCi/mg) for 20 min in a 37° water bath. The cell suspension had a packed cell volume of 50 to 60%. Immediately following incubation the suspension was washed twice with 10-ml volumes of 0.9% NaCl solution to remove the unincorporated chromium. The amount of chromium used did not exceed 0.3 µg, which was well below the amount likely to effect red cell survival (27). The uptake of ⁵¹Cr by the erythrocytes through use of this labeling procedure is reported to be 90 to 95% complete (27). The erythrocyte concentration of the cell suspension following these procedures varied from 9 x 10⁶ cells/cu mm in individual experiments. Following labeling, in selected experiments the erythrocytes contained within a stoppered tube were injured by heating in a 49.5° water bath for 20 min. The erythrocyte concentration of the suspension was not found to be altered by the heating procedure. Microscopic examination revealed the heated cells to be crenated with no normal-appearing cells evident. A 0.2-ml volume of either the labeled nonheated or labeled heated cell suspension containing from 1.8 to 2.6 x 10⁹ labeled erythrocytes was then added to each 35-ml volume of blood perfusate. Since the erythrocyte concentration in the blood perfusate was found to average 6 x 10⁹ cells/cu mm (210 x 10⁶ cells/35 ml), it meant that 0.85 to 1.2% of the total number of erythrocytes in the perfusate were labeled. At periodic intervals during the perfusion, 0.2-ml blood samples were collected from both the arterial inflow and venous outflow of blood from the spleen, added to 4 ml of 0.1% Na₂CO₃ solution, and counted for radioactivity in a well type γ scintillation counter. The radioactivity of the samples ranged from 1200 to 3700 cpm.

The percentage of ⁵¹Cr-labeled erythrocytes in the blood perfusate sequestered in the spleen was calculated by dividing the radioactivity of the spleen by the radioactivity initially present in the total volume of blood perfused through the spleen during the course of an experiment. The calculation used is shown by the formula:

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\% \text{ labeled RBC's sequestered} = \frac{\text{cpm/spleen}}{\text{Initial cpm/total volume blood perfused}} \times 100.
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The number of labeled cells sequestered was calculated by determining the percentage of the total number of labeled cells perfused through the spleen sequestered during the course of the experiment. The total number of labeled cells perfused was obtained by multiplying the number of labeled cells per unit volume of arterial blood by the total volume of blood collected through the splenic vein. The calculation used is shown by the formula:

No. of labeled RBC's sequestered = \% Labeled RBC's sequestered \times total no. of labeled RBC's perfused

At the end of each perfusion, spleen wet weight and radioactivity were determined. The spleens were fixed in 10% formalin and, with a standard paraffin histological procedure, sectioned at 6 µ and stained with hematoxylin and eosin.

RESULTS

Sequestration of labeled heat-injured erythrocytes by perfused leukemic and normal spleens was found, in all instances, to be greater than that seen for noninjured erythrocytes (Table 1), thus, demonstrating that the perfused rat spleen has the capacity of selectively removing injured cells from blood perfusates. This was indicated by
the higher levels of radioactivity in the spleens perfused with blood containing labeled injured erythrocytes, as compared to spleens perfused with blood containing labeled noninjured erythrocytes. In addition, it was also shown by consistently lower levels of radioactivity in the venous blood outflow, as compared to the arterial blood inflow in experiments where spleens were perfused with labeled injured cells. This decrease in the radioactivity of the venous blood outflow was found to correspond to the level of radioactivity in the perfused spleen. No consistent differences were noted in the level of radioactivity between the arterial inflow and venous outflow in experiments where spleens were perfused with blood containing labeled noninjured cells.

The percentage of uptake of labeled noninjured erythrocytes from blood perfused through spleens of leukemic rats ranged from 1.9 to 3.1%, which represented a total number of labeled cells sequestered of from 22 to 36 million. These values compared to a mean percentage of uptake by isolated perfused spleens of normal rats of 1.8 ± 0.2%, which represented a mean total of 17 ± 2 million labeled cells. The greater numbers of labeled normal erythrocytes found to be contained within the perfused leukemic spleens, as compared to the perfused normal spleens, was probably a reflection of the larger mass of the leukemic spleen. However, on a per mg basis spleens of highly leukemic animals (Experiment L2, L3, and L4) trapped fewer labeled erythrocytes than their normal counterparts.

The percentage of uptake of labeled heat-injured erythrocytes by the perfused leukemic spleens was variable but could be related to the stage of the leukemia as indicated by the percentage of myeloblasts in the bone marrow. Previous studies (18-20) have shown that the most reliable index of the course of the pathogenesis is the alteration noted in bone marrow cellularity. During the 2nd week of the pathogenesis, a progressive decrease in total marrow cellularity concomitant with a steady rise in numbers of leukemic myeloblasts from near 0% to in excess of 90% characterizes the disease process, and is sufficiently consistent to be used as a parameter to determine the stage of the leukemia. The percentage of uptake of injured erythrocytes from the blood perfusate ranged from 9.3 (late stage) to 37.4% (early stage). This represented a total number of injured cells sequestered of from 92 to 405 million. In contrast, the percentage of uptake and total number of injured erythrocytes sequestered in perfused spleens of normal rats was relatively consistent. The mean percentage of uptake by the normal spleen was 34.7 ± 5.2% and the mean total number of cells sequestered was 358 ± 107 million (Table 1), comparable to values noted for the early stage of the leukemic process. Of importance is the finding that the percentage of uptake of injured erythrocytes and, therefore, the number of cells sequestered in leukemic spleens, markedly decreases during the pathogenesis. Usually, as the disease progresses, splenomegaly becomes severe. The perfused enlarged spleens of the late-stage leukemic animals (Experiments L9, L10, L11, and L12) invariably demonstrated a reduced ability to sequester injured erythrocytes. In spleens weighing less than 2 g, this function was either unaltered or decreased.
As shown in Table 1, the values relating to the sequestration of labeled erythrocytes in the perfused 10-day myelogenous leukemic rat spleens, in contrast to the normal rat spleens, are presented individually for each experiment. This is done because of the variations in the size, weight, and histological condition of the leukemic spleens, which was for the most part indicative of the stage of the pathogenesis, and was found to have a relation to the sequestration of heat-injured erythrocytes. The leukemic rat spleens in this study varied in weight from 0.8975 to 3.3671 g as compared to a mean weight of 0.7350 ± 0.081 g for spleens of normal rats. The leukemic rats from which spleens were obtained for perfusion usually showed a weight loss of about 20 g, the average spleen donor rat weighing about 230 g. Spleens were found to vary in size from slightly enlarged to markedly enlarged and in consistency from normal to very soft. The capsules varied from a normal brownish-red to pink. No infarctions were observed and the spleens retained their normal contours. Histological examination revealed an altered architecture in all the leukemic spleens regardless of size or consistency (Figs. 1 to 3). In spleens weighing less than 2 g, the histological picture varied greatly between different areas within the same spleen. (Figs. 2, A and B). Thus, within one spleen an overall normal-appearing architecture was evident in some areas, while in other areas the architecture appeared abnormal as characterized by a deficiency or absence of lymphoid follicles. On the other hand, in some sections large follicles could be observed with wide mantle (marginal) zones. Numerous hematopoietic foci and myelogenous blast-type cells were present in the red pulp. In spleens weighing more than 2 g, the histological picture within the same spleen was more homogenous (Fig. 3). In these animals the splenomegaly was always accompanied by hepatomegaly. The lymphoid follicles were dispersed. Many of the follicles appeared to be compressed by the red pulp, which was expanded by the presence of numerous myelopoietic foci and cells which have been identified as being in the myeloid and erythroid series. Hematopoietic foci are observed with regularity in spleens of leukemic rats (19).

The Po2 and PCO2 values of the arterial blood perfusate at the start of each perfusion ranged from 40 to 80 and 15 to 35 mm Hg, respectively. This range of values was due in part to the degree to which the blood donor rats were anesthetized with ether at the time they were bled and in part to the variation in hematocrits, hence hemoglobin content, of the blood perfusate used for each experiment. The hematocrits of the blood among individual perfusions varied from 35 to 42%. These differences in the initial Po2 and PCO2 values did not show any relation to erythrocyte sequestration. Moreover, no discernible differences were noted in the total oxygen utilization per spleen between the perfused leukemic and normal spleens. Subsequent to the initial 10 to 15 min of perfusion, the Po2 and PCO2 values of the venous blood remained at a relatively constant level for the duration of the perfusion. In a characteristic experiment the blood Po2 values resulting from passage through the spleen were found to decrease from 60 to 20 mm Hg; the PCO2 values were found to increase from 25 to 44 mm Hg. No consistent appreciable changes were observed after the first 5 min of perfusion in the erythrocyte count between the arterial blood inflow and venous blood outflow from the spleens.

**DISCUSSION**

The mechanism by which altered erythrocytes are selectively removed from the circulating blood by the RES under normal conditions is incompletely understood. Even less understood are the changes which may occur in this function in pathological conditions such as leukemia. It is generally acknowledged that the initial stage of phagocytosis involves an adhesion reaction with macrophages (30, 35). Serum factors have been reported to have a role in red blood cell adhesion to macrophages which eventually leads to erythroclasis. The removal of aged rat erythrocytes by the RES cells in the liver and the adhesion of fresh heterologous red blood cells to mouse macrophages have been shown to be opsonin-dependent (22, 25). A deficiency in serum opsonins has also been found to impair RES clearance of colloidal gold (23), aggregated human serum albumin (9), and gelatinized RE-test lipid emulsion (33). Opsonin activity has been reported to be a major factor in regulating RE phagocytic function in the spleen and also in the liver (13, 32). In the present study the leukemic spleens were perfused with blood collected from normal nonleukemic donor rats. This precluded the possibility that the impaired ability of the leukemic spleen to clear erythrocytes from the perfusate was attributable to a deficiency in serum opsonins and/or hemoantibodies, which might have been a possibility if clearance was tested in the intact leukemic rat. Decreased levels of hemoantibodies have been reported in tumor-bearing mice immunized with sheep red cells (26, 41, 46). That oxidative and glycolytic metabolism is necessary for the isolated perfused rat spleen to remove heat-injured cells from the blood perfusate has been established in a previous study, in which the addition of metabolic inhibitors to the perfusate nearly suppressed cellular uptake completely (10). That the inhibitors almost totally suppressed the metabolism of the spleen was made evident by blood gas measurements, through use of physiological blood gas analyzer system. In the present study, with this system, differences were not noted in the total oxygen utilization and carbon dioxide production between the leukemic and normal spleens.

The results obtained from studies relating RES function and leukemia in human subjects have shown some disagreement. In a study of human acute leukemia with the use of 131I-aggregated human serum albumin, the most consistent finding was that of reduced RES phagocytic function, although normal or increased RE phagocytic function was also reported in some cases (16). In another study with labeled human serum albumin, reduced phagocytic activity was observed in 2 patients with acute leukemia, and normal or accelerated phagocytic ac-
tivity was observed in 5 patients with chronic leukemia and lymphoma (4). On the other hand, with the use of an RE-test lipid emulsion, it was observed that RES phagocytic activity was increased not only in 3 patients with leukemia (granulocytic, myelogenous, and lymphocytic) but also in patients with lymphoma and a wide variety of other human cancers (34). One possible explanation for the differences observed in phagocytic activity among these studies may be related to the different test colloids used for studying clearance (8, 28, 43). The differences observed in the human studies may also be related to the treatments administered to the individuals and to dissimilarities in the overall condition of health, age, sex, and genetic constitution of the cases studied. The most consistent finding of these studies in cases of human acute leukemia seems to be a reduction in phagocytic function which is in apparent agreement with the finding of this study performed with the acute myelogenous leukemic rat. In an animal experimentation study (29), alterations were observed, in the phagocytic capacity of the RES in mice for colloidal carbon following development of a reticulum cell leukemia induced by the Friend virus (14). In this investigation, an early depression of phagocytic activity was followed by a markedly enhanced clearance rate attributed primarily to the liver, with little or no information available as to the contribution of the spleen. In studies with mice in which neoplastic processes other than a leukemia were involved (39, 40), it was found that the most characteristic and constant phenomenon in the tumor-bearing animal was the depression of splenic phagocytosis of $^{51}$Cr-labeled sheep erythrocytes; hepatic phagocytosis was variably affected. In rats with transplanted lymphomas, splenic phagocytosis for i.p. injected colloidal gold was markedly depressed, whereas hepatic phagocytosis was significantly increased (42). In rats bearing the Walker carcinosarcoma, the phagocytosis of $^{35}$S-labeled sulfonamide derivatives was consistently decreased in the spleen. The effect of the tumor on hepatic phagocytosis was variably dependent on the route of administration of the radiochemical (1). Such studies seem to indicate that in rats and mice bearing transplanted tumors splenic phagocytosis is impaired, while hepatic phagocytosis may or may not be stimulated. A point worth mentioning, which may reflect on the differences observed in splenic and hepatic phagocytosis in leukemia, and neoplasia in general, is that in the spleen, unlike the liver, sequestration and intraorgan movements of particles are considered a stage in the phagocytic process. Thus, the mechanism for alteration of RE function in the spleen may be different from the problem encountered in Kupffer cell phagocytosis in the liver.

The clearance from the blood of senescent or injured red blood cells by the RES is recognized to involve sequentially the processes of cellular change due to either normal aging or injury, sequestration, and finally hemolysis (6, 31). It seems obvious from the results of this study that in the leukemic rat spleen the sequestration phase in this sequential chain of events is impaired. The mechanism by which this impairment occurs, although not completely definable on the basis of the results, can neverthe less be conjectured upon. Studies by others have shown that altered red blood cells and particulate matter are trapped first in the perifollicular areas and then move outward into the red pulp, where after a few hours they are eventually phagocytized (21, 38). In the leukemic rat spleen the red pulp is observed to be expanded by the presence of numerous leukemic and normal cell types. This was particularly true in the very enlarged spleens of late-staged leukemic rats, where the red pulp was greatly expanded throughout the entire organ. However, in moderately enlarged spleens the red pulp was less expansive and in some areas appeared to be almost normal. In the perfusion experiments the very enlarged spleens consistently showed a pronounced decrease in their capacity to sequester cells, whereas in the moderately enlarged spleens this function was either unaltered or decreased. This inverse relationship between sequestering capacity and degree of splenic enlargement suggests that the decrease in sequestering capacity may be due to the presence of normal and leukemic hematic elements at sites which otherwise would be available for injured cells. This in turn may serve to prevent or impede contact with macrophages in the splenic red pulp. It is similarly possible that in the leukemic spleen macrophages can function normally, but they are prevented from doing so by circulatory failure or circulatory alterations that interfere with particle or cellular movement from the perifollicular areas to the red pulp where phagocytosis occurs (21, 38). In this study infarctions and gross alterations in the intrasplenic vasculature was not observed in histological sections. However, that abnormalities in the intrasplenic vascularization may account for or contribute to the changes observed in cellular sequestration cannot be excluded. The finding that the sequestering capacity of some moderately enlarged spleens remained unaltered while that of others was profoundly decreased could further be interpreted to mean that, in addition to the types of mechanical impedance suggested, other mechanisms may also play a role.

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Fig. 1. Normal spleen perfused for 1 hr with noninjured chromium-labeled RBC is shown to illustrate normal relationship of lymphoid follicles with mantle zone (M) to surrounding red pulp (R). ×30.

Fig. 2. A, spleen of an early stage leukemic rat perfused for 1 hr with heat-injured chromium-labeled RBC. Spleen weight 0.9071 g. In this section the splenic architecture has a more normal appearance. The mantle zone surrounding the follicles appear wider and less dense than in the normal spleen. The red pulp is seen to contain numerous nucleated cell types. ×30. B, section of a different area of same spleen in A is shown to illustrate the absence of normal follicles. ×30.

Fig. 3. Spleen of a late-stage leukemic rat perfused for 1 hr with heat-injured chromium labeled RBC. Spleen weight, 3.0551 g. The lymphoid follicles are seen to be widely dispersed by the expanded red pulp. ×30.
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