Comparison of the Hemagglutination Inhibition Assay Kit for Erythropoietin (ESF) With the Fetal Mouse Liver Cell Bioassay In Vitro

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The commercially available hemagglutination inhibition (HAI) assay kit for erythropoietin (ESF) was compared with the fetal mouse liver cell (FMLC) bioassay. No correlation was obtained between ESF levels determined by both methods in a variety of pathologic sera. The HAI kit showed a great batch variability. Significant immunoreactivity was found in those fractions of a normal human serum and a human urinary ESF preparation that were not active in the FMLC bioassay. A very poor recovery of immunoreactivity was found when the international reference preparation for erythropoietin (second IRPE) was added to a normal human serum.

Many methods have been described for the measurement of erythropoietin (ESF) in biologic fluids. On the one hand, there are bioassays in vivo and in vitro, and on the other hand, immunochemical methods.

Generally, bioassays for ESF are relatively insensitive, time consuming, and expensive compared to immunoassays. However, bioassays may be assumed to be specific since they are based on the original physiologic action of the hormone, whereas a basic problem in immunoassays is the uncertain biologic activity of the antigen to be determined. Therefore, it is not surprising that major differences between bioologic and immunologic determinations of serum ESF concentrations have been found, e.g., in sera of anemic uremic patients. Consequently, it is necessary to compare immunoassays for ESF with biologic methods in a variety of clinical situations. Since 1975, a hemagglutination inhibition (HAI) assay for ESF has been commercially available and widely used. However, some investigators have reported that the kit was unsatisfactory due to the lack of reproducibility of the reagents. In this study, we have evaluated the HAI assay kit for ESF and compared it with the fetal mouse liver cell (FMLC) bioassay for ESF, which is currently in use in our laboratory.

MATERIALS AND METHODS

HAI Assay for ESF

Two HAI assay kits from J.C.L. Research Co., Knoxville, Tenn., were used, and the assays were performed according to the manufacturer’s instructions. All test sera were heated at 56°C for 30 min and absorbed with human group 0 erythrocytes. Absorption was carried out by incubation of the serum sample with packed erythrocytes for 15 min at 37°C. Subsequently, the supernatant serum was incubated with erythrocytes for 30 min at room temperature and finally for 15 min at 4°C. Dilutions of test serum and ESF standard preparation were incubated with the anti-ESF antiserum for 30 min at room temperature. ESF-sensitized human group 0 red blood cells were added, and the mixtures were incubated for another period of 30 min at room temperature. The HAI titer was recorded at the dilution that first allowed 3+ hemagglutination. Control sera with stated potency (low, normal, and elevated level) provided by the manufacturer were run and gave the expected results throughout the experiments. The 75 mIU/ml ESF standard preparation produced a 3+ hemagglutination at a titer of 1:64 in all assays. Replacement of the anti-ESF antiserum by saline did not produce agglutination in assays of normal human sera, which is in contrast to the findings of Vegt et al. All specimens assayed by the HAI kit were stored and processed in glass or polypropylene plastic tubes as recommended by the manufacturer. The two different HAI kits will be referred to as kit I and kit II.

FMLC Bioassay for ESF

A modified method of this bioassay in vitro has recently been described by de Klerk et al. 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% fetal calf serum (Flow). 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 x 10^6 cells/ml. The cultures were preincubated for 2 hr at 37°C in tubes gassed with 5% CO_2 in air. Subsequently, 1 µCi ^59 Fe-ferric citrate (Radiochemical Centre, Amersham) bound to human apotransferrin (Behringwerke) was added to each of the cultures. Following incubation for 4 hr at 37°C, heme was extracted using acid methyl ethyl ketone, and ^59 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 bioassays. Details of this method and of the statistical analysis of the results have been described previously.

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

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prior calibration against the international reference preparation for erythropoietin (second IRPE). For this, the Connaught standard was assayed against the second IRPE, using the FMLC bioassay. The mean result of 6 separate assays was taken as the potency ratio between both preparations.

Test Sera
Normal human sera were collected from healthy blood donors of the Dutch Red Cross. Pathologic sera were obtained from patients with different types of primary anemia, uremia, and primary and secondary polycythemia. All uremic sera were from patients undergoing intermittent hemodialysis. These sera were taken immediately before dialysis.

Comparison of ESF Levels Determined by the FMLC Bioassay and the HAI Kit
ESF levels were determined in 38 human sera, 19 of which were studied using the FMLC bioassay and HAI kit I and the other 19 using the FMLC bioassay and HAI kit II. The sera studied using HAI kit I were from patients with a primary anemia (10), uremia (3), primary polycythemia (4), and secondary polycythemia (2). The sera studied using HAI kit II were from patients with a primary anemia (12), uremia (5), and secondary polycythemia (2).

Fractionation of a Normal Human Serum and a Human Urinary ESF Preparation
The pooled void volume of one 2-mI sample of a normal human serum eluted from a Sephadex G25 column was fractionated on a Sephadex G100 column. In a separate experiment, a sample of a human urinary ESF preparation containing 50 U of ESF was also fractionated on a Sephadex G100 column. Elutions were carried out using phosphate-buffered saline at pH 7.4. The column effluents were assayed by the HAI technique using kit I and kit II and by the FMLC bioassay.

The human urinary ESF preparation, 27.8 U/mg, was procured by the Department of Physiology, University of Northeast, Corrientes, Argentina, processed by the Hematology Research Laboratories, Children’s Hospital of Los Angeles, and distributed by the National Heart Lung and Blood Institute (NHLBI).

Recovery of ESF Added to a Normal Human Serum by the HAI Assay
Twofold dilutions of the second IRPE were made in saline (0.9% w/v NaCl). Then, 0.25 ml of each solution containing, respectively, 75, 150, 300, and 600 mU of the second IRPE was added to 0.75-ml aliquots of a normal human serum containing 37.5 mU of ESF as determined by HAI kit II and also to 0.75 ml of saline. The ESF levels in these final solutions were determined using HAI kit II.

RESULTS
The results of measurement of ESF in sera from normal persons and anemic and uremic patients using two different HAI kits are shown in Fig. 1. Striking differences were found between the results of these kits, especially where the anemic sera were concerned. However, since the number of serum samples was small, no definite conclusions can be drawn for each group separately. ESF levels determined by kit I were 4–100-fold higher than those determined by kit II.

The results of a comparison between serum ESF levels determined by the FMLC bioassay and each of the two HAI kits are shown in Fig. 2. There was no correlation between biologic ESF levels and those obtained by kit I (r = −0.04) and kit II (r = + 0.11). With kit I, immunologic ESF levels were higher than...
biologic ESF levels in 18 of 19 sera. From these 19 sera, the 3 uremic sera showed the highest immunologic ESF levels, whereas ESF was not even detectable when using the FMLC bioassay. With kit II, immunologic ESF levels were lower than biologic ESF levels in

Fig. 2. Relation between serum ESF levels determined by the FMLC bioassay and HAI kit I (•) and HAI kit II (○), respectively.

13 of 19 sera. From these 19 sera, the 4 uremic sera showed higher immunologic than biologic ESF levels.

The results of measurement of ESF levels in sera from normal persons and uremic patients using the FMLC bioassay and HAI kit II are shown in Fig. 3. Using the FMLC bioassay, the mean ESF level was found to be 38 mU/ml in 38 normal human sera and 25 mU/ml in 14 uremic sera, as compared to 74 mU/ml in 16 normal human sera and 69 mU/ml in 12 uremic sera as determined by the HAI assay. Using this HAI kit, normal serum ESF levels ranged from 9.4 to 112.5 mU/ml. The normal range given by the manufacturer and reported by Jordan et al. was 15–59 mU/ml.

Absorption patterns at 280 nm, HAI titers, and stimulation of $^{59}$Fe incorporation into heme using the FMLC culture system of different column fractions of a normal human serum and a human urinary ESF preparation are shown in Figs. 4 and 5. Significant immunoreactivity was present in nearly all protein-containing fractions of both preparations, whereas biologic activity was present only in a few fractions of

Fig. 3. Determination of ESF levels in two groups of normal human sera and two groups of sera from anemic uremic patients using the FMLC bioassay (•) and HAI kit II (○). The uremic patients were on intermittent hemodialysis, and the two groups were quite comparable with respect to BUN levels and degree of anemia. Mean ESF levels were compared using Student's two-tailed t test.

Fig. 4. Results of fractionation of a normal human serum by gel filtration through a Sephadex G100 column. (--) Absorbance at 280 nm; (•) HAI titers obtained by kit I; (○) HAI titers obtained by kit II.
the urinary ESF preparation. No biologic activity was found in fractions of the normal human serum. Using HAI kit I, very high immunoreactivity was found in the urinary ESF fractions of low protein content eluted immediately after the void volume.

Results of experiments performed by adding known amounts of the second IRPE to a normal human serum and measuring the quantity recovered using HAI kit II are shown in Fig. 6 and revealed a very poor recovery of the added hormone.

**DISCUSSION**

Discrepancies between immunologic and biologic hormone determinations may be expected for the following reasons. First, immunoreactivity refers only to the presence of a more or less specific hormone antigen and does not necessarily imply the presence of the biologically active hormone. High levels of prohormones and/or hormone metabolites may be assumed to be responsible for some of these discrepancies. In this respect, the high immunoreactive ESF levels in
sera from anemic uremic patients\textsuperscript{1-4} deserve thorough evaluation and may provide new insights into the production and/or metabolism of ESF in the uremic patient.

Second, for many hormones, including ESF, no immunochemical standard is available. The number of immunochemical units is therefore related to preparations standardized to biologic units. The relative impurity of such preparations may lead to differences between the results of immunoassays and bioassays.\textsuperscript{11,12} The promising results obtained by Lange et al. for ESF determination in serum using an HAI technique\textsuperscript{13} have led to the development of an HAI assay kit for ESF produced by the J.C.L. Research Corporation.

In this study, we have presented evidence against the usefulness of this commercially available HAI kit for the determination of ESF in human sera. We found a great variability between the two kits tested. There was no correlation at all between the ESF levels in different pathologic sera determined by the FMLC bioassay and by each of the two HAI kits. ESF levels in normal and uremic sera determined by the HAI technique were significantly higher than those determined by the FMLC bioassay. Moreover, the normal range was found to exceed greatly the normal range given by the manufacturer.

These anomalous results may be explained by the fractionation experiments of normal human serum and human urinary ESF. Considerable immunoreactivity was found in nearly all protein-containing fractions of both preparations, whereas biologic activity was present only in a few fractions of the urinary ESF preparation. These findings demonstrate that the HAI kit is sensitive to a variety of contaminants. Furthermore, a very poor recovery of immunoreactivity was obtained when the international reference preparation for ESF (second IRPE) was added to a normal human serum. This suggests that important antigenic sites on the ESF molecule(s) may be hidden in serum. We conclude that the two HAI kits used measured mainly contaminants and hardly any ESF in human sera.

Finally, although immunoassays for ESF will undoubtedly have a great future, one has to be aware of the many problems that may be connected with such methods. In our opinion, each newly developed immunoassay for ESF must first be carefully compared with an existing biologic method.

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