Modified Method of Erythropoietin (ESF) Bioassay In Vitro Using Mouse Fetal Liver Cells. II. Measurement of ESF in Human Serum

By G. de Klerk, A. A. M. Hart, C. Kruiswijk, and R. Goudsmit

A modification of the mouse fetal liver cell bioassay for erythropoietin (ESF) that allows quantitative detection of ESF in human serum is described. The modification consists of (1) correction for the effect of serum iron on $^{59}$Fe incorporation into heme and (2) the use of an "internal standard," i.e., a standard ESF preparation dissolved in the assayed test serum. As a result of this modification the statistical method of bioassay analysis had to be changed fundamentally. The mean serum concentration of ESF measured in 20 normal males was 48 mU/ml, as compared to 29 mU/ml in 18 females. The difference was significant at $p = 0.017$. The stimulatory activity of a human serum on $^{59}$Fe incorporation into heme could be neutralized by an anti-human ESF immune serum.

DETERMINATION OF ERYTHROPOIETIN (ESF) in serum from normal human subjects to assess physiologic levels has been limited by the insensitivity of the current bioassay methods in vivo. Bioassay systems in vitro seem to be promising in this respect. The major disadvantages of these cell culture methods refer to their sensitivity to impurities and nonspecific influences of the ESF-containing biologic material.

The assay of human serum for ESF using mouse fetal liver cells in culture showed (1) a toxic effect of human serum on mouse erythroblasts due to complement-dependent IgM heteroantibodies (this could be abolished by heating the serum for 30 min at 56°C) and (2) a decrease of $^{59}$Fe incorporation into heme due to dilution of the radiolabel by iron present in the test serum (a method of correction for this effect was described previously). In addition to these factors present in each human serum, many other constituents of individual human sera may influence nonspecifically ESF-mediated stimulation of heme synthesis, e.g., steroids and substances retained in uremia.

In order to eliminate the influence of such unknown serum factors we introduced a so-called internal standard, i.e., a standard ESF preparation that was dissolved not in saline but in the assayed test serum. In this way interfering substances in the test serum equally affected the test serum cultures and the standard cultures. In this paper the consequences of these modifications on the statistical analysis are described. The reproducibility of the modified method is discussed, and the results of determination of ESF titers in normal human sera are presented.

MATERIALS AND METHODS

Each human serum is assayed against its internal standard, consisting of the assayed test serum to which is added a known concentration of an ESF standard preparation. Sheep plasma erythropoietin...
(Connaught, step III) was used as a standard by prior calibration against the International Reference Preparation (second IRPE). In parallel with the assay of a human serum, a freshly prepared solution of transferrin-bound iron (Tf-Fe) is assayed in which the concentrations of transferrin and iron are similar to those in the serum. Its dose-response curve is used as a correction curve for the influence of serum iron on the dose-response curves of both the test serum and its internal standard. Details of the basic bioassay technique and the method of correction were described previously.4

Statistical analysis. Under the assumption of linearity the corrected dose-response curves of test serum and internal standard can be written as

\[
\bar{T}_i - \bar{c}_i = a_i + b_i x_i + \epsilon_i, \quad (1a)
\]

\[
\bar{S}_i - \bar{c}_i = a_s + b_s x_i + \epsilon_s, \quad (1b)
\]

where \(\bar{T}_i\), \(\bar{S}_i\), and \(\bar{c}_i\) are the mean responses (log cpm) at the \(i\)th concentration of the test serum, its internal standard, and the Tf-Fe solution, respectively, \(x_i\) is the logarithm of the \(i\)th concentration, and the \(\epsilon_i\) are independently and identically distributed variables measuring deviations due to random fluctuations.

The left-hand terms of Eqs. (1) share a common variable \(\bar{c}_i\), which prohibits application of the standard linear regression technique. Equations (1a) and (1b) may be transformed into

\[
u_i = \left( \frac{T_i S_i}{T_i + S_i} \right)^{1/2} \left[ (1a) - (1b) \right] = \frac{T_i S_i}{T_i + S_i} \left( \bar{T}_i - \bar{S}_i \right)
\]

\[
w_i = \left( \frac{C_i}{(T_i + S_i)(T_i + S_i + C_i)} \right)^{1/2} \left[ T_i (1a) + S_i (1b) \right]
\]

\[
u_i = \left( \frac{C_i}{(T_i + S_i)(T_i + S_i + C_i)} \right)^{1/2} \left[ T_i (\bar{T}_i - \bar{c}_i) + S_i (\bar{c}_i - \bar{S}_i) \right]
\]

\[
w_i = \left( \frac{C_i}{(T_i + S_i)(T_i + S_i + C_i)} \right)^{1/2} \left[ T_i a_i + S_i a_s + (T_i b_i + S_i b_s) x_i \right] + \epsilon_w, \quad (2b)
\]

where \(T_i\), \(S_i\), and \(C_i\) are the number of replications at the \(i\)th concentration of the test serum, its internal standard, and the Tf-Fe solution, respectively, and (1a) and (1b) indicate the use of either side of Eqs. (1a) or (1b), respectively.

Given the independence and identical distribution of the replications of test serum, internal standard, and Tf-Fe solution, it can be proved that the variables \(u_i\) and \(w_i\) have equal variances and are mutually independent.

Equations (2) can be used provided that at the \(i\)th concentration at least two of the variables \(\bar{T}_i\), \(\bar{S}_i\), and \(\bar{c}_i\) are known. When \(\bar{T}_i\) or \(\bar{S}_i\) are unknown (i.e., \(T_i = 0\) or \(S_i = 0\)), Eq. (2b) can be used. When \(\bar{c}_i\) is unknown Eq. (2a) still holds, but in that case neither Eq. (1a) nor (1b) can be used.

Using Eqs. (2), least-squares estimates of \(a_i\), \(b_i\), \(a_s\), and \(b_s\) can be calculated. Calculations are facilitated by the independence and homoscedasticity* of \(u_i\) and \(w_i\).

Linearity of the corrected dose-response curves is tested by comparing the fitted values from Eqs. (2) to the observations using the analysis of variance. Parallelism \((b_i = b_s)\) is also tested by the analysis of variance.

*Observations are described as homoscedastic when at all points along the curve the observations have the same scatter (Colquhous D: Lectures on Biostatistics. Oxford, Clarendon, 1971, pp 220 222).
A matrix of least-squares weight coefficients of the estimates of \(a\), \(b\), \(a_1\), and \(b_1\) can be calculated, indicating the accuracy of these estimates and their correlation. If these variance-multiplying factors are symbolized by \(V\), then one can define

\[
\begin{align*}
\text{var} |a| &= V(a,a) \sigma^2, \\
\text{var} |b| &= V(b,b) \sigma^2, \\
\text{cov} |a,b| &= V(a,b) \sigma^2,
\end{align*}
\]

where \(\sigma^2\) is the variance of the replications.

The least-squares estimate of the common slope \(h (= b_1 - b_1)\), symbolized \(\hat{h}\), is given by a weighted average of \(\hat{b}_1\) and \(\hat{b}_s\):

\[
\hat{h} = \frac{(V(b_1,b_1) - V(b_1,b_s))\hat{b}_1 + (V(b_1,b_s) - V(b_1,b_1))\hat{b}_s}{V(b_1,b_1) - 2V(b_1,b_s) + V(b_1,b_s)},
\]

(3a)

where \(\hat{b}_1\) and \(\hat{b}_s\) are the least-squares estimates of \(b_1\) and \(b_s\), respectively.

Because of the correlation between estimates of the intercepts \((a, a_1)\) and slopes \((b_1, b_s)\), estimates of \(a_1\) and \(a_s\) must be corrected for the difference between \(\hat{b}\) and \(\hat{b}_s\), respectively. These new least-squares estimates of \(a_1\) and \(a_s\), symbolized \(\hat{a}_1\) and \(\hat{a}_s\), are given by

\[
\begin{align*}
\hat{a}_1 &= \hat{a}_1 + (1/H)(V(a_1,b_1) - V(a_1,b_s))\hat{b}_s - \hat{b}_1), \\
\hat{a}_s &= \hat{a}_s + (1/H)(V(a_1,b_s) - V(a_1,b_1))\hat{b}_s - \hat{b}_1),
\end{align*}
\]

(3b)

where

\[
H = V(b_1,b_1) - 2V(b_1,b_s) + V(b_1,b_s).
\]

The least-squares estimate of the potency ratio between test serum and internal standard, symbolized \(R\), is calculated from

\[
\hat{R} = \text{antilog} \frac{\hat{a}_1 - \hat{a}_s}{b}.
\]

The confidence limits \((\hat{R}_l, \hat{R}_u)\) are calculated using Fieller’s theorem. Finally, the unknown concentration of ESF in the test serum, symbolized \(\hat{U}\), and its confidence limits \((\hat{U}_l, \hat{U}_u)\) are calculated from

\[
\begin{align*}
\hat{U} &= \frac{\hat{R}}{1 - \hat{R}} K, \\
\hat{U}_l &= \frac{\hat{R}_l}{1 - \hat{R}_l} K, \\
\hat{U}_u &= \frac{\hat{R}_u}{1 - \hat{R}_u} K,
\end{align*}
\]

where \(K\) is the known concentration of standard ESF in the internal-standard preparation.

Reproducibility. Each of four human sera was assayed against its own internal standard on two to five occasions in different experiments. Serum I was obtained from a male blood donor (Hb 15.3 g/dl, mean ESF titer 31 mU/ml), serum II from a patient with polycystic renal disease on maintenance hemodiagnosis (Hb 14.2 g/dl, ESF 22 mU/ml), serum III from a patient with chronic myeloid leukemia (Hb 4.5 g/dl, ESF 935 mU/ml), and serum IV from a patient with a refractory anemia (Hb 6.0 g/dl, ESF 1955 mU/ml).

ESF in normal serum. Sera were collected from 38 healthy blood donors of the Dutch Red Cross.
The donors had not been phlebotomized during the preceding 4 mo. The sera were stored at -20° C until ESF assay.

Neutralization of human ESF. The neutralizing anti-human urinary ESF immune serum was kindly supplied by Dr. J. F. Garcia (Lawrence Berkeley Laboratory, Donner Laboratory, University of California). A human serum obtained from a patient with an iron deficiency (Hb 5.5 g/dl, serum iron 2 μmol/liter) containing ESF at 502 mU/ml (95% confidence limits 303-768 mU/ml) as well as a solution of the second IRPE in saline containing 500 mU/ml (stated potency) were incubated with the anti-ESF immune serum for 1 hr at 37°C and subsequently for 24 hr at 4°C. Saline (0.9% w/v NaCl) was used as control and incubated with the immune serum in parallel with the ESF preparations. After centrifugation the supernatants were assayed.

RESULTS

Modified method. The graphic expression of the modified bioassay in vitro is shown in Fig. 1. A normal human serum was assayed. The noncorrected dose-response curves of test serum and internal standard declined progressively at increasing concentrations. Following correction for the effect of serum iron on 55Fe incorporation no significant deviations from linearity (p = 0.18) or from parallelism (p = 0.76) were found. The potency ratio was 0.346, with 95% confidence limits 0.315 and 0.383. The serum ESF concentration (U) with 95% confidence limits (U, U,) was calculated at 53 mU/ml (46-62 mU/ml).

Reproducibility and precision. The results from repeated assays of four sera are summarized in Table I. Within each group of replications deviations of the logarithm of the individual potency ratios (log R) from their weighted average (log R) were compared to their standard deviations (SD log R) using an F test. Devia-
Table 1. Reproducibility of an Estimation of the Logarithm of the Potency Ratio (log R) Between Test Serum and Internal Standard

<table>
<thead>
<tr>
<th>Serum</th>
<th>SD(log R)</th>
<th>df</th>
<th>log R</th>
<th>log R</th>
<th>(log R - log R')</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.040</td>
<td>27</td>
<td>-0.5697</td>
<td>-0.6267</td>
<td>2.01</td>
<td>4.35</td>
<td>0.047</td>
</tr>
<tr>
<td></td>
<td>0.043</td>
<td>26</td>
<td>-0.6929</td>
<td></td>
<td>2.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.151</td>
<td>26</td>
<td>-0.7100</td>
<td></td>
<td>0.08</td>
<td>0.05</td>
<td>0.951</td>
</tr>
<tr>
<td></td>
<td>0.145</td>
<td>23</td>
<td>-0.7493</td>
<td>-0.7526</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.075</td>
<td>23</td>
<td>-0.7640</td>
<td></td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.022</td>
<td>48</td>
<td>-0.2056</td>
<td></td>
<td>1.65</td>
<td>1.83</td>
<td>0.159</td>
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<tr>
<td></td>
<td>0.034</td>
<td>36</td>
<td>-0.2275</td>
<td>-0.1770</td>
<td>2.24</td>
<td>1.85</td>
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<tr>
<td></td>
<td>0.014</td>
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<td></td>
<td>1.59</td>
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<tr>
<td></td>
<td>0.030</td>
<td>38</td>
<td>-0.1877</td>
<td></td>
<td>1.10</td>
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<td></td>
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<tr>
<td>D</td>
<td>0.036</td>
<td>35</td>
<td>-0.4855</td>
<td></td>
<td>0.10</td>
<td>1.74</td>
<td>0.131</td>
</tr>
<tr>
<td></td>
<td>0.034</td>
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<td>-0.4426</td>
<td>-0.5683</td>
<td>0.87</td>
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<tr>
<td></td>
<td>0.029</td>
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<td>-0.4344</td>
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<td>1.84</td>
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<tr>
<td></td>
<td>0.038</td>
<td>39</td>
<td>-0.5146</td>
<td></td>
<td>1.14</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>0.051</td>
<td>38</td>
<td>-0.5683</td>
<td></td>
<td>3.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.74</td>
<td>0.131</td>
<td></td>
</tr>
</tbody>
</table>

Each of four human sera was assayed on two to five occasions in different experiments.

Fig. 2. Concentration of ESF in 38 normal human sera.
Table 2. Kendall Rank Correlation Between Serum ESF Concentration (mU/ml), Blood Hemoglobin Concentration (g/dl), and Age in 38 Normal Human Subjects

<table>
<thead>
<tr>
<th>Variables</th>
<th>Correlation Coefficient (r)</th>
<th>Significance (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males ESF vs age</td>
<td>-0.154</td>
<td>0.342</td>
</tr>
<tr>
<td>Males ESF vs Hb</td>
<td>+0.038</td>
<td>0.818</td>
</tr>
<tr>
<td>Females ESF vs age</td>
<td>+0.159</td>
<td>0.358</td>
</tr>
<tr>
<td>Females ESF vs Hb</td>
<td>-0.061</td>
<td>0.726</td>
</tr>
</tbody>
</table>

Males: ages 20–55 yr, Hb 14.2–17.9 g/dl, n = 20; females: ages 21–53 yr, Hb 12.2–14.8 g/dl, n = 18.

Correlations were not significant (p = 0.13), indicating that the estimated potencies of the same preparation in repeated tests do not vary more than would be predicted from the internal evidence of the component assays. The index of precision (λ) for 50 assays of human sera ranged from 0.04 to 0.19 (mean 0.09), which is in accordance with previous reports on this bioassay in vitro.

ESF in normal serum. ESF titers were determined in 38 normal human sera. The results are shown in Fig. 2. The difference between the mean ESF titer in 20 males (48 mU/ml) and the mean titer in 18 females (29 mU/ml) was found to be significant at p = 0.017 using the two-sided Wilcoxon test. Within this group of normal sera no significant correlation was found between ESF titer and age or between ESF titer and blood hemoglobin concentration. This held true for both

Fig. 3. Neutralization of human ESF from serum and urine by an anti-human ESF immune serum. ESF preparations and control solutions of saline were incubated with immune serum for 1 hr at 37°C and subsequently for 24 hr at 4°C before addition to cultures. Means of three replicate cultures ± SD.
sexes. The Kendall rank correlation coefficients and their significance are shown in Table 2.

**Neutralization of human ESF.** The effect of incubation of human ESF with an anti-human ESF immune serum on $^{59}$Fe incorporation into heme is shown in Fig. 3. A human urinary ESF preparation (second IRPE) and a human serum, both with equal potency, were neutralized by the same amount of the anti-ESF immune serum. A toxic influence of the immune serum was excluded because the blank heme synthesis in the control cultures was not affected at all.

**DISCUSSION**

Quantitative bioassay depends on the validity of the assumption that the standard and unknown behave as though they were identical, apart from the concentration of the substance being assayed. However, biologic fluids are usually assayed against partially purified standard preparations dissolved in saline, which implies that the above condition is not satisfied. This may lead to erroneous results, especially in bioassay systems in vitro.

Because of the wide variety of serum constituents, including therapeutic agents, which may influence ESF-mediated stimulation of heme synthesis, it seems reasonable to assay a human test serum against a standard ESF preparation dissolved in the same test serum. In this way any nonspecific influence of the test serum on the responsiveness of the cultured cells to ESF will affect both the standard cultures and the test serum cultures. Moreover, by use of this internal-standard procedure differences in the ESF responsiveness enhancing or inhibitory properties of two sera may be studied. The two sera (e.g., a patient serum and a normal pool serum) are assayed in the same experiment, each against its own internal standard. If nonspecifically interfering material is present in the patient serum, the ratio between the individually estimated serum potencies will not equal the potency ratio calculated directly from a comparison of the corrected serum dose-response curves.

In this modified assay method the same Tf-Fe correction curve is used for the test serum and its internal standard. This implies that the corrected dose-response curves of test serum and internal standard are not independent, which requires fundamental changes in the usual statistical method of bioassay analysis. If the standard linear regression technique were used and the correlation between the left-hand terms of Eqs. (1) were not taken into account, the estimates of the intercepts and slopes of both corrected curves would be less accurate and the calculated confidence limits would be too narrow. Furthermore, the use of transformations of Eqs. (1) would lead to different results. On the other hand, applications of the general linear least-squares theory on transformations of eqs. (1) will always give the same result provided that the effect of a transformation on the variance and covariance of the left-hand terms is taken into consideration.

Confidence limits purport to predict from the results of one experiment what will happen when the experiment is repeated under the same conditions. This was found to hold good for the results obtained by the modified mouse fetal liver cell bioassay for ESF. The true error found by actual repetition of the assay did not deviate significantly from the internal estimates of error calculated in the individual assays. This compares favorably with other bioassays, where the true error is often underestimated.
The results reported in this study showed the presence of measurable concentrations of ESF in normal human serum. The mean ESF titer determined in 20 males was 48 mU/ml, compared to 29 mU/ml in 18 females. Dunn et al., using the mouse fetal liver cell bioassay, reported a mean serum ESF level of 230 mU/ml in 20 normal males, compared to 100 mU/ml in 9 females. Napier et al., using the same assay system, reported a normal range of 50-250 mU/ml. Kazal and Erslev, using bioassays in the polycythemic mouse, found that serum ESF concentrations in normal individuals were too low (<50 mU/ml) to be measured by these techniques. Garcia, using a radioimmunoassay, reported a mean level of 4.9 mU/ml in 311 males and 4.3 mU/ml in 457 females. Lertora et al., using a radioimmunoassay, estimated normal plasma levels of ESF of 52-84 mU/ml. Finally, Jordan et al., using a hemagglutination inhibition assay, reported a normal range of 6-60 mU/ml.

From these data it appears that normal ESF levels determined by the modified mouse fetal liver cell bioassay correspond rather well to the normal range of Jordan et al., and are not far from the range of Lertora et al., both of whom used immunologic methods. Furthermore, most ESF levels we found in normal sera were below 50 mU/ml, as predicted by Kazal and Erslev using bioassays in vivo. Notwithstanding the discrepancy between our normal range and that obtained by Garcia, his finding by an immunologic technique of a sex difference in normal ESF levels supports the specificity of a similar finding by bioassay.

No correlation was found between normal ESF levels and either age or blood hemoglobin concentration. Therefore it seems improbable that a causal relationship between ESF level and hemoglobin concentration is implicated in the difference between men and women in both parameters. The higher levels of ESF and hemoglobin in adult males may be explained by a dual action of androgens, which are known to increase the production of ESF as well as to stimulate erythropoiesis by a direct effect on the bone marrow.

Finally, it was shown that the stimulatory effect on Fe incorporation into heme of both a human serum and an urinary ESF preparation (second IRPE) could be completely neutralized by an anti-ESF immune serum. This may suggest a considerable immunologic specificity of the active principle in human serum determined by this method.

REFERENCES
Modified method of erythropoietin (ESF) bioassay in vitro using mouse fetal liver cells. II. measurement of ESF in human serum

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