Studies of the Hemopoietic Microenvironment.

I. Changes in the Microvascular System and Stroma During Erythropoietic Regeneration and Suppression in the Spleens of CF1 Mice

By Robert S. McCuskey, Howard A. Meineke, and Samuel F. Townsend

Specific alterations in the microvascular and connective tissue compartments of the hemopoietic microenvironment have been examined during erythropoietic regeneration and suppression in the murine spleen and bone marrow using in vivo microscopic and histochemical methods. The results have confirmed the concept of specific hemopoietic microenvironments and have demonstrated specific alterations in the microenvironment during erythropoietic stimulation and repression. Elevated erythropoiesis in the splenic red pulp is accompanied by an elevation in blood flow through the microvascular system. Both the linear velocity of flow and the number of sinusoids with blood flow in them increased significantly. In contrast, erythropoietic repression was accompanied by a decreased linear velocity of blood flow, as well as a marked increase in the amount of blood being stored in the splenic sinusoids. This also was the picture when diffuse granulopoiesis was present in the red pulp, or when granuloid or undifferentiated colonies were present. The chemical composition of the stroma in the spleen and bone marrow also varied during states of hemopoietic activity and, in addition, there were differences in the composition of the stroma between these two organs. In both organs, foci of early proliferating cells were enveloped by a coating of sulfated acid mucopolysaccharide. This coat persisted on cells in later stages of granulopoiesis but not on cells in the later stages of erythropoiesis. The latter were enveloped with a coating of neutral mucopolysaccharide. A tentative hypothesis to explain the mechanisms involved in producing these changes is discussed.

The cellular kinetics involved in the production of erythroid cells have been studied extensively. Several studies have suggested that the microenvironment in which various cellular lines differentiate into mature forms varies and is different for erythroid and granuloid proliferation; these differences constitute specific "hemopoietic-inductive microenvironments (HIM)." Alterations in the HIM lead to changes in its contained hemopoietic cellular composition. The specific nature of the HIM, however, has not been reported.

Morphologically, the splenic hemopoietic microenvironment can be sub-

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Submitted August 13, 1971; revised October 26, 1971; accepted October 29, 1971.

Supported by NIH Grant AM-10507.

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divided into: a microvascular compartment composed of arterioles, capillaries, sinusoids, and venules; a connective tissue compartment composed of fibers, ground substance, and cells; and neural elements associated with both the blood vessels and the stroma. Since few studies have attempted to analyze directly these various compartments, a series of studies have been initiated in this laboratory to study the hemopoietic microenvironmental in the spleens of mice during erythropoietic regeneration and suppression using in vivo microscopic methods, histological and histochemical techniques, as well as routine hematological procedures. Concomitantly, the bone marrows of these mice are being studied only histologically because, at the moment, methods have not yet been developed adequately to study the murine marrow in vivo, although progress has been made in this direction for the rabbit.3,4

These studies were given further impetus, since previous data in this laboratory have suggested that erythropoietin, in addition to its action on stem cells, might influence the hemopoietic microenvironment.4-7 In addition, experimental results from other laboratories cannot be explained adequately in terms of cellular kinetics, and some authors have suggested that the alterations in erythropoiesis were the result of an altered, but unspecified, microenvironment.8-11

Hemopoietic recovery of the spleen following sublethal irradiation was chosen as the experimental condition since discrete compartments of hemopoietic activity could be obtained, since the cellular population of these compartments could be regulated experimentally, and since the reduced size of the irradiated spleen would aid in evaluating the microvascular system in vivo. In vivo microscopic evaluation of the splenic microvascular system was necessary, since fixed histologic sections provide only a static as well as limited concept of the function of this compartment of the microenvironment. This initial paper reports the differences in the vascularity and the stroma of the spleen and marrow observed following erythropoietic regeneration and suppression.

MATERIALS AND METHODS

Female, CF1 mice weighing approximately 20 g were used as experimental animals. Four groups of animals were established: (1) controls, nontransfused and nonirradiated to provide spleens containing extensive but diffusely organized erythropoiesis and some interspersed granulopoiesis; (2) polycythemic, transfused on 2 successive days and then 3 days later with washed, packed red blood cells to provide spleens with erythropoiesis suppressed but containing diffusely organized granulopoiesis; (3) irradiated, 530 R whole body x-irradiation to provide spleens that 10 days after irradiation contain grossly visible, discrete, large colonies of regenerating erythroid tissue surrounded with red pulp containing little or no hemopoiesis of any type and small grossly inconspicuous colonies of granulopoiesis; and (4) irradiated polycythemic, 530 R whole body x-irradiation followed immediately by transfusion, then transfused on the subsequent day and 3 days later (both irradiated and nonirradiated cells were used) to provide spleens that ten days after irradiation contain no erythropoiesis but retain colonies of granulopoiesis.

Mice were irradiated six at a time in a revolving plexiglass holder using a 250 KVP Westinghouse machine. The amount of irradiation was calibrated prior to each day’s exposure in a paraffin phantom by using a 100 R Victoreen ionization chamber. The animals were irradiated using the following factors: 250 kV, 15 mA, 0.5 mm Cu and 1 mm Al filtration, half-value layer 1.7 mm Cu, and STD 70 cm. The total dose of whole
body irradiation to all recipients was 530 R, as measured in the paraffin phantom. CF1 retired breeders were used as blood donors for transfusion.

The spleens were studied using in vivo microscopic methods, having a resolution of 0.3 μ under optimal conditions.12,13 To accomplish this the mouse was anesthetized, and a 1 cm left subcostal incision was made to allow the tip of the spleen to be exteriorized over a window covered with Saran Wrap in a specially designed microscope stage. The window overlays the condenser of a modified Leitz Panphot microscope. The spleen then was transilluminated with monochromatic light (390–650 μ obtained from a Leitz prism monochrometer equipped with an XBO-150 Xenon lamp) through the substage condenser. Observations and measurements of the microvasculature of the living spleen were made by direct microscopy at a magnification of 750 X, using a 75 X water immersion objective and a 10 X ocular containing a calibrated micrometer scale. To obtain motion pictures of the microscopic images, the optical image was projected onto the photocathode of an image orthicon (RCA, TK-31A) television system and kinérecorded at 30 frames/sec from a 17 inch video monitor using a modified Arriflex-16S, 16 mm motion picture camera synchronized with the television system; Kodak 16-mm Tri-X reversal film was used.

The use of monochromatic light permitted the selection of wave lengths of light that were absorbed selectively by specific tissue and cellular components. This differential absorption of light by these structures enhanced their contrast with the surrounding structures and aided in their visual recognition. When such differences of absorption were sensed by the television tube and converted into an electronic image, the contrast between tissue and cellular components could be enhanced further by adjustment of the brightness and contrast controls on the video monitor. For example, patterns of blood flow could be followed easily by selecting a wave length of light that was absorbed maximally by the hemoglobin within the red blood cells (414 μ).

Using this method the following can be observed in all spleens: differentiation of red and white pulp; differentiation of blood vessels into arterioles, sinusoids, and venules; patterns of blood flow through these vessels; the shape and deformation of individual blood cells flowing through the sinusoids; the endothelium of most blood vessels; stagnation of blood (storage) in the sinusoids; and under optimal conditions some cytoplasmic and nuclear detail in blood cells developing extravascularly.

The spleens of the irradiated animals were studied 10 days after irradiation. Those of the unirradiated polycythemic animals were examined 8 days after the initial transfusion. In each spleen ten microscopic fields were selected at random in the red pulp. All vessels with blood flow in each field were counted, measured, and classified as to type. In addition, the distances between sinusoids containing blood flow were measured. In the irradiated animals the grossly visible colonies could be oriented under the objective and identified, and the differences in the vascularity between the colonies and the surrounding red pulp also were measured. The cellular composition of these colonies subsequently was determined histologically. Since only vessels with blood flow in them were measured, the resulting data indicated the relative amount of blood flow through the organ when compared to normal animals. Measurements of the distance between sinusoids with blood flow in them provided, in addition, a relative measurement of the amount of blood storage in the sinusoids when correlated with splenic weights. The linear velocity of blood flow in the vessels was estimated using a scale of 0–4+ where: 0 = no flow, there was no movement of blood cells; 1+ = very slow flow, the blood oscillated with individual cells seen; 2+ = slow flow, there was steady forward progression of blood cells with individual cells seen; 3+ = moderate flow, the blood cells were blurred; and 4+ = rapid flow, the blood cells were not detected as individual entities.

Following in vivo study, a sample of blood was withdrawn from the heart for determination of hematocrit, hemoglobin, total red blood cell count, total white blood cell count, and percentage of reticulocytes. Spleens were removed, weighed, and fixed in Bouin’s solution or 10% neutral formalin. Bone marrow also was obtained for histologic study by fixing the femurs in Bouin’s solution or formalin.

The spleens and femurs were processed routinely to obtain serial sections stained with
Fig. 1. (A) Red pulp of spleen from unirradiated mouse stained with colloidal iron-PAS-Bismarck brown and methylated to demonstrate PAS-positive coatings (arrows indicate examples) on cells in late stages of erythroid development (small cells with round solid nuclei). × 1200. (B) Red pulp of spleen from...
hematoxylin and eosin. In addition, serial histologic sections of tissues fixed in formalin
that were representative of each experimental group were processed histochemically using
the colloidal iron-PAS-Bismarck brown method of Townsend with methylation and
saponification steps to localize and differentiate the various mucopolysaccharides in the
tissues. Sections treated with alpha-amylase or Prussian blue served as controls for the
PAS and colloidal iron reactions, respectively. To insure that the same area could be
examined and compared with each of the stains, sequential serial sections were used; each
section then was stained with a different procedure.

The data accumulated from the in vivo examinations were correlated with histologic
and hematologic results to insure that the vascular data were representative of the various
experimental groups.

RESULTS

Distinct differences in the splenic microvascular system were found between
irradiated and irradiated polycythemic mice, as well as between unirradiated
and unirradiated polycythemic mice. In addition, both the in vivo microscopic
studies and histologic studies revealed that instead of the four groups initially
established six were distinguishable (Table 1). The two additional groups com-
prised irradiated animals showing no morphologic evidence of hemopoietic
recovery. The hematologic data obtained from these animals is summarized
in Table 2.

The following characteristic differences were observed between spleens in
unirradiated and unirradiated polycythemic animals. During polycythemia
there was a decreased number of sinusoids containing blood flow accompanied
by an increase in the distance between them that was occupied by an increased
number of sinusoids storing blood, and there was an increase in the mean inter-
ternal diameters of all blood vessels (Table 1). Concomitantly, a reduction in
the linear velocity of blood flow through the splenic microvascular bed was
observed. The number of arterioles and venules with blood flow in them also
was decreased. While the linear velocity of flow in unirradiated spleens was
4+ in arterioles, 2+-3+ in sinusoids, and 3+-4+ in venules, the velocity
in unirradiated polycythemic animals was 3+-4+ in arterioles, 0-1+ in
sinusoids, and 1+-2+ in venules. The net effect of increased blood storage
and reduced blood flow in polycythemic animals also was reflected grossly by
their enlarged spleens (Table 2).

Histologically, the extensive but diffusely organized erythropoiesis (not
organized in colonies) normally seen in the red pulp of the mouse spleen was
absent, and diffuse granulopoiesis was prominent. Colloidal iron intensely
stained the interstitium of the red pulp of these polycythemic animals and also
was seen in the macrophages (Fig. 1B). Only the macrophages could be stained
with Prussian blue. In addition, a colloidal iron-positive coating enveloped all

unirradiated polycythemic mouse stained with colloidal iron-PAS-Bismarck
brown to demonstrate colloidal iron-positive materials in the interstitium (C) and
colloidal iron-positive coatings on developing granulocytes (large cells with
donut-shaped nuclei) (arrows indicate examples). X 1200. (C) Red pulp of
spleen from unirradiated polycythemic mouse stained with colloidal iron-PAS-
Bismarck brown to demonstrate colloidal iron-positive coatings on developing
granulocytes (arrows indicate examples). X 1200.
### Table 1. Measurements of Mouse Splenic Microvasculature During Erythropoietic Stimulation and Repression

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>No. of Animals</th>
<th>Internal Diameters of Vessels (mean ± SE) (μ)</th>
<th>Distance Between Sinusoids (mean ± SE) (μ)</th>
<th>No. of Vessels With Flow in 10 Microscopic Fields (346,185 sq μ)† (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Arterioles</td>
<td>Venules</td>
<td>Sinusoids</td>
</tr>
<tr>
<td>Unirradiated</td>
<td>15</td>
<td>5.32</td>
<td>14.56</td>
<td>7.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.078</td>
<td>±0.59</td>
<td>±0.073</td>
</tr>
<tr>
<td>Unirradiated polycythemic</td>
<td>7</td>
<td>5.90</td>
<td>18.60</td>
<td>7.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.18</td>
<td>±0.90</td>
<td>±0.12</td>
</tr>
<tr>
<td>Irradiated with colonies (red pulp and</td>
<td>10</td>
<td>5.24</td>
<td>14.00</td>
<td>5.18</td>
</tr>
<tr>
<td>erythroid colonies)†</td>
<td></td>
<td>±0.087</td>
<td>±0.73</td>
<td>±0.05</td>
</tr>
<tr>
<td>Colony‡</td>
<td>6</td>
<td>—</td>
<td>—</td>
<td>4.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>—</td>
<td>—</td>
<td>±0.083</td>
</tr>
<tr>
<td>Red pulp‡</td>
<td>6</td>
<td>—</td>
<td>—</td>
<td>5.62</td>
</tr>
<tr>
<td>Irradiated with no colonies</td>
<td>7</td>
<td>5.60</td>
<td>19.05</td>
<td>6.42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.30</td>
<td>±2.70</td>
<td>±0.20</td>
</tr>
<tr>
<td>Irradiated, polycythemic with granuloid</td>
<td>7</td>
<td>5.38</td>
<td>23.41</td>
<td>7.90</td>
</tr>
<tr>
<td>colonies</td>
<td></td>
<td>±0.21</td>
<td>±1.48</td>
<td>±0.13</td>
</tr>
<tr>
<td>Irradiated, polycythemic with no colonies</td>
<td>5</td>
<td>5.60</td>
<td>15.37</td>
<td>7.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.20</td>
<td>±0.82</td>
<td>±0.13</td>
</tr>
</tbody>
</table>

*Total area in the spleen in which vessels were analyzed.
†Fields were selected at random without regard for location in or out of a colony.
‡Differential measurements of vessels in red pulp and erythroid colonies were made in only six of the ten animals examined in vivo.
Table 2. Peripheral Blood Data, Body Weights, and Spleen Weights From Mice Examined In Vivo and Histochemically During Erythropoietic Stimulation and Repression

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>No. of Animals</th>
<th>Hematocrit (%)</th>
<th>Hemoglobin (g/100 cc)</th>
<th>Red Blood Cell Count (M/cu mm)</th>
<th>White Blood Cell Count/ cu mm</th>
<th>Reticulocyte Count (%)</th>
<th>Body Weight (g)</th>
<th>Spleen Weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unirradiated</td>
<td>15</td>
<td>46.70 ± 1.65</td>
<td>14.83 ± 0.60</td>
<td>8.32</td>
<td>5,908 ± 0.35</td>
<td>0.94 ± 0.28</td>
<td>21.8 ± 2.2</td>
<td>89.3 ± 11.0</td>
</tr>
<tr>
<td>Unirradiated polycythemic</td>
<td>7</td>
<td>66.61 ± 1.58</td>
<td>22.29 ± 0.29</td>
<td>13.36</td>
<td>11,046 ± 0.29</td>
<td>0.0714 ± 0.076</td>
<td>22.5 ± 2.2</td>
<td>134.0 ± 11.0</td>
</tr>
<tr>
<td>Irradiated with erythroid colonies</td>
<td>10</td>
<td>32.30 ± 3.35</td>
<td>10.32 ± 1.11</td>
<td>5.97</td>
<td>425 ± 0.55</td>
<td>0.27 ± 0.22</td>
<td>22.5 ± 2.2</td>
<td>42.0 ± 2.2</td>
</tr>
<tr>
<td>Irradiated with no colonies</td>
<td>7</td>
<td>32.28 ± 2.76</td>
<td>10.57 ± 0.92</td>
<td>6.15</td>
<td>377 ± 0.92</td>
<td>0.0 ± 0.72</td>
<td>18.0 ± 2.2</td>
<td>22.7 ± 2.2</td>
</tr>
<tr>
<td>Irradiated, polycythemic with granuloid colonies</td>
<td>7</td>
<td>61.45 ± 2.82</td>
<td>20.77 ± 0.98</td>
<td>11.28</td>
<td>1,811 ± 0.87</td>
<td>0.0 ± 0.72</td>
<td>22.1 ± 2.2</td>
<td>76.7 ± 11.0</td>
</tr>
<tr>
<td>Irradiated, polycythemic with no colonies</td>
<td>5</td>
<td>68.62 ± 2.26</td>
<td>22.26 ± 0.85</td>
<td>13.57</td>
<td>1,785 ± 0.60</td>
<td>0.0 ± 0.72</td>
<td>21.5 ± 2.2</td>
<td>41.4 ± 2.2</td>
</tr>
</tbody>
</table>

Mean ± SE
the cells in foci of early erythropoiesis in nonpolycythemic animals and also surrounded cells of all stages in foci of granulopoiesis in the polycythemic animal (Fig. 1C). These coatings were not stained by Prussian blue and disappeared with methylation and did not reappear with saponification. In contrast, foci of small late erythroid cells in the nonpolycythemic animals were enveloped by a PAS-positive coating (Fig. 1A) that was not digestible by alpha-amylase. There also was considerable PAS-positive staining (not digestible by alpha-amylase) of the stroma of the red pulp. Many mature circulating red blood cells in the sinusoids, in contrast, were enveloped with a colloidal iron-positive coating.

Bone marrow within the femurs of the nonpolycythemic and polycythemic mice also differed histologically and histochemically. In the nonpolycythemic animal, foci of granulopoiesis predominated in the marrow, and cells in these foci were enveloped by a coating that was stained by colloidal iron (Figs. 2A, 2B, and 3A). There was almost no PAS-positive stroma in the marrow except within foci of late erythroid cells, and the total amount of colloidal iron staining was minimal when compared to the red pulp of the spleen; very few macrophages were seen, and a delicate stroma that was colloidal iron positive generally was seen.

In spleens observed 10 days postirradiation two groups could be distinguished—those with and those without grossly visible colonies. In those with discrete colonies, blood flow through the microvascular bed was elevated (arterioles 4+, sinusoids 2+−3+, venules 3+−4+), the mean diameters of the sinusoids were decreased, and the number of sinusoids observed was increased when compared to unirradiated controls (Table 1). Little or no storage of blood was seen in sinusoids. There was a distinct difference in the vascularization of colonies compared with the surrounding red pulp. In colonies, vessels with narrow lumens were found rather than sinusoids of larger diameter that were seen in the adjacent red pulp. Morphologically, these appeared to be capillaries but were counted and included as sinusoids in the data. The capillaries in the colonies were more widely separated than were the sinusoids (Table 1) and originated from pulp arterioles and drained into the sinusoids in the red pulp surrounding the colony. As a result, when measurements were made without reference to this difference, the mean distance between sinusoids for the whole spleen was similar to that of the unirradiated animal. Moreover, while the number of sinusoids was increased greatly compared with the red pulp of unirradiated animals (Table 1), this increase was further accentuated when measurements of the number of sinusoids in the red pulp was separated from those in the colonies (Table 1).

Histologically, the large discrete colonies that protruded from the surface of the spleen and that were observed in vivo were erythroid; smaller foci of granulopoiesis were also found in the red pulp. The latter did not protrude from the surface, and, as a result, the vessels in these small foci of granulopoiesis would have been included in the data obtained for the red pulp. Thus, measurements of vessels in colonies presented in Table 1 represented only erythroid colonies. In the erythroid colonies a rim of colloidal iron-stained
Fig. 2. (A) Bone marrow from unirradiated mouse stained with colloidal iron-PAS-Bismarck brown to demonstrate colloidal iron-positive coatings on granuloid cells (large cells with donut-shaped nuclei) (arrows indicate examples). Small late erythroid cells (E) are not enveloped by such a coating. × 1200. (B) Bone marrow from unirradiated mouse stained with colloidal iron-PAS-Bismarck brown to demonstrate colloidal iron-positive coatings on granuloid cells (large cells with donut-shaped nuclei) (arrows indicate examples). × 1200.
material (not stained by Prussian blue) was found to surround almost every cell (Fig. 4B). Those cells that did not have a coating of colloidal iron-positive material were small, late erythroid cells, probably normoblasts. The latter were enveloped by a PAS-positive cell coating (not digestible with alpha-amylase). The total amount of extracellular PAS-positive material in these colonies was small compared to that in the surrounding red pulp. The red pulp surrounding the erythroid colonies contained considerable amounts of colloidal iron-positive material (Fig. 4A).

The bone marrow of the sublethally irradiated animals contained foci of both erythro- and granulopoiesis in approximately equal numbers. Early erythroid cells, most granuloid cells, and undifferentiated cells were enveloped by a coating stained by colloidal iron (Fig. 3B). In foci containing small, late erythroid cells, the cells lacked a colloidal iron coating (Fig. 3B) and were coated with a PAS-positive material. There was a slight increase in the number of macrophages containing material stained with colloidal iron and Prussian blue.

Those spleens that had no grossly visible colonies, had little or no blood flow, and most sinuosids were collapsed or contained no blood. As a result, there was little or no storage of blood, and the splenic weights were reduced (Table 2). Histologically, these spleens showed no evidence of recovery of either erythropoiesis or granulopoiesis and thus appeared to have been irradiated lethally. The red pulp of these spleens contained considerable amounts of material stained positively with colloidal iron.

Two groups could be distinguished in spleens observed 10 days post-irradiation in animals that were polycythemic—again, those with and those without grossly visible colonies. When colonies were present, they were small and did not protrude from the surface of the spleen as did the erythroid colonies. There was no appreciable difference in the splenic microcirculation observed between these two groups and the spleens of unirradiated polycythemic mice. Histologically, the colonies that were observed were composed of undifferentiated or granuloid cells. No evidence of erythropoiesis was found. The red pulp of these spleens stained intensely with colloidal iron; the staining appeared similar to that in the red pulp of the unirradiated, polycythemic animals. Cell coatings that stained with colloidal iron were found to envelope most cells in the undifferentiated or granuloid colonies. The bone marrow of these animals appeared similar histochemically to that of the unirradiated polycythemic mouse.

DISCUSSION

The results of this study have confirmed the concept of specific hemopoietic microenvironments proposed by Trentin\(^2\) and have demonstrated specific alterations in two of the components of the hemopoietic microenvironment that occur during erythropoietic stimulation and repression. These results may be helpful in explaining the results of several other studies that can not be explained adequately in terms of cellular kinetics and that suggest a role for the microenvironment in regulating erythropoiesis. For example, blood concentration of erythropoietin peaks within 24 hr after initiation of hypoxia.
Fig. 3. (A) Bone marrow from unirradiated polycythemic mouse stained with colloidal iron-PAS-Bismarck brown to demonstrate colloidal iron-positive coatings on granuloid cells (large cells with donut-shaped nuclei) (arrows indicate examples). Note lack of colloidal iron-positive material in the interstitium as compared to the spleen, Figs. 1B and C, × 1200. (B) Bone marrow from irradiated mouse stained with colloidal iron-PAS-Bismarck brown to demonstrate colloidal iron-positive coatings on granuloid cells (large cells with donut-shaped nuclei) (arrows indicate examples). Note lack of this staining on the cells in a foci of erythropoiesis (E). × 1200.
Fig. 4. (A) Red pulp from the spleen of an irradiated mouse stained with colloidal iron-PAS-Bismarck brown to demonstrate colloidal iron-positive material (C) in interstitium. Note lack of hemopoiesis in this area. × 1200. (B) Early erythroid colony from spleen of an irradiated mouse (same spleen as in A) stained with colloidal iron-PAS-Bismarck brown to demonstrate colloidal iron-positive coatings (arrows indicate examples) enveloping early developing erythroid cells. × 1200.
and then falls to normal levels within 48 hr, yet erythropoiesis continues for several days apparently without an additional requirement for erythropoietin.\(^9\) In addition, growth of erythroid-committed cells has been shown to occur without erythropoietin.\(^10\) Administration of erythropoietin to mice prior to lethal irradiation improves the splenic plating efficiency of injected stem cells, while preirradiation decreases the plating efficiency.\(^11\) Could it be that an additional function of erythropoietin is to condition the microenvironment so that optimal erythropoiesis can occur, or alternatively, could the microenvironment influence the effectiveness of erythropoietin?

A number of investigations suggest that the composition of the connective tissue may influence cellular proliferation. Variations in the content and type of mucopolysaccharides have been shown to influence mitosis, and hormones that also influence hemopoiesis, such as estrogens, can influence the composition of these substances and thus may influence cellular proliferation.\(^15,16\)

Studies in this laboratory and elsewhere have indicated that the microvascular system varies with the functional state of the hemopoietic organ. In the rabbit bone marrow and in the transplants of mouse splenic tissue in ectopic sites, the vascularity increased in response to erythropoietic stimuli.\(^4,5,17\) These results suggest that erythropoietin may have a vasoproliferative effect. In addition, erythropoietin caused sphincters in the fetal hepatic sinusoids to dilate and thus to release stored mature or maturing red blood cells;\(^6\) this again suggests a vasoactive function for erythropoietin. Normoblasts adherent to the sinusoid wall also were seen to be released.\(^7\) Reports from other laboratories also have suggested that erythropoietin may effect reticulocyte release.\(^18\) The work of Knopse and Crosby\(^9\) suggest that alterations in the normal sinusoidal architecture of the bone marrow influence hemopoiesis and may be responsible for aplastic anemia.

Consistent with the above, in the present study a distinct difference has been found in the microvascular component of the splenic hemopoietic microenvironment during erythropoietic stimulation and repression. Elevated erythropoiesis in the splenic red pulp is accompanied by an elevation of blood flow through the microvasculature of the red pulp. Both the linear velocity of flow and the number of sinusoids with blood flow in them increased significantly. In contrast, erythropoietic repression was accompanied by a decreased linear velocity of blood flow, as well as a marked increase in the amount of blood being stored in the splenic sinusoids, a condition that would suggest that the hemopoietic tissue is hypoxic. This was also the picture when diffuse granulopoiesis was present in the red pulp (unirradiated, polycythemic animals) or when granuloid or undifferentiated colonies were present (irradiated polycythemic animals). There was little or no blood flow in the red pulp of spleens that contained no hemopoiesis and appeared to have been lethally irradiated.

The histochemical results also demonstrated that the composition of the stroma in the spleen and bone marrow varies during states of hemopoietic activity and, in addition, that there are differences in the composition of the stroma between these two organs. In both organs foci of early proliferating
cells (erythroid and granuloid) were enveloped by a coating of material that stained with colloidal iron. This coating persisted on cells in later stages of granulopoiesis but not on cells in the later stages of erythropoiesis. The latter were enveloped with a coating of material that was PAS-positive. Since the staining with colloidal iron was removed by methylation of the tissue sections and the staining did not return after saponification, the coating of early erythroid and granuloid cells was judged to be sulfated acid mucopolysaccharide. The stroma that was colloidal iron positive also was judged to be sulfated acid mucopolysaccharide, whereas most of the colloidal iron-positive material seen in macrophages was judged to be hemosiderin or other iron-containing breakdown products of phagocytized red blood cells, since this material was stained with Prussian blue. The PAS-positive coating that surrounded late erythroid cells was thought to be neutral mucopolysaccharide, since it was not digestible by alpha-amylase. The origin of these cell coatings remains speculative. However, these may represent chalones and antichalones. Such substances are reported to influence the proliferation of hematopoietic precursors and are reported to be produced by the hematopoietic cells themselves.

These results suggest that stem cells committed to the erythroid cell line will complete this development only in a microenvironment that is highly vascularized, has high rates of blood flow, and that contains neutral mucopolysaccharides. This may constitute, at least in part, the hematopoietic-inductive microenvironment for erythropoiesis (HIM-E) that has been suggested by Trentin. In contrast to the HIM-E, the hematopoietic-inductive microenvironment for granulopoiesis (HIM-G) has lower rates of blood flow, although it may be well vascularized and contains sulfated acid mucopolysaccharides. Such a microenvironment appears to be inhibitory to erythropoiesis in agreement with the concept of specific microenvironments proposed by Trentin.

While this study has identified alterations in two of the components of the hematopoietic microenvironment that occur during erythropoietic stimulation and repression, the mechanisms involved in producing these changes remain speculative. Nevertheless, a preliminary working hypothesis has evolved in this laboratory based upon the data presented in this report, previous work in this laboratory, as well as the work of others. The validity of this hypothesis currently is being tested in this laboratory. It is proposed that hypoxia of hematopoietic tissue (resulting from anemia, irradiation, polycythemia, disease, etc.) initiates a change in the composition of the mucopolysaccharides present in the organ. In the murine spleen this results in a large increase in sulfated acid mucopolysaccharides. In the murine bone marrow this shift is not so prominent. Such an environment favors mitotic activity of undifferentiated stem cells of both the erythroid and granuloid line. In the anemic animal there is a concomitant release of erythropoietin from the hypoxic kidney that initiates vasodilation and/or vasoproliferation in the spleen and marrow, resulting in increased blood flow and improved oxygenation. Whether the vasodilation is due to a direct action of erythropoietin on the vessels or is the result of the release of a metabolite from the stimulated stems remains to be
HEMOPOIETIC MICROENVIRONMENT

determined. With the increase in \( P_{O_2} \) (and possibly a shift in \( pH \)), there is a shift in production of mucopolysaccharide toward neutral types. Such an environment is favorable for erythropoiesis and permits erythroid-committed cells to differentiate and mature. In the polycythemic animal, blood flow and tissue \( P_{O_2} \) do not return rapidly, since erythropoietin release is suppressed and thus the concentration of sulfated acid mucopolysaccharides remains elevated, an environment in which granuloid cells are capable of differentiating.

That the shift in mucopolysaccharides is dependent on blood flow and tissue oxygenation and not the release of a metabolite from stimulated erythropoietin-sensitive stem cells is suggested by the observation of similar shifts in mucopolysaccharides in the ulcerated stomach where, following the lesion, sulfated acid mucopolysaccharides are found in the stroma until the ulcerated area revascularizes when a shift to neutral mucopolysaccharide occurs (Townsend, unpublished observations). Similar shifts also are seen following revascularization of ectopic kidney transplants in the hamster cheek pouch (Oestermeyer, McCuskey, and Townsend, unpublished observations) and during the revascularization of the uterus following menstruation (Townsend, unpublished observations). In addition, the concentration of acid mucopolysaccharides increases in the aortic wall following hypoxia.

The differences in the erythroid responses between the murine spleen and bone marrow may be related to the differences in the microvasculature and stroma between the two organs. In the spleen, erythroid colonies are fed directly by arterioles, whereas in the bone marrow a "portal system" exists and the majority of the arterial blood must first flow through capillaries in the bone before entering the venous sinusoids. This would suggest that the \( P_{O_2} \) in the blood circulating through the hemopoietic tissue was lower than that which might be expected in the spleen. This might explain the lack of stroma containing neutral mucopolysaccharides in the marrow and the occurrence of a delicate stroma that is colloidal iron positive (sulfated acid mucopolysaccharides). In contrast, the red pulp of the normal spleen contains considerable stroma that stains positive for neutral mucopolysaccharide with PAS and contains little sulfated acid mucopolysaccharide. In addition, the differences in oxygenation may effect the production of chalones and anti-chalones that in turn influence the proliferation of cells in the hemopoietic tissue. If so, the presence of a colloidal iron-positive cell coating on many mature circulating red cells in the unirradiated polycythemic and in the normal animal is not too surprising.

REFERENCES

Studies of the Hemopoietic Microenvironment. I. Changes in the Microvascular System and Stroma During Erythropoietic Regeneration and Suppression in the Spleens of CF1 Mice

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