The regenerative potential of chronically iron-deficient rat marrow was studied in extramedullary marrow autotransplants and intramedullary cavity after ablation. Subcutaneous implantation of normal marrow results consistently in establishment of a marrow nodule with a mean weight ratio of 30.7% of the implanted tissue. In iron deficiency, the mean weight ratio was 7.1%, and the take was 11%. After ablation of femoral marrow, the regenerative process proceeds distally from the uninjured marrow at the femoral head. The process of regeneration reached the midshaft after 11 days in iron-deficient animals but took only 5 days for normal animals. Iron repletion accelerated the process toward normal. These results are consistent with biochemical data from iron-deficient marrow (low nucleic acid content, decreased incorporation of $^3$H-thymidine into DNA, and decreased utilization of $^{59}$Fe and $^{14}$C-glycine for heme synthesis) and suggest that the reported hypercellularity of the marrow in iron deficiency may reflect sequestration of erythroid precursors, rather than the compensatory mechanism of increased cell proliferation.

Chronic iron deficiency often results in hypercellularity of the bone marrow. Some evidence, however, suggests that this hypercellular marrow is metabolically less active than normal marrow. Therefore, the hypercellularity may not be a dynamic process compensating for the anemia, but rather a static process due to sequestration of erythroid precursors within the marrow.

Marrow regeneration after injury has not been studied in chronic iron deficiency. Information from such a study might demonstrate an accelerated regeneration if the hypercellularity is a reactive process. On the other hand, if the marrow is metabolically torpid, the regenerative process would be retarded.

Earlier reports have outlined the regeneration of normal bone marrow following autotransplantation to s.c. tissues and after physical destruction within the marrow cavity. In both sites, the marrow regenerates similarly,
leading to the formation of a s.c. marrow nodule and restoration of intra-medullary marrow tissue. The present work demonstrates the effect of chronic iron deficiency on marrow regeneration, both in intra- and extramedullary sites.

MATERIALS AND METHODS

Male Wistar rats, after weaning, were divided into four groups. Two groups were fed a synthetic iron-deficient diet (TD 6835, General Biochemicals Laboratory, Chagrin Falls, Ohio) ad lib until they were iron depleted. Two other groups were fed Purina lab chow ad lib and were used as the control. All groups received tap water ad lib. Body weight, hematocrits, and reticulocyte counts were determined at intervals. Peripheral blood smears were stained with Wright's stain; marrow smears were stained with Wright-Giemsa and with Prussian blue. After 4 mo, animals were operated on using aseptic conditions.

Group 1 (34 rats) was maintained on an iron-deficient diet. They developed a severe iron deficiency anemia with a mean hematocrit of 27 and a reticulocyte count ranging from 5 to 22% (mean 15%). Red cells on peripheral smear were markedly microcytic and hypochromic. Serum iron, determined on a sample of pooled sera was 19 μg/100 ml with a total iron binding capacity of 711 μg/100 ml. There was no stainable iron in bone marrow smears. These iron-deficient rats developed a moderate to severe diarrhea and grew more slowly than the controls.

Group 2 (41 rats) was iron deficient at the time of surgery, but was given 5 mg of iron dextran i.m. immediately postoperatively and then every 4 days thereafter for a total of 15 mg. Following surgery, the rats were fed the normal diet. These rats had 5–15% reticulocytosis, and 3–4 days after iron therapy began, the hematocrit gradually rose and stabilized at 44–57% after 1 wk.

Group 3 (40 rats) was maintained on a normal diet for the duration of the study and was used for control. These animals had a hematocrit of 44–52% at the time of surgery, and serum iron, determined on a sample of pooled sera, was 186 μg/100 ml with a total iron binding capacity of 444 μg/100 ml.

Group 4 (12 rats) was maintained on the normal diet but was subjected to repetitive bleeding in order to induce anemia. Two to four milliliters of blood were removed two to three times weekly by inserting a standard hematocrit tube in the orbital vein. When the orbital vein could no longer be used, bleeding was continued by cardiac puncture or through the jugular veins. The volume and the rate of bleeding were adjusted to maintain the hematocrit level below 27%. The amount of iron removed in the red cells was replaced by injections of iron dextran. The blood was used for hematocrit and reticulocyte count determinations. Many rats did not tolerate this treatment or did not become sufficiently anemic and subsequently were excluded from this group. Included in this

<p>| Table 1. Regeneration of Marrow Tissue in Chronic Iron Deficiency |
|---------------------------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Extramedullary Autotransplants</th>
<th>% of Take</th>
<th>Mean Weight*</th>
<th>Intramedullary Growth†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Iron-deficient)</td>
<td>11</td>
<td>7.1</td>
<td>11</td>
</tr>
<tr>
<td>Group 2 (Iron-deficient, iron-repleted)</td>
<td>60</td>
<td>14.5</td>
<td>7</td>
</tr>
<tr>
<td>Group 3 (Normal control)</td>
<td>100</td>
<td>30.7</td>
<td>5</td>
</tr>
<tr>
<td>Group 4 (Anemic, iron-repleted)</td>
<td>100</td>
<td>38.2</td>
<td>5</td>
</tr>
</tbody>
</table>

* Expressed as percentage of initial weight.
† Measured by the day when the regenerative boundary reached midshaft of the injured femur.
Fig. 1. Regeneration of marrow tissue in femoral cavity of iron-deficient rat. Organizing blood clot is seen in lower part of figure. Note distinct boundary between regenerative zone and clot (double arrows). This boundary is made of cells, some of which can be recognized as osteoblasts. Within interstices of osteoid (B), which comprises most of regenerative zone, large sinuses (S) are reconstructed, and hemopoietic proliferation begins and expands in perisinus area (single arrow); 11 days after evacuation. Hematoxylin and eosin stain. X 50.

group were those animals who developed anemia with a hematocrit consistently below 32%. These rats all had variable but persistent reticulocytosis, and stainable iron was detected in their bone marrow and liver when the animals were killed.

RESULTS

Extramedullary Marrow Implants

The sequence of regeneration of implants in normal rats has already been described. During the first 4 days, blood vessels from the surrounding tissue invade the implant. This is associated with proliferation of primitive mesenchymal cells (fibroblasts) and is followed by production of osteoid substance turning into trabecular bone. The marrow sinusoids appear, whereupon hemopoiesis begins. Proliferation of hemopoietic cells is associated with resorption of bone except for a thin shell that encases the reconstituted marrow.

A similar sequence of events was observed in our control groups (3 and 4). In iron-deficient rats (group 1) only a few vessels were seen during the first 4 days. Proliferation of primitive cells was absent in most implants. Only in an occasional implant, a cluster of fibroblasts or osteoid tissue was present.
Fig. 2. Histologic section of femoral marrow in iron-depleted rat. Note intense cellularity mostly due to increase in number of erythroid precursors. Fat cells, generally present in variable number in femoral marrow, have been replaced by hemopoietic cells. Few sinusoids are present and contain red cells. A megakaryocyte is seen in right upper corner. Hematoxylin and eosin stain, × 50.

By the end of the first week necrosis and phagocytosis supervened, and by the end of the second week the implant was replaced by fibrous tissue. In this group only two of the 18 transplants were successful after 35 days. This contrasted with 15 of 25 in group 2, 20 of 20 in group 3 (control), and eight of eight in group 4 (anemic but not iron-deficient). The percentage (by weight) of recovered tissue to implanted tissue was also significantly different in these groups with a mean of 7.1 for group 1, 14.5 for group 2, 30.7 for group 3, and 38.2 for group 4 (Table 1).

**Intramedullary Marrow Regeneration**

Following ablation, clot fills the marrow cavity. The regenerative process begins from the uninjured proximal end of the femur with the proliferation of blood vessels that penetrate the clot and move distally. Later, primitive cells gradually replace the clot, and restoration of the marrow resembles that described above in the implants. The greatest density of these cells forms a distinct boundary between the zone of proliferation and remaining clot (Fig. 1). This boundary gradually moves distally. In normal rats, the boundary reaches the midfemur by day 5. In group 1 (iron-deficient), the boundary was delayed and did not reach midfemur until day 11, whereas group 2 (iron-deficient, iron-repleted) reached midshaft by day 7 (Table 1). However, in all groups, the sequence of regeneration was similar.

In groups 1, 2, and 4 smears and sections of marrow were hypercellular, with an estimated M:E ratio of 1:1 and predominance of late normoblasts in the erythroid series (Fig. 2). Similarly, the implants in these groups were hypercellular, and the imprints from these nodules were indistinguishable from those of femoral marrow.
DISCUSSION

The results presented here indicate that in chronic iron deficiency the regeneration of bone marrow is inhibited. In extramedullary (subcutaneous) sites, the marrow nodule often fails to develop and, if present, is quite small. Intramedullarily, the regenerative process is significantly slowed, but the final volume is always the same as preablation. In this case, regeneration occurs within the rigid confines of a bony shaft; the volume is fixed. When implanted s.c., the regeneration of the nodule competes for space with the reconstruction of surgically traumatized s.c. tissue. If the latter can recover more quickly than the metabolically sluggish iron-deficient marrow, this may result in a small or absent nodule.

Anemia, per se, does not appear to inhibit the regeneration of marrow; the inhibition was not observed in anemic rats who were not iron deficient (group 4). In fact, regeneration of extramedullary marrow implants appeared to be accelerated in this group. However, the duration of anemia was significantly shorter in this group as compared to animals with iron deficiency anemia. In the latter group, the systemic effects of long-standing anemia might have played a part in inhibition of marrow regeneration.

Our results are in agreement with biochemical studies demonstrating hypoactivity of iron-deficient marrow, as indicated by subnormal nucleic acid content, decreased rate of incorporation of $^3$H-thymidine into DNA, and decreased utilization of $^{59}$Fe and $^{14}$C-glycine for heme synthesis. These findings suggest that hypercellularity of the marrow in a state of iron deficiency may reflect stagnation and sequestration of erythroid precursors within the marrow, rather than the compensatory mechanism of increased cell proliferation.

Attention has recently been focused on tissue changes in iron deficiency. Morphologic changes have been described in mitochondria. Mitochondria are larger and more numerous than usual and occupy a greater proportion of the cytoplasm. Many mitochondria are completely divided, often at a point of constriction.

Biochemical changes also occur. Iron-containing enzymes are variably reduced. Synthesis of other molecules, such as apoferritin, porphyrin, and myoglobin, is also reduced.

Iron-depleted rats have recently been found to have marked alteration in tissue protein and DNA content, to varying degrees in different tissues. These changes were greatest in those tissues not fully developed at the induction of iron deficiency. The regenerating marrow in our model is similar to developing tissues in that synthesis of protein and DNA is required for proliferation of the fibroblast (primitive mesenchymal cells). In certain microorganisms, deficiency of iron has been shown to be associated with retardation of protein and DNA synthesis. Beutler has pointed out a distinct possibility that the tissue effects of iron deficiency may be mediated through a similar mechanism, resulting in reduction in the rate of protein and DNA synthesis. Likewise, in regenerating marrow tissue, the reduction in the rate of protein and DNA synthesis may inhibit the proliferation of fibroblasts and
compromise the sequence of events necessary for marrow regeneration. The exact pathogenetic mechanism responsible for inhibition of cellular proliferation remains to be elucidated.

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Regeneration of Marrow Tissue in Chronic Iron Deficiency in Postweanling Rats

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