Control of Erythropoiesis in Rats With Adjuvant-induced Chronic Inflammation

By John N. Lukens

In order to characterize the defect in erythroid homeostasis in chronic inflammatory states, the relationship between erythropoietin production and erythropoietic response was examined in rats with adjuvant disease. Exposure of adjuvant-injected rats to graded levels of lowered barometric pressure induced increases in plasma erythropoietin which were significantly less than those measured in normal animals similarly stimulated. Erythropoietin inhibitors were not detected by in vitro or in vivo assay techniques: the biological activity of ovine erythropoietin was not modified by incubation with plasma from rats with adjuvant disease; the erythropoietic response of ex-hypoxic polycythemic mice to exogenous erythropoietin elicited nearly identical increases of radioiron incorporation in normal and adjuvant-injected rats whose endogenous erythropoietin was suppressed by hypertransfusion. It is concluded that the diminished erythropoietic response to anemia in adjuvant-induced chronic inflammation results from a relative failure in the production of biologically active erythropoietin.

Despite the heterogeneity of pathological conditions with which it is associated, the anemia of chronic disorders is remarkably uniform with respect to severity, red blood cell kinetics, and iron metabolism. As defined by Cartwright,1,2 the essential diagnostic features of this mild, refractory anemia are a decrease in plasma iron, a decrease in the total iron-binding capacity of plasma, a decrease in the saturation of transferrin with iron, a decrease in bone marrow sideroblasts, and normal or increased stainable iron within bone marrow reticulum cells. The mechanisms whereby a variety of chronic inflammatory states induce similar changes in the erythron have not been fully characterized. A decrease in red blood cell survival time, although well established, is not quantitatively impressive.3 Anemia develops because the erythroid marrow fails to increase its output sufficiently to compensate for shortened red blood cell survival. The present study was undertaken in order to characterize the disturbance of erythroid homeostasis in chronic inflammation.

Adjuvant disease in the rat was used as an experimental model for chronic inflammation. A single intradermal injection of Freund’s complete adjuvant

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induces in rats a generalized inflammatory reaction which is regarded as a delayed type of hypersensitivity to disseminated mycobacterial antigen. Between the third and seventh wk following adjuvant administration, rats exhibit a mild, stable anemia which has the morphologic, kinetic, and biochemical features of the anemia of chronic disorders in man.

**MATERIALS AND METHODS**

**Experimental Animals**

Male Holtzman rats, 300–400 g in weight, were used in all experiments. They were housed in individual metal cages and were fed Purina laboratory chow and water ad libitum. Adjuvant disease was induced with a single injection of Freund's complete antigen. All experiments were conducted 21–28 days following adjuvant administration.

**Assay of Erythropoietin (ESF)**

The biologic assay of Cotes and Bangham was used to measure plasma ESF. A minimum of five assay animals was used for each sample. Results are expressed as the percent radioiron incorporation by red blood cells per milliliter plasma injected.

**Hematologic Measurements**

The red blood cell mass was measured with 
$^{51}$Cr-labeled isologous red blood cells. In studies of red blood cell iron incorporation in rats, freshly prepared ($^{59}$Fe) ferrous citrate was added to normal rat serum. The mixture was incubated at room temperature for 30 min and then stored at 4°C for 18 hr prior to use. Each animal received 0.8 to 1.0 μg iron (including unlabeled iron) and 0.5 μCi of the isotope in 0.5 ml serum i.v. Three days following the injection, blood was aspirated under ether anesthesia from the abdominal aorta. Two milliliters of heparinized blood was hemolyzed in distilled water and counted with a standard in a well-type scintillation counter. Packed cell volumes were determined in duplicate. A blood volume of 5.75% of the body weight was assumed in calculating iron incorporation.

Standard methods were used for routine hematologic studies. The significance of group differences was determined by Student's t test.

**RESULTS**

**The Anemia of Adjuvant Disease (Table 1)**

The anemia associated with adjuvant disease is mild in degree and slightly microcytic and hypochromic. Normal reticulocyte numbers and normal red blood cell utilization of transferrin-bound radioiron reflect normal, rather than increased, erythropoietic rates. These observations parallel those made in human subjects with chronic inflammation.

<table>
<thead>
<tr>
<th>Observation</th>
<th>Controls</th>
<th>Adjuvant Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV (ml/100 ml)</td>
<td>46.7 (±0.5)*</td>
<td>44.1 (±0.9)</td>
</tr>
<tr>
<td>RBC mass (ml)</td>
<td>11.3 (±0.6)</td>
<td>7.8 (±0.4)</td>
</tr>
<tr>
<td>MCV (μl)</td>
<td>55.8 (±1.9)</td>
<td>50.0 (±1.5)</td>
</tr>
<tr>
<td>MCHC (%)</td>
<td>29.7 (±0.7)</td>
<td>27.5 (±1.3)</td>
</tr>
<tr>
<td>$^{59}$Fe Utilization (%)</td>
<td>60.4 (±5.7)</td>
<td>58.1 (±8.6)</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>2.9 (±0.3)</td>
<td>2.8 (±0.5)</td>
</tr>
</tbody>
</table>

*Values are means ± SEM for groups of 25 rats. PCV, packed cell volume; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration.
Effect of Adjuvant Disease on Hypoxia-Provoked ESF Production

At atmospheric pressure, the erythropoietic stimulating activity of plasma from control animals (1.4 \( \pm \) 0.7\% ml plasma injected, mean \( \pm \) SEM) was not significantly different from that measured in the plasma of rats with adjuvant disease (0.6 \( \pm \) 0.1\% ml). The effect of adjuvant disease on endogenous ESF formation was assessed by exposing rats to 0.5 atm for 6, 9, 12, or 15 hr. The immediate post-hypoxic ESF content of control rat plasma correlated with the duration of hypobaric exposure (Fig. 1). Although animals with adjuvant disease also demonstrated an increase in plasma ESF, the response was consistently less than that observed in normal rats. The differences in plasma ESF activities between control and adjuvant-injected rats are significant (\( p < 0.05 \) for the 9-hr groups; \( p < 0.01 \) for the 12- and 15-hr groups).

ESF-Inhibiting Activity of Plasma in Adjuvant Disease: in vitro Neutralization Test

The observed differences in plasma ESF levels could result either from differences in ESF production or from neutralization of ESF by a factor or factors in the plasma of adjuvant-injected rats. The capacity for plasma from animals with adjuvant disease to neutralize ESF was evaluated in the following experiments.

Known amounts of ovine ESF (Connaught Medical Research Laboratories, Toronto, Canada) were incubated with sodium chloride (0.9 g/100 ml), plasma pooled from five healthy rats, or plasma pooled from five rats with adjuvant disease. The mixtures were incubated with slow rotation for 1 hr at 23\(^\circ\)C and then at 3\(^\circ\)C for 72 hr. The erythropoietic activities of the mixtures were measured in posthypoxic polycythemic mice. Each assay animal received 0.1, 1.0, or 10.0 units ESF in 0.4 ml sodium chloride solution or plasma. The measured erythropoietic response (Table 2) was proportional to the dose of ESF injected for all incubation mixtures. Under the conditions of this experiment, plasma of rats with adjuvant disease did not compromise the biologic activity of ESF.

Fig. 1. Effect of hypobaric exposure on levels of plasma ESF in control rats and rats with adjuvant disease. Points and bars refer to means and SEM for groups of five rats.
Table 2. Erythropoietic Activity of 0.1, 1.0, and 10.0 Units of Ovine ESF after Incubation with Saline, Normal Rat Plasma, or Adjuvant-Disease Plasma

<table>
<thead>
<tr>
<th></th>
<th>0.1 units</th>
<th>1.0 units</th>
<th>10.0 units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0.9 (±0.3)†</td>
<td>5.0 (±0.8)</td>
<td>10.6 (±2.2)</td>
</tr>
<tr>
<td>Control Plasma</td>
<td>0.5 (±0.2)</td>
<td>5.7 (±1.6)</td>
<td>12.9 (±1.8)</td>
</tr>
<tr>
<td>Adjuvant-plasma</td>
<td>0.5 (±0.1)</td>
<td>7.4 (±0.5)</td>
<td>9.4 (±1.1)</td>
</tr>
</tbody>
</table>

*Ovine ESF obtained from Connaught Medical Research Laboratories, Toronto.
†Means ± SEM for groups of five mice.

ESF-Inhibiting Activity of Plasma in Adjuvant Disease: in vivo Neutralization Test using Exogenous ESF

On the sixth post-hypoxic day, polycythemic mice received an i.p. injection of plasma pooled from normal rats or from rats with adjuvant disease. Five hours after the plasma injection, 1.5 units of ovine ESF were given i.p. The erythropoietic response to these injections was quantitated by measuring the red blood cell incorporation of radioiron, given on the eighth post-hypoxic day. Groups of mice received 0.5, 0.75, or 1.0 ml of test plasma. The erythropoietic response to exogenous ESF was not consistently or significantly modified by plasma from rats with adjuvant disease (Table 3).

ESF-Inhibiting Activity of Plasma in Adjuvant Disease: in vivo Neutralization Test using Endogenous ESF

ESF elaboration was activated in posthypoxic polycythemic mice by re-exposure to 0.5 atm for 10 hr on the fifth posthypoxic day. In prior experiments, a 10-hr exposure was found to fall within the linear portion of the dose-response curve. Radioiron was injected on day 8 and mice were bled for determination of red blood cell utilization of tracer iron on day 10. A single i.p. injection of 0.75 ml pooled rat plasma was given 30 min before hypobaric exposure, immediately after exposure, or 24, 48, or 72 hr after removal from the high-altitude chamber. It was reasoned that if a plasma inhibitor were active during the stage of stem cell differentiation, inhibition with plasma given just before or just after the erythropoietic stimulus would be observed. On the other hand, inhibition of the action of ESF on erythroid

Table 3. Effect of a Single Injection of Plasma on the Erythropoietic Response of Ex-hypoxic Polycythemic Mice to 1.5 Units Ovine ESF

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Vol. (ml)</th>
<th>**Fe-RBC Incorporation (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.5</td>
<td>9.3 (±1.1)*</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Adjuvant</td>
<td>0.5</td>
<td>7.6 (±3.2)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.75</td>
<td>7.1 (±1.3)</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Adjuvant</td>
<td>0.75</td>
<td>7.3 (±1.9)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.0</td>
<td>9.0 (±3.0)</td>
<td></td>
</tr>
<tr>
<td>Adjuvant</td>
<td>1.0</td>
<td>7.0 (±2.0)</td>
<td>&lt;.05</td>
</tr>
</tbody>
</table>

*Mean ± SEM for groups of five mice.
Table 4. Erythropoietic Response of Polycythemic Mice Following Injection of Plasma at Various Intervals Before and After Exposure to Hypoxia

<table>
<thead>
<tr>
<th>Type</th>
<th>Plasma</th>
<th>Time given</th>
<th>$^{58}$Fe-RBC Incorporation (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Control</td>
<td>—</td>
<td>18.8 (±2.1)*</td>
<td>—</td>
</tr>
<tr>
<td>Control</td>
<td>Adjuvant</td>
<td>Prior to hypoxia</td>
<td>14.1 (±3.0)</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Adjuvant</td>
<td>Control</td>
<td>0 hr after hypoxia</td>
<td>16.5 (±2.5)</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Control</td>
<td>Adjuvant</td>
<td>24 hr after hypoxia</td>
<td>12.5 (±1.4)</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Adjuvant</td>
<td>Control</td>
<td>48 hr after hypoxia</td>
<td>12.1 (±1.8)</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Control</td>
<td>Adjuvant</td>
<td>72 hr after hypoxia</td>
<td>19.3 (±2.4)</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Adjuvant</td>
<td>Control</td>
<td></td>
<td>16.5 (±2.6)</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>12.1 (±1.5)</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Adjuvant</td>
<td>Control</td>
<td></td>
<td>17.3 (±3.6)</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Control</td>
<td>Adjuvant</td>
<td></td>
<td>13.3 (±4.9)</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Adjuvant</td>
<td></td>
<td></td>
<td>12.2 (±3.4)</td>
<td>&lt;.05</td>
</tr>
</tbody>
</table>

*Mean ± SEM for groups of five mice.

differentiation might be detected only with plasma injected 1 to 3 days after hypobaric exposure.

The erythropoietic response to endogenous ESF was not significantly modified by pooled plasma given either before or after hypobaric exposure (Table 4).

Response of Erythroid-Committed Stem Cells to ESF in Adjuvant Disease

Endogenous ESF was suppressed by the intravenous infusion of 2 ml packed isologous red blood cells per 100 g body weight on 2 consecutive days. Two days following the second transfusion the PCV of normal rats and rats with adjuvant disease were 66.9 ± 1.2% and 63.8 ± 1.7%, respectively (means ± SEM for groups of 25 rats). Peripheral blood reticulocytes in control and adjuvant-injected animals were 0.3 ± 0.1% and 0.7 ± 0.1%, respectively. On the third and fourth posttransfusion days, rats were given a measured dose of ovine ESF i.p. The erythropoietic response to injected ESF was quantitated by measuring the red blood cell incorporation of transferrin-bound radioiron given on the fifth day after transfusion.

The relationship between erythropoietic response and dose of ESF is presented in Fig. 2. A linear dose-response relationship is observed for both control rats and for rats with adjuvant disease. No significant difference in the erythropoietic response of adjuvant-injected and control animals was demonstrated. On the other hand, the mean responses of adjuvant groups were uniformly less than those of control groups. The consistency of the latter observation suggests that a real—albeit small—difference in erythropoietic response might be demonstrated with larger groups.

DISCUSSION

Current concepts of erythroid homeostasis ascribe to the kidney a mechanism for the detection of unmet tissue oxygen needs. In response to a
decrease in the effective delivery of oxygen by hemoglobin, the kidney releases ESF or a factor (renal erythropoietic factor) which acts on an α2 globulin substrate in the plasma to generate ESF. The erythropoietic hormone, in turn, effects differentiation of erythroid-committed stem cells into recognizable red cell precursors. In addition, erythrocyte maturation is accelerated by ESF. In states of chronic inflammation, the decrease in hemoglobin concentration which results from shortened erythrocyte survival is not attended by an appropriate increase in erythropoiesis. The compromised erythropoietic response may result (1) from failure of the anemia to elicit an increase in ESF biosynthesis; (2) from inhibition of the biologic activity of ESF; (3) from a relative insensitivity of erythroid-committed stem cells to ESF; or (4) from deficiency of a factor or factors necessary for optimal rates of hemoglobin synthesis. The present investigations permit assessment of the integrity of the ESF-erythroid marrow axis (considerations 1, 2, and 3).

Because of the insensitivity of currently available assay techniques for ESF, diminished plasma levels of ESF cannot be detected with confidence. Failure to demonstrate a significant decrease in the ESF activity of unchallenged animals with adjuvant disease, therefore, is of little significance. With exposure to graded decreases in barometric pressure, rats with adjuvant disease demonstrated consistently lower levels of plasma ESF than did their normal counterparts. It is reasonable to extrapolate this difference in ESF production to normal atmospheric pressure and to suggest that the mild anemia of adjuvant disease does not provoke an appropriate increase in ESF formation. This observation complements measurements of ESF in human subjects with chronic disease. In contrast to the direct relationship between severity of anemia and plasma ESF observed in patients with iron deficiency, the plasma concentration of ESF is not increased in anemic patients with rheumatoid arthritis, chronic infections, and malignant neoplasms. Although these findings suggest that ESF biosynthesis is quantitatively deficient in chronic disorders, a qualitative abnormality of the erythropoietic hormone cannot be excluded.

Inhibitors to ESF have been identified in a variety of pathological states as well as in normal man and animals. These demonstrations suggest
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that both activators and inhibitors may participate in the normal regulation of erythropoiesis. A shift of the dynamic balance in favor of ESF inhibition has been suggested on theoretic grounds as a cause for the relative erythroid hypoplasia in patients with chronic disorders. Catabolic products of tumor cells\(^{12}\) and neuraminidase release during infectious illness\(^{18}\) have been proposed as potential candidates with ESF-inhibiting properties. However, neither Ward and associates\(^{10,11}\) nor Firat and Banzon\(^{12}\) were able to demonstrate inhibition of the biological activity of sheep ESF by plasma from patients with rheumatoid arthritis, chronic infection, or malignant neoplasms. The assay techniques used by these investigators may not have detected an ESF antagonist if the mechanism of inhibition involved interference with ESF utilization. In the present study, methods were developed to detect inhibition of endogenously elaborated ESF as well as neutralization of heterologous ESF. Plasma from rats with adjuvant disease failed to compromise the erythropoietic response of assay mice to either endogenous or exogenous ESF. Because the mouse assay system may not be sufficiently sensitive to detect low levels of ESF antagonism, these studies do not conclusively exonerate ESF neutralization as a factor in the relative deficiency of ESF activity.

The effect of chronic inflammation on the functional integrity of red blood cell precursors has not previously been examined. Indirect measures of erythropoiesis in animals with turpentine abscesses suggest that the response of the erythroid marrow to ESF may be compromised. Although the anemia associated with sterile abscesses in rats has been abolished by injections of cobalt,\(^{19}\) exposure to hypoxia,\(^{20}\) and by the administration of human ESF,\(^{20}\) the maximal values for PCV achieved by rats with inflammation were consistently less than those realized by normal rats. Since the magnitude of stimulation was sufficiently great to annul differences in pre-treatment levels of ESF, it has been argued\(^ {1,20}\) that the final PCV values should be approximately equal in abscessed and normal animals. On the other hand, the increment of PCV increase prompted by a given erythropoietic stimulus was the same in inflamed and normal rats. The experiments of Erslev and Lavietes\(^ {21}\) might also be cited in support of quantitatively abnormal marrow response in chronic inflammation. These investigators observed that serum from phlebotomized rabbits induced less of a reticulocytosis in rabbits with sterile abscesses than in healthy animals. The impurity of the erythropoietic stimulus, the insensitivity of relative reticulocyte numbers as a measure of erythropoiesis, and probable differences in pre-transfusion ESF production rates obviate unqualified conclusions. In the studies presented here, a normal quantitative relationship between ESF stimulation and erythropoietic response was demonstrated in adjuvant-injected rats in whom endogenous ESF formation was suppressed.

These studies do not clarify the pathogenesis of aberrant iron metabolism in chronic inflammatory states. Adjuvant disease in rats, like chronic inflammatory disorders in man, is associated with a decrease in plasma iron, an expansion of the splenic iron pool, a decrease in bone marrow sideroblasts, and an increase of stainable iron within marrow reticulum cells.\(^ {9}\) Since the flow of iron from the reticuloendothelial system is finally adjusted to the iron
needs of the erythroid marrow, it is reasonable to suggest that mobilization of iron from the reticuloendothelial system and erythropoietic rates are controlled by related mechanisms. The observation that ESF production and iron distribution are both altered in chronic disorders is compatible with this thesis.

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REFERENCES

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