Studies of the Reticuloendothelial Mass and Sequestering Function of Rat Bone Marrow

By Willis R. Keene and James H. Jandl

It is believed that bone marrow contains a relatively large mass of reticuloendothelial (RE) cells. These provide a lining for the marrow sinusoids and are potentially capable of phagocytosing injected foreign particles such as carbon and electronegative colloidal dyes, much as do the RE cells of liver and spleen. However, there is little quantitative information concerning this population of RE cells or the ability of the marrow to sequester defective red cells, a function conspicuously evident in the spleen and liver. Previous publications from this laboratory have described the sequestration by spleen and liver of Cr51-labeled rat red cells that have been coated in vitro with rabbit antirat red cell antibody. This red cell preparation was used to evaluate the capacity of bone marrow to sequester cells. Other measurements were made with Fe59-labeled, immature, isologous red cells and with colloidal suspensions of carbon particles. Comparisons were made between normal rats and animals whose marrow was made hypocellular by protein deprivation, by hypertransfusion, or by treatment with either mechlorethamine (nitrogen mustard) or 6-mercaptopurine. A preliminary report of the findings has been published.

Method

General Procedures

Caesarian-derived male rats of the Sprague-Dawley strain were used. Animals of uniform age were divided randomly into the desired number of groups; each group initially contained 4 or more animals, but occasionally accidental death occurred during experimental procedures which resulted in groups of unequal numbers.

Injections were made into tail veins in the following volumes: (a) 2 ml. of a 40 per cent suspension in normal saline of Cr51-labeled antibody-treated red cells; (b) 2 ml. of a serum suspension of Fe59-labeled immature erythrocytes, which usually contained about 100 x 10⁶ nucleated cells; (c) 1 ml. of rat serum containing less than 1 µg. of added ferric iron and 5-7 µc. of Fe59; and (d) mechlorethamine hydrochloride about 0.5 ml. of a solution containing 1 mg. per ml. The mechlorethamine was injected within 15 minutes after dissolving the dry powder in saline. The dose per animal was 1 mg/Kg.

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This work was supported by research grants HE-07652-01 and T1-AM-5391-01 from the National Institutes of Health, Bethesda, Md.

Submitted July 17, 1964; accepted for publication Dec. 20, 1964.

*Charles River Breeding Laboratories Inc., N. Wilmington, Mass.

†Merck Sharp and Dohme, West Point, Pa.
body weight unless specifically stated otherwise. Experiments were made with mechlorethamine-treated animals 48 hours later at which time the treated animals were usually 10 to 15 Gm. lighter than controls, while blood counts were 5000 per mm.³ or less, and hematocrits usually had not changed. All animals appeared healthy 48 hours after treatment, but if allowed to survive longer, all would die 4–5 days after treatment.

The sodium salt of 6-mercaptopurine* was dissolved in saline to give a concentration of 10 mg. per ml. Subcutaneous injections of 12 mg./Kg. were made daily for 4 weeks, prior to the experimental observations. Treated rats gained weight less rapidly than controls and at the time of study usually weighed 50 to 75 Gm. less. White blood counts were less than 8000 per mm.³ and hematocrits were 40 to 45 per cent.

In experiments involving Fe⁵⁹-labeled immature erythrocytes, animals were sacrificed by cervical dislocation 30 minutes after injection, an interval that allowed about 80 per cent of the Fe⁵⁹ to be cleared from circulating blood. Cr⁵¹-labeled, antibody-coated red cells were cleared more slowly and tissue uptake was therefore measured 3 hours after injection, an interval which normally allowed removal of about 70 per cent of the injected Cr⁵¹.

Erythropoiesis was suppressed in certain experiments either by hypertransfusion or protein deprivation. The polycythemia of hypertransfusion was achieved by injecting intravenously 4 ml. of isologous whole blood within 1 week. Studies were made in these animals 2 days after the last transfusion at which time the hematocrit levels ranged between 58 and 65 per cent and reticulocyte counts were less than 0.1 per cent. Rats weighing 200 to 250 grams were deprived of protein by ad libitum feeding of a protein-free diet which contained: dextrin, 84 per cent; corn oil, 9 per cent; agar, 2 per cent; cod liver oil, 1 per cent; and vitamins and salts, 4 per cent.† This diet was fed for 14 to 16 days prior to study. Control animals of similar weights and from the same shipment date were maintained on standard laboratory ration. During this period control animals gained about 100 Gm. and animals on the protein depletion diet lost about 50 Gm.

Liver, spleen, lung, left kidney and right femur were dissected cleanly, rinsed in tap water, and blotted with gauze sponges before being weighed. Radioactivity measurements were made on approximately 1 Gm. of each tissue, one femur, and 1 ml. of fluids by counting Cr⁵¹ or Fe⁵⁹ in a well-type scintillation counter for a minimum of 10 minutes or 10,000 counts. Disappearance of Cr⁵¹-labeled red cells and Fe⁵⁹-labeled immature red cells from circulating blood was studied by counting radioactivity present in 0.1 ml. of whole blood in 1.0 ml. of water at selected intervals after injection. To study plasma disappearance of transferrin-bound Fe⁵⁹ 0.1 ml. of whole blood was pipetted into 1 ml. of heparinized physiologic saline. After centrifuging to separate the cells, radioactivity was measured in 0.5 ml. of the supernatant. The differences in geometry involved in these several samples caused less than 2 per cent difference in counting efficiency.

Special Studies of Bone Marrow

Bone marrow radioactivity was measured in several experiments by counting the entire skeleton. This was made possible by soaking the carcass 48 hours in 5 per cent KOH, which stripped the bones of surrounding muscle and fibrous tissue. The bones were then washed in tap water, dried at 37 C., and counted for radioactivity in a well-type scintillation counter. From these measurements the distribution of radioactivity in the entire skeleton was determined, making it possible to derive a percentage of total skeletal radioactivity present in one femur (table 1).

Marrow cellularity was measured in samples collected from the femoral shaft by excising both ends and flushing out the marrow cavity with 2 ml. of isologous rat serum injected into one end with a snug-fitting #18 needle. Clumps of cells were broken up

*Lot NSC-755, Burroughs Wellcome & Co., Tuckahoe, N. Y.
†Nutritional Biochemicals Corporation, Cleveland, Ohio.
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Table 1.—Distribution of Skeletal Radioactivity after Injection of Transferrin-Bound Fe¹⁵ or Antibody-Treated Erythrocytes

<table>
<thead>
<tr>
<th>Material Injected</th>
<th>No. of Animals</th>
<th>Femurs</th>
<th>Tibia-Fibulas</th>
<th>Long Bones of Forelegs</th>
<th>Mandibles</th>
<th>Pelvis</th>
<th>Vertebrae</th>
<th>Skull</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transferrin-bound Fe¹⁵</td>
<td>8</td>
<td>19</td>
<td>16</td>
<td>11</td>
<td>4</td>
<td>9</td>
<td>24</td>
<td>6</td>
</tr>
<tr>
<td>Cr³⁰-Labeled</td>
<td>4</td>
<td>17</td>
<td>12</td>
<td>8</td>
<td>2</td>
<td>11</td>
<td>33</td>
<td>6</td>
</tr>
<tr>
<td>Antibody-treated erythrocytes</td>
<td>4</td>
<td>3.4</td>
<td>1.3</td>
<td>1.2</td>
<td>0.6</td>
<td>0.3</td>
<td>3.6</td>
<td>1.3</td>
</tr>
</tbody>
</table>

P value of difference by t test

- .7 < .05 < .3 < .1 < .2 < .001

Mean (Standard Deviation). Per cent of total skeletal radioactivity.

by vigorous agitation with a Vortex Jr. mixer. After a uniform suspension was obtained, smears were made for differential cell counts and a total nucleated cell count was made using the Sanborn-Frommer Cell Counter† by adding 0.02 ml. of marrow suspension to 10 ml. of white cell diluent. In some experiments, animals were injected with Fe⁵¹ so that marrow red cell precursors were labeled with Fe⁵¹ at the time of sacrifice. The portion of total femoral marrow collected from the femoral shaft could then be calculated by counting the radioactivity in the excised femoral ends, the marrow suspension, and the empty femoral shaft. Data from 8 femurs (8 animals) showed that the marrow suspension contained 33.3 ± 2.4 per cent of the Fe⁵¹ present in the entire femur. Since the studies described above and in table 1 demonstrated that 9.5 per cent of the entire body marrow was present in one femur, the total marrow cellularity was estimated by multiplying the number of cells collected from the femoral shaft by 31.8. Because of the admitted difficulty with direct cell counts, the nucleated cell count of marrow suspensions obtained by direct cell counting was compared with DNA content of the same suspension. DNA was measured by pentose analysis using salmon sperm DNA as standard. In performing DNA analyses on these serum-suspended cell preparations it was found initially that serum itself contained substances that reacted with diphenylamine reagent to form a red color which interfered colorimetrically at 600 mu. Accordingly, the marrow cell suspensions were washed once, and then resuspended in normal saline prior to their extraction for DNA, a precaution which eliminated such interference. In estimating nucleated cell count from DNA content, it was assumed that each nucleus contained 6 x 10⁻¹² Gm. DNA, although this figure may be somewhat in error with respect to the mature granulocytes present.

Antibody Treatment of Erythrocytes

Freshly collected heparinized whole blood was labeled with high specific activity Cr⁵¹ as Na₂Cr⁵¹O₄ in the amount of 5 μc. per ml. of whole blood. After 30 minutes of gentle agitation at room temperature the labeled cells were washed twice in an equal volume of saline, and restored to a 50 per cent cell suspension in saline. An equal volume of an appropriate dilution of rabbit antirat red cell antiserum (previously heated 2 hours at 56 C.) was added to the red cells and incubated with gentle agitation at 37 C. for 1 hour. The antiserum-red cell mixture was then centrifuged and washed twice to remove excess antibody. The cells were then restored to a 50 per cent suspension with saline before injection. Red cells sensitized in this manner. showed little or no agglutina-

*Scientific Industries, Inc., Queens Village, N. Y.
†Sanborn Company, Waltham, Mass.
¶Abbott Laboratories, North Chicago, Ill.
tion in saline, but strong agglutination in polyvinylpyrrolidone (P.V.P.). viscosity grade K-44.17

Preparation of Fe59-labeled Immature Erythrocytes

Rats weighing 300 to 400 Gm. were bled 4 ml. of whole blood by cardiac puncture on two successive days. Three days after the first bleeding each was injected intravenously with 30 to 40 μc. of Fe59 that had been added as Fe59Cl3* to rat serum transferrin in vitro. Four to 6 hours later, Fe59-labeled marrow was collected by washing the marrow out of each femoral shaft with 2 ml. of rat serum. Each femur yielded 120 to 150 x 10⁶ nucleated cells.

Methods Involving the Use of Colloidal Carbon Particles

Reticuloendothelial blockade was produced by injecting intravenously colloidal carbon,† 25 mg. per 100 Gm. body weight. Antibody-coated red cells were injected 1 hour after the carbon injection in order to determine the effect of RES blockade on red cell sequestration.

Clearance of carbon particles from the circulation was measured by collecting whole blood samples 2, 5, 15, 30, 45, and 60 minutes after injection. Carbon content of blood samples was determined by lysing 0.02 ml. of whole blood in 4 ml. of 0.1 per cent Na₂CO₃. The optical density of this suspension was read at a wave length of 675 against a blank prepared with 0.02 ml. whole blood collected before carbon injection. Carbon content was then computed from a standard curve prepared by serially diluting the original carbon suspension. In order to permit identification of marrow RE cells, mechlorethamine-treated and normal animals were injected with carbon, 25 mg. per 100 Gm. body weight and 75 mg. per 100 Gm. body weight, respectively. The latter quantity was divided into three separate injections given about 8 hours apart. The larger dose was necessary because only minimal carbon appeared in marrow phagocytes of normal animals until liver and spleen RE cells were saturated with carbon. Marrow cover slip smears were made 1 hour after the last injection of carbon and stained with Wright's stain. Cells containing carbon were then enumerated by counting 1000 nucleated cells.

Carbon contained in liver, spleen, and bone marrow was extracted by the alcohol-alkali digestion and extraction procedure described by Halpern, Biozzi, Mene and Benacerraf.18 The carbon extracted from about 1 Gm. of spleen and liver and from marrow collected from one femoral shaft was resuspended in 5 ml. of purified calf skin gelatin.19 10 per cent. An appropriate aliquot of the gelatin suspension was added to 4 ml. of 0.1 per cent Na₂CO₃ and read for optical density at 675. Carbon concentration was then read from a standard curve.

Calculations

Radioactivity present in each organ was determined by counting the radioactivity in a weighed sample (about 1 Gm.) of tissue and multiplying by total organ weight/sample weight.

Blood volume determinations were made in normal animals ranging in weight from 200 to 500 Gm. using Cr⁵¹-labeled isologous red cells. Based on these measurements, which confirm previous figures,11 the following percentages were used to estimate blood volume from body weight:

*Abbott Laboratories, North Chicago, Ill.
†Gunther-Wagner, Pelikan, Lot #C11/1431a.
‡Eastman Organic Chemicals, Rochester, N. Y.
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<table>
<thead>
<tr>
<th>Body Weight, Gm.</th>
<th>Per Cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>201–300</td>
<td>6.0</td>
</tr>
<tr>
<td>301–375</td>
<td>5.5</td>
</tr>
<tr>
<td>376–450</td>
<td>5.0</td>
</tr>
<tr>
<td>451-up</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Total bone marrow uptake of radioactivity was calculated by multiplying the net counts per minute from one femur by 11.8 for labeled cells and 10.6 for transferrin-bound Fe59 (table 1). Measurements were made which indicated that there was no significant difference between counts of the right and left femurs, though the right femur was used routinely.

Differences between means were tested for statistical significance by the t test. Tabular data are presented, for the most part, as mean (standard deviation) and the approximate P value of the difference is tabulated in a separate column. In the text and in a few tables mean ± standard error is used to facilitate rapid estimation of both variability and probability values.

RESULTS

A. Measurements of Size and Distribution of Bone Marrow RES

The distribution of Marrow RE cells, as reflected by the uptake of Cr51-labeled antibody-coated red cells, was compared with the distribution of transferrin-bound Fe59. Data in table 1 show that antibody-coated cells are distributed throughout the skeleton and that the distribution is generally similar to that of transferrin-bound Fe59 and accordingly to the distribution of red cell precursors. The only important exception to this parallelism between the distribution of RE cells and of red cell precursors is found in comparing Fe59 and Cr51-labeled cells present in vertebrae. Using the per cent of total marrow radioactivity present in two femurs, factors were derived for calculating total marrow radioactivity from the activity present in one femur; these factors for Cr51-labeled cells and transferrin-bound Fe59 are 11.8 and 10.6, respectively. The factor for Fe59 is only slightly less than a factor of 14.4 computed by Finch from data obtained using different methods.19

As determined by direct cell counts, there were 425 ± 21 x 10⁶ nucleated cells present in one femur from each of 6 animals. Using this figure and the factor above, 10.6, it was calculated that rats weighing 360 to 420 Gm. possess about 4.5 x 10⁸ nucleated marrow cells. However, DNA measurements made on the same marrow samples gave a mean DNA content of 5.11 mg. per femur; computation of total body bone marrow DNA, using the 10.6 factor, gave an estimate of 54.2 ± 3.0 mg. If the DNA content per nucleus is 6 x 10⁻¹²,15 the calculated total number of nuclei would be 9.03 x 10⁶. The sizeable discrepancy between direct cell counts and those calculated from DNA analyses appears largely to result from the fact that it is not possible to dissociate fully the aggregates of marrow cells into an even cell suspension. Thus, the direct cell counts reported here patently underestimate the actual counts, whereas it is believed that the DNA estimates do not overestimate by more than about 5 or 6 per cent as the result of polyploidy and the presence of cells undergoing active DNA synthesis. Accordingly, the total marrow cellularity in these
Fig. 1.—Photomicrograph of Wright's stained smear of bone marrow from mechlorethamine-treated rat which was injected with colloidal carbon particles 1 hour before sacrifice. Carbon particles can be seen in RE cells, but not in lymphocytes, granulocytes, or platelets.

Rats would approximate $8.5 \times 10^9$ cells. This represents a total nucleated cell population between 4 and 5 times that of normal rat spleen, the DNA content of which was found to be $11.5 \pm 1.1$ mg., and exceeds even that of rat liver.$^{15}$

There are major difficulties in determining the number of RE cells in any organ, but in marrow these problems are multiplied because of difficulties in measuring total organ size and in determining uniformity of cell population in various bones.$^{20}$ Even when these difficulties are mastered or circumvented one is faced still with almost insoluble semantic problems arising from observations that certain RE cells selectively phagocytose small particles under similar conditions, while other cells only phagocytose large particles.$^{21}$

Even more perplexing is the observation that one morphologic RE cell type may undergo transformation into another cell type and in so doing change its phagocytic specificity.$^{22}$ Nevertheless, for purposes of the experiments reported herein marrow RE cells were defined as those having the appearance of histiocytes or reticulum cells and which phagocytose intravenously injected carbon particles.

Marrow smears made from animals previously injected with colloidal carbon particles showed that $12.6 \pm 1.74$ per cent of the nucleated cells contained carbon. Virtually all of these cells appeared to be histiocytes or reticulum cells. No carbon was phagocytosed by granulocytes and only a very occasional monocyte contained carbon (fig. 1). It is estimated, therefore, on the basis of the estimated total cell content, that the rat bone marrow contains approximately $10^9$ RE cells.
B. Sequestration of Antibody-Treated Red Cells by Bone Marrow

Blood stream clearance and organ uptake of red cells coated with antibody was studied in normal rats and in animals treated with mechlorethamine or 6-mercaptopurine. Surprisingly, altered red cells were cleared from whole blood more rapidly in the treated than in normal animals (table 2) and the increase in rate of clearance was attributable to a striking increase in bone marrow sequestration.

It was previously shown that disappearance of antibody-treated red cells from circulating whole blood was directly proportional to the amount of antibody used in coating the red cells. The more rapid clearance of red cells coated with greater quantities of antibody was due to an increase in hepatic uptake, whereas splenic sequestration diminished. These observations have been confirmed in both normal and mechlorehathamine-treated animals (fig. 2). It was shown, further, that at all antibody levels the rate of blood stream clearance in animals previously treated with mechlorehathamine or 6-mercaptopurine exceeded the rate of clearance in normal animals. The difference in rate of clearance was due to an increase in sequestration of cells by the hypocellular marrow of the treated animals. Whereas normal bone marrow sequestered only 4 per cent of the injected cells, from 12 to 17 per cent was sequestered in the marrows of treated animals—an amount comparable to that in the spleen. As the quantity of antibody was increased, bone marrow radioactivity increased and in this respect bone marrow resembled liver rather than spleen.

Although the bone marrows from mechlorehathamine-treated animals were hyperactive as sequestering organs, they showed marked hypocellularity, with a decrease in all blood forming elements. The femoral shaft marrow from treated animals could be seen macroscopically as well as microscopically to contain a greater number of non-nucleated, mature red cells than marrow samples from normal controls. This was confirmed by injecting Cr51-labeled normal mature red cells into 4 rats; three times the normal amount of Cr51 activity was recovered in the marrows of mechlorehathamine-treated animals 3 hours after injection, indicating increased marrow vascularity. The mean total marrow nucleated cell counts calculated from direct cell counts of suspensions of femoral shaft marrow from 6 normal and 5 mechlorehathamine-treated animals were 4.5 ± 0.2 x 10⁶ and 1.3 ± 0.2 x 10⁶, respectively. In the same marrow samples the normal total content of DNA was found to be 54.2 ± 3.0 mg. and that of mechlorehathamine-treated rats was only 18.1 ± 1.9

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**Table 2.—Sequestration of Cr51-Labeled, Antibody-Treated Red Cells; Effect of Pretreatment with Mechlorethamine or 6-Mercaptopurine**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Animals Studied</th>
<th>Cr51 Activity, Per Cent of Injected, 3 Hours Postinjection*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Whole Blood</td>
</tr>
<tr>
<td>Normal controls</td>
<td>18</td>
<td>38 ± 3.2</td>
</tr>
<tr>
<td>Mechlorethamine</td>
<td>20</td>
<td>30 ± 3.3</td>
</tr>
<tr>
<td>6-Mercaptopurine</td>
<td>4</td>
<td>16 ± 2.8</td>
</tr>
</tbody>
</table>

*Mean ± Standard Error
mg. Using phagocytosis of carbon particles to define RE cells, it was shown that 12.6 ± 1.7 per cent of the cells from normal animals and 31.0 ± 1.9 per cent of nucleated marrow cells from treated animals contained carbon particles. Therefore, whereas the nucleated cell count was decreased to about 30 per cent of normal in mechlorethamine-treated animals, the absolute number of RE cells was only reduced to about 70 per cent of normal.

Histologic examination of marrow from 6-mercaptopurine-treated animals revealed changes quite similar to, albeit less marked than, those observed in bone marrow from mechlorethamine-treated animals. Although diminished cellularity was apparent, the actual number of marrow RE cells was not quantitated in the 6-mercaptopurine group.

Suppression of erythropoiesis, achieved by maintaining animals in a hypertransfused polycythemic state for as long as 10 days, did not alter blood stream clearance or spleen and bone marrow sequestration of antibody-coated red cells (table 3). It should be noted, however, that, although hypertransfusion considerably suppressed erythropoiesis, erythropoietic elements comprise only a minor fraction (about 15-20 per cent) of the marrow and total marrow cellularity is diminished but little. Actual cell counts were performed in only 2 of the hypertransfused animals; these showed approximately a 17 per cent reduction in cellularity compared to their normal controls. However, no statistical difference can be attributed to these few counts.

Protein deprivation for 2 weeks produced a highly significant (P < .001) reduction in splenic sequestration and a twofold increase (P < .01) in bone marrow uptake (table 4). The nucleated cell counts, as indicated by DNA
RE MASS AND SEQUESTERING FUNCTION OF RAT MARROW

Table 3.—Sequestration of Cr\textsuperscript{51}-Labeled, Antibody-Treated Red Cells; Effect of Hypertransfusion and Bleeding

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Animals Studied</th>
<th>Cr\textsuperscript{51} Activity, Per Cent of Injected, 3 Hours Postinjection*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>5</td>
<td>Whole Blood: 45 ± 5.0, Liver: 19 ± 2.7, Spleen: 31 ± 2.6, Bone Marrow: 5 ± 0.4</td>
</tr>
<tr>
<td>Hypertransfused</td>
<td>9</td>
<td>Whole Blood: 45 ± 2.8, Liver: 25 ± 2.0, Spleen: 28 ± 2.2, Bone Marrow: 3 ± 0.5</td>
</tr>
<tr>
<td>Bled</td>
<td>4</td>
<td>Whole Blood: 46 ± 8.4, Liver: 24 ± 4.0, Spleen: 22 ± 4.0, Bone Marrow: 2 ± 0.3</td>
</tr>
</tbody>
</table>

*Mean ± Standard Error

Table 4.—Sequestration of Cr\textsuperscript{51}-Labeled Antibody-Treated Red Cells in Protein-Deprived Versus Normal Animals

<table>
<thead>
<tr>
<th>Diet</th>
<th>No. of Animals Studied</th>
<th>Cr\textsuperscript{51} Activity, Per Cent of Injected, 3 Hours Postinjection*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>6</td>
<td>Whole Blood: 42, Liver: 20, Spleen: 32, Bone Marrow: 4</td>
</tr>
<tr>
<td>Protein-free</td>
<td>6</td>
<td>Whole Blood: 55, Liver: 17, Spleen: 18, Bone Marrow: 8</td>
</tr>
<tr>
<td>P Value of Difference By t Test</td>
<td>&lt;.1</td>
<td>&lt;.5</td>
</tr>
</tbody>
</table>

*Mean (Standard Deviation)

Table 5.—Weights and Bone Marrow DNA of Animals Fed Protein-Free Versus Standard Diet

<table>
<thead>
<tr>
<th>Diet</th>
<th>No. of Animals Studied</th>
<th>Weight in Grams</th>
<th>Bone Marrow DNA, mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(188) (.114) (.78) (.078)</td>
<td>(5.1)</td>
</tr>
<tr>
<td>Standard</td>
<td>16</td>
<td>Body: 346, Spleen: .948, Liver: 15.82, Femur: .828</td>
<td>47.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(256) (.841) (.174) (.085)</td>
<td>(9.2)</td>
</tr>
<tr>
<td>P Value of Difference By t Test</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

*Mean (Standard Deviation)

content, were reduced in protein deprived animals to 41 per cent of normal (table 5). There were, however, certain differences between the results of this study and the observations made in experiments with mechlorethamine-treated animals. First, marrow hypoplasia during protein deprivation involves a disproportionately severe effect on erythropoiesis rather than being a perfectly uniform pancytopenia. Second, there was a difference in bone size as shown by femur weights (table 4). Third, in contrast to animals treated with cytotoxic chemicals, blood stream clearance in protein deprivation was slower than normal. Thus the increased marrow uptake in the latter group may have been in part secondary to impaired sequestration in the under sized spleens rather than necessarily indicating a primary increase in the sequestering capacity of marrow reticuloendothelial cells.
**C. Studies with Fe\textsuperscript{59}-Labeled Immature Red Cells**

Comparative studies were made of the ability of the bone marrow of normal, hypertransfused, and mechlorethamine-treated, and protein-deprived animals to sequester living immature red cells.

In normal animals red cells labeled with Fe\textsuperscript{59} as described under METHODS were sequestered primarily by liver and spleen, with bone marrow taking up less than 10 per cent of the injected Fe\textsuperscript{59} in the 30-minute period of observation and about 15 per cent of that sequestered in the RES (table 6). Suppressing erythropoiesis by hypertransfusion did not suppress the uptake of labeled cells by bone marrow, an observation that also confirms that Fe\textsuperscript{59} uptake did not reflect reutilization of iron released as such from the injected cells. In mechlorethamine-treated animals, on the other hand, blood stream clearance was accelerated due to the heightened activity of the bone marrow, which sequestered 25 per cent of the nucleated cells injected and 45 per cent of the total cells sequestered in the RES.

Observations made in protein-deprived animals were similar in every respect to the observations described above for mechlorethamine-treated animals. Though the total recovery in the tissues reported in table 6 was only 60 per cent of that injected, at least 15 per cent of the Fe\textsuperscript{59}-labeled cells injected, and about 43 per cent of those sequestered, were sequestered in bone marrow. The lower total recovery in the five tissues assayed in protein-deprived animals is not explained and may depend in part upon a change in blood volume. Nevertheless, it is obvious from inspection of table 6 that there was a pronounced increase in marrow avidity for labeled, immature cells. In normal animals the marrow took up 28 per cent as much activity as did the liver and 50 per cent as much as the spleen; in protein-deprived animals the marrow uptake almost equalled that of the liver (92 per cent as much) as was 375 per cent that of the spleen. Despite the profound decrease in splenic sequestration, blood stream clearance of labeled cells was increased by virtue of this marked increase in the avidity of the marrow sequestering mechanism.

**D. Studies with Carbon Particles**

In normal animals liver and spleen were the organs of primary importance in blood stream clearance of colloidal carbon, confirming observations made previously by others.\textsuperscript{18,25} Although normal marrow took up relatively little of the injected carbon, the marrow of mechlorethamine-treated rats showed a
striking increase in carbon uptake (fig. 3), as was the case with antibody-coated red cells. Examination of marrow smears from mechlorethamine-treated animals showed that greater than 90 per cent of marrow histiocytes and reticulum cells were engorged with carbon particles while smears from normal animals showed carbon in only 50 per cent of such cells, each of which contained fewer particles.

Plasma carbon clearance was unchanged by mechlorethamine: The half-clearance time was 41.0 ± 3.2 minutes in normal and 40 ± 4.8 minutes in treated animals.26

Reticuloendothelial blockade produced by pretreatment with colloidal car-

Table 7.—Sequestration of Antibody-Treated Red Cells in Animals “Blockaded” with Carbon Particles

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Animals Studied</th>
<th>Cr11 Activity, Per Cent of Injected, 3 Hours Postinjection*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Whole Blood</td>
</tr>
<tr>
<td>Normal control</td>
<td>9</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(7.6)</td>
</tr>
<tr>
<td>Carbon blockaded</td>
<td>7</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(15.8)</td>
</tr>
<tr>
<td>P value of difference by t test</td>
<td>–</td>
<td>.001</td>
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*Mean (Standard Deviation)
Fig. 4.—Effect of colloidal carbon on red cell sequestration. Simultaneous injection of carbon particles and antibody-treated red cells resulted in unaltered carbon clearance, but red cell clearance was delayed for about 1 hour while carbon was being cleared.

bon, 25 mg. per 100 Gm. body weight, inhibited the sequestration of antibody-treated red cells by liver and spleen (table 7). Marrow sequestration, on the other hand, was enhanced twofold. The same experiment made with mechlorethamine-treated animals, whose marrow had greater than normal affinity for carbon particles to begin with, demonstrated an even greater enhancement of marrow sequestration during carbon blockade of liver and spleen. Pretreatment with 2 ml. of a 50 per cent suspension of antibody-treated red cells did not delay plasma carbon disappearance; indeed, there tended to be an acceleration. Simultaneous injection of carbon particles and Cr\(^{51}\)-labeled antibody-coated red cells resulted in unaltered carbon clearance and retarded red cell clearance (fig. 4).

**DISCUSSION**

**The Bone Marrow as an Erythroclastic Organ**

The normal rat bone marrow appears to contain approximately one billion cells capable of phagocytosis and having the morphologic characteristics of RE cells. This number is rather similar to that estimated for the littoral cells of the liver (1.2 billion) and for nonlymphocytic elements of the spleen.\(^{27}\) Nevertheless, it is evident from these studies that in normal rats the bone marrow plays a rather minor role in the acute removal of particulate materials from circulating blood. This may be due, in part at least, to the fact that blood flow to marrow is probably not more than 5 per cent of cardiac output, as estimated in man.\(^{28}\) On the other hand, splenic blood flow is estimated in man
to represent roughly a similar or lesser fraction of the cardiac output, although, as with marrow, the estimates are based on indirect measurements. Surely, at any event, the dramatically greater ability of the spleen to sequester altered red cells is not accountable on the basis of a difference in blood flow.

Marrow RE cells appear to be considerably less avid for particles than RE cells of liver and spleen. In the previously normal animal, the marrow participated importantly in particle sequestration only when RES function in the liver and spleen was impaired. This was illustrated in these experiments by demonstrating that marrow sequestration of antibody-coated red cells was enhanced when animals were pretreated with a large dose of carbon, which impaired the sequestering function of liver and spleen. Furthermore, only minimal carbon appeared in marrow phagocytes of normal animals after a single injection of 25 mg. per 100 Gm. body weight. If, however, several injections of carbon were given during 24 hours, which resulted in relative saturation of spleen and liver phagocytic capacity, marrow RE cells became heavily impregnated with carbon. Thus, with respect to altered red cells and foreign particles, the marrow appears to function as a reserve or supplementary portion of the RES that is brought into play when the liver and spleen are pre-empted. Furthermore, the studies with carbon suggest that the sequestration of sensitized red blood cells by bone marrow may involve a mechanism that is fundamentally different from that in the spleen and liver. Since phagocytosis is known to play an important role in the sequestration of carbon particles, it appears that marrow sequestration of sensitized red cells is not dependent upon such phagocytic activity. It is not known at present whether cells that phagocytose carbon are entirely identical with those involved in trapping (sequestering) altered red cells; although RE cells capable of phagocytosis are known to be intimately involved in the sequestering process, it is doubtful that phagocytosis per se is involved as an initial step of red cell destruction. As in most previous studies in vivo of experimental hemolysis, erythrophagocytosis was minimal in tissues examined early after injection of injured red cells. Presumably clearance of particulate matter involves various degrees of interaction between the flattened RE cells that are relatively weakly phagocytic and that provide much of the sinus lining and the more rounded or stellate cells that are more actively phagocytic. Thus, it is possible that, as compared with the spleen, the marrow possesses a more spacious sinusoidal bed for entrapping particles but this bed is lined by RE cells of lesser phagocytic activity.

In contrast to the normal situation, the bone marrow assumes a major role in clearing cells and other particulate materials when pancytopenia exists, as in animals treated with mechlorelamine or 6-mercaptopurine. These agents mainly deplete the marrow of its parenchymal cells, but were found to have little effect on the population of RE cells. As a result of this selective removal of cells inactive in sequestration or in phagocytosis, the marrow was found to be more vascular, i.e., to contain more circulating peripheral blood. Previous studies by others of the histologic appearance of marrow after starvation...
depict graphically an organ largely stripped down to its vascular (reticuloendothelial) components. By electron microscopy it is evident that a similar effect is produced as a result of the cell-depleting properties of drugs. It is proposed that the enhanced ability of the hypocellular marrow to clear cellular and particulate matter from the blood results from the fact that marrow RE cells are allowed to function in a less crowded, more vascular sinus bed with less mechanical interference and possibly greater blood flow.

“Recolonization” of Bone Marrow During Marrow Hypoplasia

Whereas normal marrow possessed a modest ability to sequester living, Fe\textsuperscript{59}-labeled, immature red cells, an ability uninfluenced by the level of erythropoiesis, the spleen and liver were more active in this respect. However, when the recipient marrow was rendered hypoplastic by mechlorethamine or protein deprivation, its ability to trap injected, immature red cells was considerably enhanced. Previous studies by others\textsuperscript{,10,31} have suggested an enhanced receptiveness for transfused marrow cells in irradiated animals. It appears from the present data that during marrow hypoplasia, even during the relatively physiologic stress of protein deprivation, a homeostatic mechanism exists whereby the uptake by the marrow of immature cells is favored. It is presumed that this mechanism, which might facilitate recolonization, depends upon the same enhancement of reticuloendothelial trapping function described above. To what extent the trapped erythropoietic cells prospered in their new environment was not elucidated in the present studies.

Recolonization of depleted marrow might be accomplished by trapping stem cells that are migrating from undepleted areas of the RES, including areas elsewhere in the marrow. That colonizing cells of this sort are continuously in circulation is strongly inferred by a number of experimental studies\textsuperscript{,32,33} The apparently low phagocytic activity of marrow RE cells alluded to above presumably would permit stem cells trapped in the marrow to survive and proliferate, whereas in the spleen and liver the environment for trapped cells is presumably more hostile. In support of this view is the fact that neoplastic implants proliferate notoriously well in the bone marrow, whereas metastatic “takes” in the spleen are known to be surprisingly infrequent for so important a sequestering organ—occurring in only 3 to 4 per cent of patients dying of malignancy.\textsuperscript{34} Accordingly it may be hypothesized that a marrow emptied of its parenchymal cells but retaining its vascular structure provides a better filter and receptacle for immature cells just as it does for altered cells or foreign particles. In contrast to the latter situations, however, the immature autogenous cell, if not seriously injured, may be tolerated by the comparatively benign RE cells of the marrow and allowed to proliferate.

The extent to which normal red cells in their senescence are sequestered and destroyed in the liver, spleen and bone marrow is presently obscure. Although there are reports concerning such physiologic sequestration\textsuperscript{,35-38} the methods that have been available for exploring this issue are inadequate and the calculations employed rest on precarious assumptions. Data obtained by the use of Cr\textsuperscript{51} in the long-term studies required is vitiated by the facts that...
the label is variably eluted and the fate of Cr$^{51}$ deposited in the several tissues is largely obscure. Labeled iron is unsatisfactory for such studies because of the extensive reutilization of red cell iron, particularly by the marrow, even despite repeated parenteral injections of "cold" iron.$^{37}$

The Relative Activities of Various Components of the RES

Previous studies have indicated that spleen and liver differ markedly in their avidity for injured red cells. Thus the spleen is extraordinarily effective in removing cells only slightly altered by such agents as incomplete antibodies, metalloprotein complexes,$^{39}$ gentle heat,$^{40}$ and sulfhydryl inhibitors.$^{41}$ With quantitatively greater injury to the cells, as with complete antibodies or larger doses of sulfhydryl inhibitors or in the absence of the spleen, the liver is the predominant sequestering organ.$^{5,23,41,42}$

The present studies indicate that the RE tissue of the bone marrow constitutes a third functional order of the RES having its own unique attributes. These in general are as follows: (a) marrow sequestration of most cells or particles is comparatively minor unless the spleen and liver have been saturated or blockaded; (b) marrow RE function is strikingly stimulated by depletion of the blood-forming elements; (c) bone marrow has a relatively great avidity for hemoglobin$^{12,43}$ and for immature red cells.

A feature of bone marrow sequestering function that is of additional interest is that, in its reactions to the various experimental conditions imposed, bone marrow and spleen usually responded in reciprocal fashion (fig. 5).
On the other hand, there is a tendency for bone marrow and liver to respond in parallel (fig. 6). Whereas protein deprivation, mechlorethamine administration, and carbon injection all depressed splenic RE function, they all stimulated marrow function. Substances such as hemoglobin or severely injured red cells that are taken up well by the liver are also taken up well by bone marrow, but poorly by spleen. Though the minimal positive correlation between liver and bone marrow (fig. 6) is not statistically significant, the correlation coefficient for data presented in figure 2, where liver sequestration was studied over a wider range, was +0.585. The correlation coefficient for spleen versus bone marrow uptake, calculated from the data in figure 2, was −0.517.

It would appear that the sequestering function of the bone marrow assumes major importance in situations of stress as when the blood stream is heavily saturated with foreign particles or in depleted or hypoplastic states. In the latter instance, heightened trapping function theoretically should serve to favor colonization and repopulation of bone marrow.

**SUMMARY**

Quantitative studies were made in rats of the total reticuloendothelial cell content of bone marrow and of the ability of marrow to sequester antibody-treated red cells, immature red cells and carbon particles. Although normal marrow played a relatively minor role in clearing cells and particles from the blood stream, its clearing activity increased markedly when the marrow was
depleted of its blood-forming cells. The greatly enhanced ability of hypoplastic marrow to trap immature red cells suggests that this may be a homeostatic mechanism for repopulating the depleted marrow.

Carbon blockade resulted in decreased liver and spleen uptake of sensitized red cells, but enhanced the marrow uptake. This suggests that marrow sequestration of cells, in contrast to that in the liver and spleen sequestration, is not affected by phagocytic activity.

In their functions and reactions the RE cells of the marrow usually acted in parallel with those of the liver and reciprocally with those of the spleen.

SUMMARIO IN INTERLINGUA

Esseva executate in rattos studios quantitative del total contento de cellulas reticuloendothelial in le medulla ossee e del capacitate del medulla de sequestrar erythrocytos tractate con anticorpore, immatur erythrocytos, e particulas de carbon. Medulla normal habeva un rolo relativamente minor in eliminar cellulas e particulas ab le circulation del sanguine. Tamen, le activitate eliminatori del medulla cresceva marcatemente quando illo esseva private de su cellulas hematopoietic. Le grandemente intensificate capacitate del medulla hypoplastic de trappar erythrocytos immatur suggere que isto es possibilemente un mechanismo homeostatic pro repopular le deplete medulla.

Bbocage a carbon resultava in un reducite acceptation de sensibilisate erythrocytos per le hepate e le splen sed intensificava le acceptation de tal cellulas per le medulla. Isto suggere que sequestration medullari de cellulas—per contrasto con illo in le hepate e le splen—non es afficite per activitate phagocytic.

In lor functiones e reactiones le cellulas reticuloendothelial del medulla ageva usualmente in parallela con illos del hepate e in reciprocitate con illos del splen.

REFERENCES

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Studies of the Reticuloendothelial Mass and Sequestering Function of Rat Bone Marrow

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