Radioimmunooassay of Erythropoietin: Analytical Performance and Clinical Use in Hematology

Marie-Hélène Schlegeter, Marie-Elisabeth Toubert, Marie-Pierre Podgorniak, and Yves Najean

We report here the performance of a recently commercialized radioimmunooassay kit for determining erythropoietin (EPO) in serum or plasma. The lower detection limit of the method was 3 U/L. Precision, analyzed by the variation coefficients between different assay runs and in the same experiment, was always <10%; accuracy was assessed by recovery and dilution tests. In anemic patients (hematocrit 18–39%), the concentration of EPO was logarithmically related to hematocrit. A relatively large dispersion of the results was noted, as reported by others with various RIAs. Patients with severe renal failure demonstrated a very low EPO value, whatever the degree of their anemia. In some chronic anemias resulting from malignancy, EPO concentrations were also relatively low. In the polycythemia vera group, the EPO mean was below normal for >95% of the patients, whatever their clinical stage (first evaluation, relapse, or remission). In contrast, 91% of the patients with pure erythrocytosis had a normal or increased EPO value, even when the etiology was unknown. Measurement of EPO concentration may be useful for the clinical differentiation of myeloproliferative disorders and, subsequently, for their prognosis and choice of treatment.

Additional Keyphrases: production of standards by recombinant DNA • myeloproliferative disorders • polycythemia • erythrocytosis • anemia • renal failure

Erythropoietin (EPO) is the first hormone described as a hematopoietic growth factor, even though Carnot and Deflandre (Fr Acad Sci 1906;148:384) suggested, rather than demonstrated, the activity of the so-called "hematopoietin." EPO was rediscovered in the USA in the 1950s by Jacobsen et al. (1); its physiological significance has been objectively studied and its biochemical and genetic identification has been completed. Recently, EPO has been used for clinical trials in some types of severe anemia (2–4).

Determining EPO concentration by bioassays has always been difficult, long, and expensive: incorporation of $^{59}$Fe into polycythemic (post-transfusion, or exophytic) mice, in vitro incorporation of $^{59}$Fe in a suspension of liver cells from newborn mice, or in vitro growth of erythocyte colonies of normal or erythroblastic leukemia cell lines (5–8). The early radioimmunoassays (RIAs) developed were faulty, mainly because of incomplete purification of the antigen (9).

Recently, EPO produced in large amounts by cloning and expression of the human EPO gene in mammalian cells has allowed both the treatment of chronic anemias from renal failure and the development of immunological methods for measuring EPO. An RIA of EPO in plasma has been described in which highly purified recombinant EPO is used as tracer and standard (10). Such a method enables the large-scale study of EPO values in human diseases and evaluation of the clinical usefulness of this assay. We report here the use of a commercially available RIA kit to measure EPO in various hematologic disorders.

Materials and Methods

Methods

The RIA for EPO is based on the competition between EPO in serum (or plasma) and pure recombinant iodinated EPO, for a rabbit antibody raised against purified human urinary erythropoietin. We used the RIA kit developed by Incstar Corp. (Stillwater, MN) and marketed by Baxter France (B.P. 56 78311 Maurepas, France), according to the manufacturer's procedure. The standard in this kit is calibrated against the World Health Organization 2nd International Reference Procedure erythropoietin, HUM Urinary/Bioassay, 67/343. The EPO concentrations are expressed as units per liter (U/L).

Briefly, duplicate 200-μL samples of standard human recombinant EPO (10–260 U/L) or of unknown serum are preincubated with a rabbit-specific antibody (100 μL) for 2 h at room temperature. After adding $^{125}$I-labeled recombinant EPO and incubating the mixture for 16–24 h at 4 °C, we added the precipitating reagent (containing normal rabbit serum, goat anti-rabbit serum, and surfactant) and then, 30 min later, centrifuged the tubes and their contents. The amount of radioactivity in the pellet is counted for 1 min with a gamma-scintillation counter.

As previously shown (9), either serum or plasma samples can be processed, whatever the anticoagulant used; EPO can be determined within three months after collection, if samples are stored at −20 °C. Because only 1 mL of serum or plasma is required, studies in pediatric departments may be feasible.

Patients

We studied 51 normal subjects without anemia (hematocrit (Ht) 39–49%) and 77 patients with polycythemia vera (PV), diagnosed according to the Polycythemia Vera Study Group criteria (11): some (n = 51) at diagnosis or at relapse (Ht 51%) and some (n = 26) in remission after chemoradiotherapy (Ht 40–50%). In all cases we measured erythrocyte and plasma volumes at the time that we collected venous blood for determining EPO concentration.

Fifty-seven patients sent to the Nuclear Medicine De-
partment for blood volume measurement had Ht >51%, but did not meet any PV criteria. They are considered as exemplifying pure erythrocytosis (PE).

In addition, we studied 79 patients with chronic anemia, excluding those with malignant and renal diseases. For comparison with these 79 patients, we added 14 patients with a chronic renal deficiency, who were undergoing chronic hemodialysis. In an additional 27 cases, erythrocyte and plasma volumes were measured in patients with myeloma, macroglobulinemia, or lymphoma, to discriminate between plasma volume excess and erythrocyte volume deficit in the mechanism of the anemia; we measured the EPO titer on the same day.

Results

Analytical Performances

Sensitivity. We established the lower detection limit by determining the mean counts/min value minus 2SD observed upon repetitive testing of the zero standard. We found a value of 3 U/L, comparable with that reported for other RIAs.

Data reduction and precision of the assay. A typical dose–response curve is shown in Figure 1 (right). A spline curve-fitting method is used for data evaluation.

Figure 1 (left) depicts the precision profile of the standard curve. Within- and between-assay CVs are <10% for EPO values between 12 and 150 U/L and 12 and 100 U/L, respectively.

Table 1 shows that intra- and interassay CVs for EPO measurement in serum are ≤9% over the whole range of standardization (10–250 U/L). For this study, normal and pathological sera were pooled at different concentrations of EPO and then aliquoted and stored at −20 °C. The aliquots were measured over a three-month period. Congealing of sera at −20 °C does not affect the measured EPO concentration.

Linearity. We assessed linearity of the test by dilution and recovery tests. Three sera with different concentrations of EPO were diluted in zero standard; in addition, one serum was diluted in a serum with an undetectable value of EPO (Figure 2). Dilutions in zero standard give linear curves that go through the x- and y-axis intercept. For samples diluted in a serum with an undetectable EPO value, the linearity of the dilution curves was still acceptable.

Analytical recovery tests consisted of the addition of known amounts of recombinant EPO (from Amersham International, Amersham, Bucks., U.K.) or of various volumes of a serum with a high EPO concentration to a serum

with a known low EPO value. Under both conditions, the curves are linear with a slope near 1 and a y-axis intercept near 0 (Figure 3).

Clinical Results

Normal subjects. For 51 normal subjects (Ht 39–49%), the mean EPO value in serum was 13.5 U/L (range 10–20 U/L). The 95% confidence interval (i.e., mean ± 2SD) was 8.7–18.3 U/L. We did not find any difference related to age or sex.

Normal values of EPO measured by RIA and reported elsewhere are listed in Table 2.

Chronic anemias. In 79 patients with chronic anemia but without malignant or renal diseases, we observed a logarithmic relation between the plasma EPO values and the Ht value (Figure 4). As reported by others (5,10) the values were widely dispersed around the calculated line. The least squares analysis gave a logarithmic regression curve expressed by the equation:

<table>
<thead>
<tr>
<th>Table 1. Precision of EPO Measurements in Five Pooled Sera</th>
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<tbody>
<tr>
<td><strong>Intra-assay, n = 10 each</strong></td>
</tr>
<tr>
<td>EPO, U/L</td>
</tr>
<tr>
<td>12.4</td>
</tr>
<tr>
<td>12.6</td>
</tr>
<tr>
<td>68.7</td>
</tr>
<tr>
<td>72.7</td>
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<tr>
<td>24.5</td>
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</table>

Fig. 1. Precision profile of the standard curve and typical dose–response curve

Each calibration point was measured 10 times in the same assay run (intra-assay, ○) or on eight different days (interassay, •). CVs were calculated for B/B0 ratios and reported as a function of EPO value (left). In addition, a typical dose–response curve is represented (right).

Fig. 2. Dilution curves for four sera

Three sera were diluted in zero standard (○, □, and +); one serum was diluted in a serum with a low EPO value (<).

Fig. 3. Analytical recovery of EPO

(Left) Increasing volumes of a serum with high EPO value were added to two sera with a known EPO value (25 and 44 U/L), yielding superimposable curves (only one is represented here). (Right) Increasing, known amounts of pure recombinant EPO (from Amersham) were added to a serum with a low EPO content (25 U/L.)
Table 2. Summary of Reported Normal Values for EPO Measured by RIA

<table>
<thead>
<tr>
<th>References</th>
<th>Mean (SD) EPO, U/L</th>
</tr>
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<tbody>
<tr>
<td>12</td>
<td>14.9 (4.2)</td>
</tr>
<tr>
<td>13</td>
<td>29 (17)</td>
</tr>
<tr>
<td>14</td>
<td>13.3 (1.6)</td>
</tr>
<tr>
<td>9</td>
<td>15.2 (5.5) (male)</td>
</tr>
<tr>
<td></td>
<td>15.8 (4.6) (female)</td>
</tr>
<tr>
<td>15</td>
<td>16.5 (3.4)</td>
</tr>
<tr>
<td>16</td>
<td>14.9 (3.3)</td>
</tr>
<tr>
<td>17</td>
<td>25.6 (6.6)</td>
</tr>
<tr>
<td>18</td>
<td>25 (5)</td>
</tr>
</tbody>
</table>

![Table 2](image_url)

log EPO = \(-0.059 \times \text{Ht} + 3.45\)

where EPO is expressed in U/L and Ht in percentage. Calculation of the slope of the curve (\(-0.059\)) had a standard error of 0.007 (\(r = 0.69\)).

In the patients with severe renal failure, EPO was low or even very low (<6 U/L), whatever the degree of anemia (Figure 4). For about half of the patients with malignant diseases, EPO values were statistically lower than expected from the Ht values (not shown).

**PV patients.** We studied 51 cases with Ht between 51% and 70%. In this group, the mean ± SD EPO concentration was 7.2 ± 2.4 U/L, with a single case >12 U/L. No correlation was found between EPO concentration and either Ht excess or blood volume.

We investigated 26 PV patients in clinical remission (induced by \(^{32}\)P or hydroxyurea), with Ht between 40% and 50%. The mean ± SD EPO value was 8.9 ± 3.6 U/L. EPO concentrations were >12 U/L in only three cases.

No statistically significant difference was noted between EPO mean values in these two groups (PV in remission and PV in progression). In contrast, the difference between EPO mean values for PV patients in clinical remission and normal controls was statistically significant (\(P < 0.001\)), although Ht values were identical (Fisher’s test).

In both groups of PV patients, the EPO value never exceeded 17 U/L (Figure 5).

**Patients with PE.** Fifty-seven patients sent to the Nuclear Medicine Department were investigated for "so-called" PE, i.e., excess of erythrocyte volume with or without a well-defined etiology. The primary affliction (e.g., tumor, cardiopathy, respiratory disease) has been characterized in only 16 of 57 cases studied over the last six months.

As shown in Figure 5, of the results were widely dispersed, with only five cases exhibiting values <14 U/L and 33 of the 57 cases displaying values >17 U/L (the highest value observed in PV). Differences between the values of these erythrocytoses and those of the three other groups (normal patients, active PV, and PV in remission) were statistically significant.

**Discussion**

Because bioassays for EPO measurement are time-consuming and expensive, they have not been developed on a large scale for use in diagnosing various pathologies. Immunassays for EPO determination are much easier to perform and less expensive. The first assays reported were criticized mainly for two reasons (9). First, there was incomplete purification of the antigen. (Generally EPO for standards and tracer was purified from the urine of anemic patients.) Second, the mass of the immunoreactive protein might reflect imperfectly its biological efficiency. However, further data demonstrated a good correlation between bioassays and immunassays. For immunassays, a pure recombinant EPO is currently used as standard and tracer, as was done in our method.

The physiological range can be defined in patients with Ht between 39% and 49%: 10–20 U/L (range) or 8.7–18.3 U/L (mean ± 2SD), comparable with values found with other RIAs (Table 2).

The method has a good sensitivity; it can detect an EPO concentration of 3 U/L in serum, which is less than the EPO concentration in normal serum. This method is also accurate, with recovery and dilution tests exhibiting a good correlation between the calculated and the observed EPO concentrations. Furthermore, CVs were always <10%.

For both methods (bioassay and radioimmunoassay), a logarithmic relationship was found between the EPO titer and Ht, or hemoglobin concentration (5, 9, 10, 12–20), with a large scatter around the regression line. Our results also exhibit logarithmic regression, with a slope similar to the data published elsewhere (6, 9, 10, 20).

Some anemias, especially in renal deficiency, may be related to a decreased production of EPO. As expected, in
14 cases of chronic renal failure, whatever the severity of anemia, a low EPO value was observed. What is the clinical benefit of EPO determination in renal failure anemia? Could the concentrations of EPO be used to distinguish between anemias caused by a defect in EPO production and other mechanisms (21) and therefore to help forecast the clinical effectiveness of EPO therapy? Only a large-scale study, possible with a simple and fast RIA like the one described here, can answer these questions.

A possible defect in EPO production has been suggested as one of the main causes of anemia in malignant diseases (3, 10), in chronic rheumatic disorders (22, 23), and in patients infected with human immunodeficiency virus (24). Few cases have been investigated, with discrepant results. Thus, the clinical applications of EPO measurement in these situations are still undefined.

In some cases, abnormal delivery of oxygen to the tissues, caused by a shift in the oxygen equilibrium curve, may impair EPO secretion (20). In anemic patients, renal blood flow is affected by increased viscosity (25) or by acquired renal defect from hematological diseases (20); these might also account for inappropriate EPO secretion.

EPO concentrations have primarily been determined in polycythemic states, in which the role of this hormone has been extensively discussed. At present, a pathophysiological discrimination is possible between PV and PE. PV is a multi-lineage myeloproliferative disorder (11) with an abnormal clonal hypersensitivity to EPO (26). PE is considered a secondary disease involving hyperproduction of EPO related either to chronic hypoxia or to secreting tumors. According to this classification, other investigators have demonstrated low concentrations of EPO in PV and high concentrations in PE (4, 12, 19).

Nevertheless, PE is not a homogeneous syndrome, except in well-characterized "secondary" cases. Many of these cases follow a chronic evolution, with further appearance of one or another symptom similar to those observed in PV. In some patients with PE, the kinetics of the stem cells exhibit the same EPO hypersensitivity as is observed in PV (27–29). In PE patients, EPO concentrations generally are much higher than in the normal group, but sometimes lower (or subnormal), suggesting a "primary" erythrocyte production disorder. From these data the EPO concentrations should be determined by RIA in every new case of PE. The growth kinetics of stem cells should be investigated in cases with no hypoxia or tumor, but presenting normal or low EPO values.

None of the identified PV cases (i.e., the patients with classical criteria of this disease) exhibited EPO values >17 U/L, even when a slight anemia occurred secondary to their treatment. This contrasts with a previous suggestion that EPO values increase after therapy (5). Koeffler and Goldwasser (12) suggested a cutoff value of 30 U/L to discriminate between primary and secondary erythrocytosis. From our present data, a limit of 14 U/L includes 96% of the PV cases (100% of the cases with Ht >50%) and excludes 86% of the PE cases.

Figure 6 depicts a receiver–operating characteristic curve with various decision points. More cases of myeloproliferative disorders are currently being investigated (30).

Finally, rare congenital and (or) familial syndromes of PE have been defined as abnormal EPO secretion and (or) abnormal sensitivity of the erythroid progenitors (31). RIAs for EPO will certainly be of interest in the investigation of erythrocytosis and erythroblastopenias in young patients.

In conclusion, the measurement of the EPO concentration in serum is useful in the biological discrimination between polycythemia vera and erythrocytosis. A large-scale study of EPO concentration is now possible with radioimmunoassays, to assess its clinical usefulness in various pathologies.

References