Erythropoiesis-inhibiting Factor(s) (EIF): Methodologic Studies

By Rolf Lindemann

Erythropoiesis-inhibiting factors (EIF) have been demonstrated in plasma from hypertransfused animals and from polycytemic individuals during periods of hyperoxia, but there is a decided discrepancy in the data published. In the present paper methodologic variations of a bioassay for demonstrating the erythropoiesis-inhibiting factor are discussed. In these studies no inhibitor of erythropoiesis could be demonstrated in plasma from hypoxia-induced polycythemic mice (HPM) on posthypoxic day 5. Injections of RBC or an equal amount of hemolyzed RBC were capable of suppressing the stimulatory effects of ESF, indicating that a red cell constituent may be responsible for the inhibitory effect observed. Transfusion-induced polycythemic mice (TPM) were therefore considered to be less suitable for demonstrating erythropoiesis inhibitors. Our results from testing several doses of a urinary EIF in normal mice, TPM and HPM, indicated that the HPM provided the most sensitive assay system. A similar effect was obtained with hypoxia-induced polycythemic rats. The most marked effect was seen in HPM when the EIF was injected shortly before administering the ESF, while the effect was less pronounced when the EIF was injected 24 hr before or after the ESF.

The finding of an erythropoiesis-inhibiting factor in urine from normal individuals suggests that erythropoietin is not the sole regulator of erythropoiesis. Furthermore, erythropoiesis inhibitors have been demonstrated in the plasma of hypertransfused animals during the period following induced polycythemia and also in individuals where polycythemia has been induced by long exposure to low oxygen pressure. Although using the same type of test material, the assay systems have varied, and some investigators have been unable to demonstrate these inhibitors. It seems important therefore to develop a more standardized assay system. Few attempts have been made to standardize a method for the demonstration of erythropoiesis inhibitors.

Questions arise as to whether or not a circulating inhibitor is already present in the polycythemic or normal test animals, if the quantity of this inhibitor varies, and, consequently, if the injection of washed red blood cells or hemolysates can inhibit erythropoiesis.

In the present investigation several methodologic variations are compared, and the importance of the test system chosen for the assay of an inhibitor of erythropoiesis is established. A sensitive method for the demonstration of erythropoiesis inhibitors is presented.

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MATERIALS AND METHODS

Preparation of Erythropoiesis-inhibiting Factor (EIF) and Erythropoietin Standard (ESF)

The EIF was prepared from pooled, dialyzed, and lyophilized urine from normal newborn babies. Previous studies have demonstrated that urine from newborn infants contained no detectable ESF, while EIF was present in large amounts. The urine was therefore used unfraccionated. The amount injected was related to the amount of creatinine in the urine. A volume corresponding to 5 mg creatinine was used in most studies.

The ESF preparation was dialyzed and lyophilized urine from a patient with aplastic anemia. An amount of the laboratory standard equivalent to 0.5 IU IRP of erythropoietin (WHO Expert Committee on Biological Standardization) was used for stimulation of erythropoiesis.

In order to exclude a local interaction of the EIF and the ESF, the EIF was injected intraperitoneally, while the ESF was given subcutaneously. The injection volumes were usually 1 ml.

Test Systems

Throughout the study, female mice of the NMRI/BOM strain were used. They weighed between 25 and 32 g. The mice were allowed to eat and drink ad libitum. At least five mice were used in each assay group.

Exhypoxic polycythemic mice (HPM) with a PCV of 66.5 ± 1.2% served as recipient animals in the inhibition assay, as described in detail elsewhere. The injections of the test material and ESF were usually started on day 5 after termination of the hypoxic period, with a second injection the following day (i.e., 0.5 ml x 2). 59Fe was injected intraperitoneally on day 7 and 72-hr 59Fe uptake was determined. Erythropoiesis was stimulated with an injection of ESF simultaneously with the EIF. The decrease in 59Fe incorporation into RBC of the mice receiving test material plus ESF, compared to the control mice receiving ESF alone, represented the degree of inhibition.

Normal, untreated mice (NM) and transfusion-induced polycythemic mice (TPM) were also used as recipient animals in one assay. The polycythemia was induced by two intraperitoneal injections of 0.8 ml syngeneic packed RBC on days 1 and 3, thus giving a PCV of 66.6 ± 0.9%. The injections of test materials were started on day 5; otherwise the time schedule was the same as that used for the HPM.

Inhibitory Effect of Plasma From Posthypoxic Polycythemic Mice

Mice prepared as the HPM were used. Blood was collected in heparin by decapitation and pooled on day 5 following the hypoxic period. The plasma was removed and kept frozen at -20°C until investigated. Blood samples were collected from four different series of HPM. One milliliter was injected per mouse and tested for inhibitory effect on erythropoiesis.

Inhibitory Effect of Washed RBC

Blood was collected in heparin by decapitation of the mice. The RBC were washed twice in cold 0.9% NaCl. Before injection, 0.2, 0.4, 0.6, and 0.8 ml of packed cells were diluted in 0.9% NaCl to provide a final volume of 1 ml per mouse. In one experiment, a larger volume of cells was used: 1.2 ml RBC plus 0.3 ml 0.9% NaCl. The cell suspensions were injected intraperitoneally simultaneously with the ESF.

Inhibitory Effect of Hemolyzed RBC

The same procedure was used for collecting and washing the cells. The same concentration of cells was diluted in cold distilled water and subsequently frozen and thawed three times in order to obtain complete hemolysis. The same final volumes as mentioned for the RBC were used. The hemolysates were injected intraperitoneally as described for the RBC.

Dose Response of EIF in Normal and Polycythemic Mice

The dialyzed, lyophilized urine preparation having EIF activity was tested for inhibitory effect on erythropoiesis in both NM and HPM or TPM. Amounts of this preparation acquired from
volumes of urine having a creatinine content of 0.5, 2.5, 5.0, and 12.5 mg were injected. The results were calculated both as decreased incorporation of $^{59}$Fe and as percent inhibition.

**Inhibitory Effect of EIF in Exhypoxic Polycythemic Rats**

Female Wistar rats weighing approximately 150 g at the time of investigation were used. They were exposed to intermittent hypoxia for 3 wk, as described for the HPM. EIF was injected in a dose corresponding to 15 mg creatinine, equivalent to a dose of 2.5 mg EIF to the HPM (i.e., 0.1 mg per g body weight in both species). The amounts of ESF (1.5 IU IRP of erythropoietin) and $^{59}$Fe (0.5 $\mu$Ci $^{59}$Fe) were about three times higher than those used in the mice.

**Variation in the Time Interval Between Injections of EIF and ESF**

In this assay, a single injection of EIF preparation was given 24 and 3 hr before and simultaneously with, or 3 and 24 hr after the ESF. The ESF was injected on posthypoxic day 5 and the $^{59}$Fe 48 hr later.

**RESULTS**

Table 1 shows the lack of inhibitory effect on erythropoiesis of plasma collected from the polycythemic mice on day 5 after the termination of the hypoxic period. No inhibitory effect could be observed with the four different plasma samples using our test system.

Table 2 shows the inhibitory effect of RBC and of an equivalent amount of hemolyzed RBC. An increasing inhibitory effect was seen with increasing amounts of RBC or hemolysates injected either as intact cells or as the hemolysate. This effect reached a maximum of about 60% inhibition with 0.8 ml cells.

Table 3 shows the inhibitory effect of different doses of EIF tested in HPM, TPM, and NM. Both TPM and NM proved less sensitive for demonstration of EIF than HPM. The maximum inhibition obtained with a dose of 12.5 mg was 46% and 56% for the TPM and NM, respectively. In HPM the inhibitory effect of an equal dose was 62%. The difference between the three groups was even more marked when the effect of a lower dose of EIF was compared. In a later assay, it was possible to depress further the degree of inhibition by increasing the amount of EIF to 30 mg, although the individual differences among the three groups remained constant.

Figure 1 shows the percent inhibition of different doses of EIF tested in HPM, TPM, and NM. The results are plotted on semilogarithmic paper, as a log dose-response curve, illustrating the data in Table 3.

**Table 1. The Inhibitory Effect on ESF-induced Erythropoiesis of 1 ml Plasma From Exhypoxic Polycythemic Mice**

<table>
<thead>
<tr>
<th></th>
<th>Assay 1</th>
<th>Assay 2</th>
<th>Assay 3</th>
<th>Assay 4</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$^{59}$Fe Uptake in RBC ± SE (No. of Mice)</td>
<td></td>
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</tr>
<tr>
<td>NaCl</td>
<td>0.20 ± 0.02 (5)</td>
<td>0.27 ± 0.04 (10)</td>
<td>0.30 ± 0.05 (3)</td>
<td>0.56 ± 0.09 (5)</td>
</tr>
<tr>
<td>ESF</td>
<td>3.06 ± 0.38 (5)</td>
<td>4.64 ± 0.99 (6)</td>
<td>8.41 ± 0.72 (5)</td>
<td>11.55 ± 1.52 (9)</td>
</tr>
<tr>
<td>ESF + posthypoxic plasma</td>
<td>3.50 ± 0.34 (5)</td>
<td>4.08 ± 0.46 (6)</td>
<td>8.68 ± 1.43 (5)</td>
<td>11.57 ± 1.60 (5)</td>
</tr>
</tbody>
</table>

*Collected on day 5 after termination of the hypoxic period, expressed as the $^{59}$Fe incorporation in RBC of hypoxia-induced polycythemic mice.
Table 3. The Inhibitory Effect of Different Doses of ElF on the $^{59}$Fe Incorporation Following ESF Injection in RBC of Hypoxia-induced Polycythemic Mice (HPM), Transfusion-induced Polycythemic Mice (TPM), and Normal Mice (NM)

<table>
<thead>
<tr>
<th></th>
<th>HPM</th>
<th>TPM</th>
<th>NM</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$^{59}$Fe Uptake in RBC ± SE (No. of Mice)</td>
<td>$^{59}$Fe Uptake in RBC ± SE (No. of Mice)</td>
<td>$^{59}$Fe Uptake in RBC ± SE (No. of Mice)</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.38 ± 0.04 (3)</td>
<td>0.28 ± 0.01 (4)</td>
<td>27.39 ± 3.36 (4)</td>
</tr>
<tr>
<td>ESF</td>
<td>7.07 ± 0.18 (10)</td>
<td>8.78 ± 0.58 (7)</td>
<td>26.32 ± 1.88 (7)</td>
</tr>
<tr>
<td>ESF + 0.5 mg ElF</td>
<td>5.23 ± 0.41* (5)</td>
<td>6.24 ± 0.17* (5)</td>
<td>18.99 ± 3.24 (5)</td>
</tr>
<tr>
<td>ESF + 2.5 mg ElF</td>
<td>3.18 ± 1.41† (5)</td>
<td>5.49 ± 0.75* (4)</td>
<td>16.67 ± 2.02* (4)</td>
</tr>
<tr>
<td>ESF + 5.0 mg ElF</td>
<td>2.81 ± 0.16† (5)</td>
<td>5.16 ± 0.69* (4)</td>
<td>13.89 ± 1.71† (3)</td>
</tr>
<tr>
<td>ESF + 12.5 mg ElF</td>
<td>2.71 ± 0.84† (3)</td>
<td>4.76 ± 0.73* (4)</td>
<td>11.67 ± 1.57† (4)</td>
</tr>
</tbody>
</table>

*0.01 > p > 0.001.
†p < 0.001.
10.05 > p > 0.01.

The doses of ElF are related to the amount of creatinine in the urine, i.e., 5 mg ElF equals 5 mg creatinine.
A dose equivalent to 0.5 and 1.5 IU ESF was used in the mice and rats, respectively.

Table 4 shows the inhibitory effect of EIF on erythropoiesis in exhypoxic polycytemic rats compared with the effect in HPM. The dose tested was 0.1 mg per g body weight in both species. The inhibitory effect was 55% in the mice and 47.5% in the rats. The mean PCV was approximately 63% in the mice and 55% in the rats at the time of investigation.

Table 5 shows the inhibitory effect of EIF when injected 24 and 3 hr before, simultaneously with, or 3 and 24 hr after the ESF. The most marked effect was seen when the injection was given immediately before or simultaneously with the ESF. The injections given 24 hr before or after produced no or less inhibition. The injection given 3 hr after the ESF also had less inhibitory effect than the injection given 3 hr before.

### Table 4. The Inhibitory Effect of 0.1 mg EIF per Gram Body Weight on the ^59^Fe Incorporation in RBC Following ESF Injection, the Per Cent Inhibition, and the Mean PCV in Exhypoxic Polycytemic Mice and Rats

<table>
<thead>
<tr>
<th></th>
<th>Per Cent ^59^Fe Uptake in RBC ± SE (No. of Animals)</th>
<th>Inhibition (%)</th>
<th>Mean PCV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NoCl</td>
<td>ESF</td>
<td>ESF + EIF</td>
</tr>
<tr>
<td>Mice</td>
<td>0.83 ± 0.04</td>
<td>7.07 ± 0.18</td>
<td>3.18 ± 1.41*</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>(10)</td>
<td>(5)</td>
</tr>
<tr>
<td>Rats</td>
<td>6.55 ± 1.22</td>
<td>18.05 ± 1.17</td>
<td>9.48 ± 2.95*</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>(9)</td>
<td>(5)</td>
</tr>
</tbody>
</table>

*0.05 > p > 0.01.

A dose equivalent to 0.5 and 1.5 IU ESF was used in the mice and rats, respectively.
Table 5. Inhibitory Effect of EIF on the $^{59}$Fe Incorporation in RBC in Exhypoxic Polycythemic Mice When Injected 24 and 3 hr Before (−24 and −3 hr), Simultaneously With (Simult.), or 3 and 24 hr After (+3 and +24 hr) Stimulation With ESF

<table>
<thead>
<tr>
<th>Assay</th>
<th>Per Cent $^{59}$ Fe Uptake in RBC ± SE (No. of Mice)</th>
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<tbody>
<tr>
<td></td>
<td>Assay 1</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.39 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>(5)</td>
</tr>
<tr>
<td>ESF</td>
<td>6.98 ± 0.72</td>
</tr>
<tr>
<td></td>
<td>(5)</td>
</tr>
<tr>
<td>ESF + EIF − 24 hr</td>
<td>3.57 ± 0.46</td>
</tr>
<tr>
<td></td>
<td>(5)</td>
</tr>
<tr>
<td>ESF + EIF − 3 hr</td>
<td>1.58 ± 0.30†</td>
</tr>
<tr>
<td></td>
<td>(5)</td>
</tr>
<tr>
<td>ESF + EIF simult.</td>
<td>3.56 ± 0.27†</td>
</tr>
<tr>
<td></td>
<td>(5)</td>
</tr>
<tr>
<td>ESF + EIF + 3 hr</td>
<td>6.08 ± 0.72</td>
</tr>
<tr>
<td></td>
<td>(5)</td>
</tr>
<tr>
<td>ESF + EIF + 24 hr</td>
<td>2.82 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>(5)</td>
</tr>
</tbody>
</table>

*0.05 > p > 0.01. 
†p < 0.001. 
‡0.01 > p > 0.001.

DISCUSSION

The activity of erythropoiesis-inhibiting factors has been demonstrated in several bioassay systems using red cell iron incorporation as the index of erythropoiesis. Conflicting reports regarding the existence of these inhibitors have been published.10–12 The investigators used similar models, but with great variations in the assay systems. Both rats and mice have served as recipients, either untreated, starved, or made polycythemic by transfusion or hypoxia.

An erythropoiesis-inhibiting factor has been demonstrated in plasma from hypertransfused sheep,3,4 rats,4 mice,5 and rabbits.5 Similar findings have also been reported in polycythemic individuals during a period with a surplus of oxygen, i.e., natives with high-altitude polycythemia moved to sea level,8 normal newborn babies during their adjustment to extrauterine life,6,7 or in patients with polycythemia vera in relapse14. It is therefore possible that a circulating erythropoiesis inhibitor is already present in the polycythemic recipient animals during the test period, thereby rendering them less sensitive.

Urine from normal human individuals has been shown to contain both ESF and EIF, indicating a dual regulation of normal erythropoiesis.12 A circulating erythropoiesis inhibitor may therefore be present in normal, untreated, or starved recipient animals. These types of animals are also probably less sensitive, since the erythroid cells in the bone marrow are not synchronized, such as the erythropoiesis seen in polycythemic mice after ESF stimulation.

When the exhypoxic polycythemic mouse bioassay was used for erythropoietic studies, the injections were usually started on day 5 following the hypoxic period. No inhibitory effect could be obtained with plasma collected on posthypoxic day 5. In this test system, erythropoiesis decreased to a negligible level of activity about posthypoxic day 5. Following an ESF stimulus, the growth of the erythroid cells became synchronized.15,16 The HPM do not have
an endogenously produced inhibitor on day 5 posthypoxia and are therefore well suited for testing inhibitory effects of test materials.

Injections of RBC or hemolysates in our assay system were also found to inhibit ESF stimulation. Intact cells produced the same degree of inhibition as an equivalent amount of hemolyzed RBC, indicating that a cell component was responsible for the inhibitory effect. Since TPM probably had a high level of inhibiting factor, they were less suitable for testing small amounts of inhibitor. High concentrations of test material, however, were inhibitory even in TPM.

The sensitivity of the different types of recipient animals to the erythropoiesis inhibitors was also studied. The HPM appeared to be the most sensitive to the inhibitors, as has been demonstrated previously. In the TPM and NM it may be necessary to inject an amount of EIF which exceeds the endogenously produced inhibitor before an effect can be demonstrated.

Two different species of recipient animals, mice and rats, have also been studied in regard to their response to the inhibitor. The same degree of inhibition was found with equal doses of EIF in mice and rats. However, different results have been reported using different types of recipient animals. In the present study, polycythemia was induced by hypoxia, and the same time schedule was used as in the HPM. However, the findings of a PCV of 55% at the time of investigation and a high 59Fe uptake in the control rats were analogous to those experienced by others and indicated that rats were less suitable than polycythemic mice. Rats also developed a high incidence of gastrointestinal hemorrhage following hypertransfusions.

Table 5 emphasizes the importance of the time interval between injections of EIF and ESF. The most markedly inhibitory effect was found when the EIF was injected shortly before or simultaneously with the ESF. When EIF was injected 24 hr before or after the ESF stimulation, the inhibition was diminished or absent. Even a time difference of 3 hr after the ESF injection caused less inhibition. According to Jepson and Lowenstein this time factor also applies to estrogens. They demonstrated an inhibition of erythropoiesis only when the estrogens were injected 4 hr before or simultaneously with the ESF stimulus. If injected later, an inhibitory effect was not obtained. Moriyama et al. obtained an inhibitory effect on erythropoiesis when uremic plasma was injected 6 hr before, simultaneously with, or 12 hr after ESF stimulation. However, injections of uremic plasma 24 or 36 hr after ESF were without effect.

The lack of uniformity in the time schedules used by the various authors makes the comparison of these studies difficult. The interval between the termination of the hypoxic period or the transfusion of RBC and the injection of the test materials varies, as well as that between the injections of EIF and ESF.

The biologic half-life of both ESF and EIF should be considered in the mechanism of action of EIF. A short half-life for ESF and EIF could account for an absent or diminished effect after a 24-hr interval. However, if this factor was of significance the variations would not occur with the injections given 3 hr before, simultaneously with, or 3 hr after the ESF.

Since intact RBC, hemolyzed RBC, and the small molecular EIF fraction contain some amount of elemental iron, it was conceivable that the decreased incorporation of radioactive 59Fe measured in the assay system was due to a
dilution of the $^{59}$Fe with unlabeled iron. Using our inhibition assay it was found that neither Fe$^{2+}$ nor Fe$^{3+}$ inhibited the $^{59}$Fe incorporation into newly formed RBC. Thus it can be assumed that ferrokinetics (i.e., a dilution of the radioactive iron with cold iron present in the test materials) did not influence the inhibitory effect measured with the EIF, the RBC, or hemolyzed RBC.

One can only speculate at this time about the mode of action of the EIF. In the posthypoxic or post-transfusion polycythemic mice, very few erythroid cells are present in the bone marrow, and ESF gives rise to a wave of erythroid cells, all at approximately the same stage of development. The most marked inhibitory effect is found when the EIF is injected shortly before or simultaneously with ESF. It is most likely that the inhibitor acts at the erythropoietin-responsive cell (ERC) level, either as an antagonist to ESF or as a chalone (i.e., a tissue-specific inhibitor of mitosis produced by the same cells exerting its effect by negative feedback). When injected later, the development of erythroid cells has already started and continues unaffected by the inhibitor. An inhibitory effect on heme synthesis should be demonstrable in the HPM even if the EIF is injected several hours after ESF stimulation. Using either serum from uremic or polycythemic rabbits, an inhibiting effect on heme synthesis has been demonstrated in bone marrow cultures. An inhibitory effect on the ERC level cannot be entirely excluded, since the $^{59}$Fe incorporation has been the only parameter used. A toxic effect of the EIF seems illogical, since the effect is obtained only when given within a short time interval.

The present study shows that there are several factors that have to be taken into consideration when inhibition of erythropoiesis is studied. The exhypoxic polycythemic mouse bioassay seems to be the most sensitive test system, with the start of the injections on day 5 following the hypoxic period. The injections of the inhibitor should be given shortly before ESF stimulation. Variations in these conditions may explain the conflicting data in the literature regarding erythropoiesis inhibitors.

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