**Serum Erythropoietin (ESF) Titers in Anemia**

By G. de Klerk, P. C. J. Rosengarten, R. J. W. M. Vet, and R. Goudsmit

Erythropoietin (ESF) titers were determined in sera from patients with different types of anemia using the fetal mouse liver cell bioassay. An inverse relationship was found between hemoglobin concentration and ESF titer. However, ESF titers differed markedly between patients at comparable degrees of anemia. Several groups of patients were distinguished on the basis of the activity of their erythroid bone marrow. In each of these groups, a significant negative correlation was found between the hemoglobin concentration and the logarithm of the ESF titer. ESF titers in patients with pure red cell aplasia were fourfold higher than those in patients with iron-deficiency anemia and tenfold higher than those in patients with megaloblastic anemia and homozygous sickle cell anemia at comparable hemoglobin concentrations. Following the initiation of specific therapy in patients with pernicious anemia and patients with iron-deficiency anemia, serum ESF titers were found to decrease prior to any substantial rise in hemoglobin concentrations. In the patients with pernicious anemia, the lowest ESF levels were found 1 day after administration of vitamin B12, whereas in the patients with iron-deficiency anemia, the lowest ESF levels were reached in the second week of oral iron therapy. On the basis of these data it was concluded that serum ESF titers in anemic patients are not only inversely related to the hemoglobin concentration but also to the activity of the erythroid bone marrow.

**ERYTHROPOIETIN (ESF)** is a renal hormone that stimulates red cell production. It is generated in response to tissue hypoxia either due to increased tissue oxygen requirement or to decreased tissue oxygen delivery. One of the major determinants of tissue oxygen delivery is the hemoglobin concentration in the blood. Consequently, in anemias caused by a primary dysfunction of red cell production or destruction, serum ESF titers are increased above normal, generally in proportion to the severity of the anemia.

Most investigators have found great differences in serum ESF titers in anemic patients at comparable hemoglobin concentrations. This has been attributed to lack of precision of ESF measurements using the current in vivo bioassay for ESF in the polycythemic mouse. Another source of variation may be the presence in crude sera of different amounts of inhibitors or nonspecific stimulators of erythropoiesis. However, the finding of different ESF levels at comparable degrees of anemia may also be considered as a real phenomenon related to the heterogeneity of the anemias included in these studies. If this is true, it would be important to find out which types of anemia are associated with high ESF levels and which types with low ESF levels. This requires an assay for ESF with an interassay variability less than the variability in ESF levels found in these patients at comparable hemoglobin levels. Furthermore, ESF measurements should not be affected by nonspecific serum factors.

In this study we have investigated the relationship between hemoglobin concentration and serum ESF titer in anemic patients using the sensitive and accurate fetal mouse liver cell bioassay for ESF. This method was used in a modified form as described previously. With this modified method, the problem of nonspecific serum substances influencing the results of serum ESF measurement is evaded by assaying each test serum against a standard ESF preparation dissolved in the same test serum. So, standard and test serum are identical apart from the concentration of ESF, which allows quantitative and specific measurement of the serum ESF titer. Considering the hypothesis of Stohlman and Brecher that serum ESF levels may be influenced by the proliferative activity of the erythroid bone marrow, we have looked for differences in serum ESF titers between patients with anemias associated with erythroid hyperplasia and those with anemias associated with erythroid hypoplasia. Furthermore, we have investigated the influence of an increase in activity of the erythroid bone marrow on serum ESF titers in patients with pernicious anemia and in patients with iron-deficiency anemia following the initiation of specific therapy. These studies demonstrate that serum ESF levels in anemic patients are related to the degree of anemia as well as to the activity of the erythroid bone marrow.

**MATERIALS AND METHODS**

**Fetal Mouse Liver Cell Bioassay for ESF**

Fetal liver cells obtained from mouse embryos after 13–14 days of gestation were suspended in Eagles minimal essential medium (Wellcome), buffered with 0.22% w/v sodium bicarbonate and containing 15% v/v fetal calf serum (Flow, Detroit, Mich.). The cell suspension was added to a range of dilutions of test serum, ESF...
standard preparation dissolved in the test serum, and a solution of transferrin-bound iron in which the concentrations of transferrin and iron were similar to those in the test serum. The final cell concentration was $5 \times 10^6$ cells/ml culture. The cultures were preincubated for 21 hr at 37°C in tubes gassed with 5% CO$_2$ in air. Subsequently, 1 $\mu$Ci $^{57}$Fe-ferric citrate (Radiochemical Centre, Amersham) was added to each of the cultures. Following incubation for 4 hr at 37°C, heme was extracted using acid ethyl-methyl ketone, and $^{57}$Fe incorporation into heme was determined by measuring the radioactivity in aliquots of the solvent layer in an automatic gamma counter.

The dose–response curve of the solution of transferrin-bound iron was used as a correction curve for the influence of serum iron on the dose–response curves of the test serum and the ESF standard preparation dissolved in an aliquot of the test serum. The corrected dose–response curves of test serum and standard were compared using the analysis of variance technique applicable to parallel line assays. Details of this method and of the statistical analysis of the results have been described previously.$^{9,10}$

All human test sera were heated at 56°C for 30 min before use. Sheep plasma ESF (Connaught, Step III) was used as a standard by prior calibration against the international reference preparation for erythropoietin (2nd IRPE).

Relationship Between Hemoglobin Concentration and Serum ESF Titer

We studied 62 patients with different types of anemia. Patients with renal diseases and nonhematologic malignancies were excluded. None of the patients received red cell transfusions for at least 10 days before an ESF assay.

The patients were divided into 4 groups on the basis of the activity of the erythroid bone marrow known to be associated with their specific type of anemia. Group A consisted of 16 patients with anemias associated with erythroid hyperplasia, including megaloblastic anemias either due to vitamin B12 deficiency (7) or to folic acid deficiency (4) and homozygous sickle cell anemia (5). Group B consisted of 11 patients with iron-deficiency anemia. In this condition, erythroid activity is known to be only moderately increased above normal.$^{11,12}$ Group C consisted of 14 patients with pure red cell aplasia. Group D consisted of 21 patients with different types of anemia associated with varying degrees of activity of the erythroid bone marrow, including various leukemias (5), monoclonal gammapathies (2), myelofibrosis (2), dysmyelopoietic syndromes (7), aplastic anemias, (2) and unclassifiable anemias (3). In all patients with pernicious anemia the Schilling tests were abnormal and the serum vitamin B12 levels below normal. In the patients with megaloblastic anemia due to folic acid deficiency, folic acid levels were below normal and vitamin B12 levels in the normal range.

Serum vitamin B12 levels and folic acid levels were determined by radioimmunoassay (Diagnostic Products Corporation, Los Angeles, Calif.). Bone marrow aspirate studies in these patients showed a megaloblastic erythropoiesis with marked erythroid hyperplasia. In the patients with iron-deficiency anemia, serum iron concentrations were low ($17.8 \pm 11.0 \mu g/100 \text{ml}$, mean $\pm$ SD) and the levels of saturation of transferrin were below 10% (3.3% $\pm$ 2.2%, mean $\pm$ SD). The patients with megaloblastic anemia and iron-deficiency anemia had not received specific therapy at the time of the ESF assay. All patients with homozygous sickle cell anemia had a considerably increased erythropoiesis as appears from their elevated uncorrected reticulocyte counts (9.1% $\pm$ 3.6%). They were maintained on a daily oral supplement of folic acid. Their renal function was normal. In the patients with pure red cell aplasia, bone marrow aspirate studies showed markedly diminished to absent erythroid precursor cells but normal myelo- and megakaryocytopenia. Reticulocytes in the peripheral blood were nil. Among the patients with pure red cell aplasia, three were undergoing immunosuppressive therapy with glucocorticoids at the time of the ESF assay.

In each of these groups the regression of the logarithm of the ESF titer on the hemoglobin concentration was determined using linear regression analysis. The slopes of the regression lines and the mean values of log ESF at the overall mean hemoglobin concentration were compared by modified $t$ tests.

Serum ESF Titers in Patients With Pernicious Anemia and Iron-Deficiency Anemia Following the Start of Specific Therapy

Four patients with pernicious anemia were studied. Therapy was started on day 0 by giving 1 mg of cyanocobalamin i.m. After 24 hr, a second dose of 1 mg was given. In four patients with iron-deficiency anemia, oral iron therapy was started on day 0. It consisted of ferrous fumarate, 1200 mg daily.

In these patients, hemoglobin concentrations, reticulocyte counts (uncorrected values), and serum ESF titers were determined immediately before and at different times following the start of therapy. ESF levels below 10 mU/ml (the lower limit of sensitivity) were arbitrarily assigned a value of 5 mU/ml.

RESULTS

Figure 1 presents serum ESF titers in relation to the hemoglobin concentration for 62 patients with different types of anemia. Statistical data are presented in Table 1. ESF titers tended to increase at decreasing hemoglobin concentrations. However, there were great differences in ESF titers at comparable hemoglobin concentrations. In each of the groups A–D, comprising patients with anemias associated with comparable activity of the erythroid bone marrow, a significant negative correlation was found between the hemoglobin concentration and the logarithm of the serum ESF titer. The slopes of the regression lines did not differ significantly ($p = 0.74$). There was no significant difference between ESF titers in patients with megaloblastic anemia and those with sickle cell anemia at comparable hemoglobin concentrations ($p = 0.29$). ESF titers in group C, consisting of patients with pure red cell aplasia, were fourfold higher than those in group B, consisting of patients with iron-deficiency anemia, and tenfold higher than those in group A, consisting of patients with megaloblastic anemia and sickle cell anemia at comparable hemoglobin concentrations. ESF titers in group D, consisting of patients with a variety of anemias, were in between those of group A and group C.

Figure 2 and Table 2 present ESF titers, hemoglobin concentrations, and reticulocyte counts in 4 patients with pernicious anemia before and after the start of therapy with vitamin B12. In all patients a remarkable fall of ESF levels was found 1 day after...
the start of therapy prior to any significant rise in hemoglobin concentration. The mean ESF level on day 1 was only 35% of the level on day 0. Between the second and fourth day after the start of therapy ESF levels increased.

Figure 3 and Table 3 present the results of starting oral iron therapy in four patients with iron-deficiency

Table 1. Composite Data on the Relationship Between the Hemoglobin Concentration (g/dl) and the Logarithm of the Serum ESF Titer (mU/ml)

<table>
<thead>
<tr>
<th>Group</th>
<th>Variance of log ESF at Given Hb</th>
<th>Slope</th>
<th>ESF Titer at Hb = 7.3 g/dl</th>
<th>Correlation Coefficient (r)</th>
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<td>A</td>
<td>0.0843</td>
<td>-0.1563 ± 0.0611*</td>
<td>87 (62-123)†</td>
<td>-0.68 (p &lt; 0.001)</td>
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<td>B</td>
<td>0.0843</td>
<td>-0.2247 ± 0.0630</td>
<td>209 (137-319)</td>
<td>-0.78 (p &lt; 0.001)</td>
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<td>C</td>
<td>0.0752</td>
<td>-0.2434 ± 0.0539</td>
<td>832 (581-1191)</td>
<td>-0.79 (p &lt; 0.001)</td>
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<tr>
<td>D</td>
<td>0.1055</td>
<td>-0.2357 ± 0.0665</td>
<td>355 (264-476)</td>
<td>-0.64 (p &lt; 0.01)</td>
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<tr>
<td>A-D</td>
<td>0.44 (p &lt; 0.001)</td>
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*Mean ± SD.
†Mean ESF titer with 95% confidence limits in parentheses.
Variances did not differ significantly (p = 0.77, Bartlett test); slopes did not differ significantly (p = 0.74, F test).
anemia. In one patient the ESF level already declined during the first days following initiation of therapy. In the other patients it took more than 6 days before the lowest ESF levels were reached. The mean ESF level between the sixth and tenth day was 37% and that between the 11th and 15th day only 32% of the level on day 0. Hemoglobin concentrations appeared to have risen only slightly in this period.

**DISCUSSION**

Increased ESF production is an appropriate and physiologic response to decreased tissue oxygen supply. Therefore, in anemic patients the production of ESF may be assumed to be closely related to the degree of anemia.

In accordance with this concept, we have demonstrated in this study that serum ESF titers tend to be higher at increasing degrees of anemia. However, the relationship between hemoglobin concentration and serum ESF titer was not found to be close at all. Serum titers differed markedly between patients at comparable hemoglobin concentrations.

With respect to this finding, it must be recognized that serum ESF levels do not represent ESF production directly, but are the result of ESF production and its disappearance from the blood. The hypothesis of Stohlman and Brecher that ESF may disappear from the blood by “utilization” by the bone marrow and that this process is proportional to the proliferative activity of the erythroid tissue,9 may provide an explanation for differences in ESF levels found in patients with different types of anemia at comparable hemoglobin concentrations.

In this study we have distinguished several groups of
Table 3. Composite Data on the Effect of Initiation of Iron Therapy in Patients With Iron-Deficiency Anemia

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* Serum ESF titer (mU/ml) with 95% confidence limits.
† Hemoglobin concentration (g/dl).
‡ Reticulocyte count (%). uncorrected value.

Although these data seem to fit in with the hypothesis of Stohlman and Brecher rather well, “utilization” of ESF by the bone marrow is not yet a universally accepted concept.

Support for this hypothesis came from the studies of Hammond et al.2,13 They found higher ESF levels and a slower rate of disappearance of ESF after blood transfusion in patients with congenital hypoplastic anemia than in patients with hemolytic anemia. On the contrary, Alexanian did not find a significant difference between urinary ESF excretion in patients with aplastic anemia and patients with hemolytic anemia.4 However, four of his eight patients with sickle cell anemia had rather low ESF levels for the measured hematocrit value.4 Naets and Wittek found similar half-disappearance times for endogenous ESF in dogs with normal and increased bone marrow activity.14 The half-disappearance time of exogenous ESF in rats made aplastic with cyclophosphamide was found to be identical to that in control rats, but it was prolonged in rats made aplastic by irradiation.15 In order to explain this discrepancy, the authors suggested an inhibitory effect of irradiation on ESF metabolism that was not related to the effect of irradiation on the bone marrow. Bozziini’s experiments with dogs,16 support the work of Naets and Wittek.14 Although these contradictory results are not fully understood, they may be related to the use of different bioassays for ESF, the measurement of serum ESF levels versus urinary ESF excretion, the use of exogenous versus endogenous ESF in the studies on the half-disappearance time, and the use of different experimental animals. The recent study of Weiss and Goldwasser on binding of fluorescent ESF to rat bone marrow cells in vitro supports the concept of “utilization” of ESF in vivo by the bone marrow. Their results suggest that ESF binding is specific and reflects the erythropoietic state of the marrow.17 If ESF is indeed “utilized” by the erythroid bone marrow while exerting its stimulatory influence on precursor cells, an increase of erythropoiesis in anemic patients should be accompanied by a fall in the serum ESF level. Hammond et al. have reported a sharp decline in serum ESF levels during recovery from aplastic crisis in patients with hereditary hemolytic anemias.3 In the present study we have reported the results of serial ESF measurements in patients with pernicious anemia and in patients with iron-deficiency anemia following the start of specific therapy.

A characteristic feature of pernicious anemia is a hypercellular bone marrow with a marked increase of erythropoietic precursor cells associated with an increased erythropoietic turnover. However, a signifi-
ESF TITERS IN ANEMIA

A significant proportion of the erythroid activity is ineffective, and many erythroid cells are destroyed before they can enter the circulation. Kinetic studies in patients with pernicious anemia have revealed the presence of at least two populations of erythroblasts resembling each other morphologically but differing in their capacity for proliferation. One population appears to progress normally from one stage of differentiation and maturation to the next, the other is arrested as regards cell proliferation but continues to mature. It is assumed that the inefficiency of megaloblastic erythropoiesis lies in the malfunction and high death rate in this nondividing cell population. We found a marked decline of the serum ESF titer in all four patients with pernicious anemia 1 day after the start of vitamin B12 therapy. Since the hemoglobin concentration had not changed significantly at that time, the fall of the serum ESF titer cannot be attributed to increased tissue oxygen supply. In fact, the rapid decline of ESF levels coincides with the conversion of the bone marrow from ineffective to effective erythropoiesis with restoration of normal proliferation and maturation of erythropoietic cells. This conversion is known to occur within 24 hr of vitamin B12 therapy. Our data are in accordance with the findings of Haavardsholm, Finne et al., who demonstrated a decrease in urinary ESF excretion in patients with pernicious anemia following treatment with vitamin B12. In their study, the lowest levels of ESF excretion were reached on the third day after the start of therapy. The predominant abnormality in iron-deficient erythropoiesis is restricted erythroid proliferation. Administration of iron to patients with iron-deficient erythropoiesis gradually restores the impaired proliferation rate of the erythroid marrow to levels that are normal for the degree of anemia. This is manifested by a reticulocytosis with a peak value occurring between the 7th and 12th day of therapy.

In all four patients with iron-deficiency anemia we found a decline of the serum ESF titer following the start of daily iron administration. The lowest ESF levels were reached in the second week of therapy, when hemoglobin concentrations were still only slightly above pretreatment levels. Although in accordance with the findings of Ortega et al., a decline of ESF levels may be expected to occur in these patients as a result of the rise of the hemoglobin concentration, the observed fall of the serum ESF titer does not seem to be proportional to the only small increase of the hemoglobin concentration and may therefore not completely be explained by increased tissue oxygenation.

In the evaluation of the above data it must be recognized that vitamin B12 deficiency and iron deficiency also lead to disturbances in cellular metabolism and function in other tissues than the bone marrow. Therefore, it might be argued that the decline of ESF levels found in these patients after the start of therapy is caused by normalization of an impaired metabolism of ESF in other tissues than the bone marrow. This explanation does not seem to be very likely. If normalization of ESF metabolism had been responsible for the observed fall of ESF levels, rather high ESF levels for the degree of anemia should have been found in the presence of an impaired ESF metabolism before treatment. However, ESF levels in these patients before the start of therapy were lower than those in patients with other types of anemia at comparable hemoglobin concentrations. Therefore, it seems reasonable to assume that the fall of ESF levels observed in these patients following the start of specific therapy is directly related to the concomitant increase in activity of the erythroid bone marrow. In conclusion, data reported in this study demonstrate that serum ESF titers in anemic patients are not only inversely related to the hemoglobin concentration, but also to the proliferative activity of the erythroid bone marrow. Although these results are consistent with the concept of "utilization" of ESF by the bone marrow, the exact mechanism of the interaction between ESF and erythropoietic tissue cannot be inferred from this study.

ACKNOWLEDGMENT

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REFERENCES


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