In Vitro Assay for Erythropoietin: Erythroid Colony Formation in Methyl Cellulose Used for the Measurement of Erythropoietin in Plasma

By Per Hågå and Bjarne Falkanger

Erythroid colony formation in methyl cellulose has been used for the measurement of erythropoietin in plasma. Livers from newborn mice less than 24 hr old were found to provide convenient target cells. Newborn mouse liver contains a substantial number of erythroid colony-forming cells (CFU-e) that have a high sensitivity to erythropoietin. The dose-response curve for erythropoietin reaching a plateau at 50 mU/ml. As little as 0.5 mU/ml of the hormone is detectable. Removal of cells that adhered to glass prior to culturing doubled the number of colonies formed in the presence of erythropoietin. Addition of untreated plasmas that showed high erythropoietin titers in the exhypoxic polycythemic mice assay gave variable results. Some of the plasmas stimulated colony formation actively and in a linear fashion. However, the majority of the plasmas were toxic to the cultures. Dialyzing the plasmas for 3 days against distilled water effectively removed the toxicity. Results obtained with the method are in good agreement with the values found using the exhypoxic polycythemic mice assay.

Currently the most widely used assay for erythropoietin employs polycythemic mice as the assay animals, and the erythropoietic stimulatory effect of the test material is measured as radioiron incorporation into red blood cells. However, the method suffers from several shortcomings. The biologic variation among the animals gives the assay a fairly low degree of accuracy, and its sensitivity is low. However, the most serious limitation is the large volume of test material required.

When grown in a semisolid medium in vitro, erythroid precursor cells (CFU-e) divide to form colonies. This colony formation is erythropoietin-dependent, and partially purified preparations of the hormone give a linear dose-response curve. The system thus seems suitable for measuring the erythropoietin levels of tissue fluids, requiring small amounts of the test material.

The aims of the present study were to standardize the growth of CFU-e from newborn mouse liver in methyl cellulose and determine if the system is applicable for measurement of erythropoietin concentrations in plasma, as well as to determine if any pretreatment of the plasmas is necessary and, if so, what procedures are effective and practical.

MATERIALS AND METHODS

A modification of the method described by Iscove et al. was employed. Livers from newborn mice (less than 24 hr old) were removed aseptically into alpha medium (Flow Laboratories) containing 20% fetal calf serum (GIBCO). Mice of the strain WLO were used. The liver was disrupted with a glass rod.
and a single-cell suspension was obtained by gentle aspiration through a Pasteur pipette. The suspension was then filtered twice through an acid-fast metal mesh (pore size 87 μm) to retain particles. After centrifugation (5 min at 1000 rpm), the cells were washed in the medium, centrifuged, and resuspended in alpha medium. The cells were plated in 35-mm plastic petri dishes (C.A. Greiner, West Germany) containing 0.8% methyl cellulose (Dow Methocel purum, 4000 cps) and fetal calf serum. α-Thioglycerol was used at a concentration of 10^{-4} M, where indicated in the legends. Sheep plasma erythropoietin step-III (Connaught Medical Research Laboratories, Canada) dissolved in alpha medium was used as the erythropoietin standard. Alpha medium and plasma/serum to be tested were added to give a final volume of 1 ml. All amounts are expressed as final concentrations. The fetal calf serum was inactivated at 56°C for 30 min before use. The alpha medium (containing nucleosides) and the methyl cellulose were prepared according to Iscove. 

Removal of adherent cells was done as described by Messner et al. Nucleated cells (2–3 × 10^6) in 10 ml of alpha medium containing 20% fetal calf serum were placed in a 100-ml glass petri dish and incubated for 30 min at 37°C in a humidified atmosphere containing 5% CO₂. The supernatant was removed after gentle swirling and then incubated for another 2.5 hr in a new petri dish under the same conditions. The nonadherent cells in the supernatant were removed, centrifuged, resuspended in alpha medium, and then plated.

The plasmas and sera with high erythropoietin titers in the ex hypoxic polycythemic mice assay were from rabbits exposed to 6 hr of hypoxia (0.5 atm) immediately prior to venipuncture and from patients with different anemic conditions. The normal adults were hospital personnel. The plasmas from newborn mice less than 24 hr old were obtained by collecting blood into microhematocrit tubes after decapitating the animals. All plasmas were heparinized with 20–30 lU/ml blood. Dialysis of the plasmas was done according to Chan et al. Samples were dialyzed in dialysis tubing (inflated width 0.25 inch, A. H. Thomas Co., Philadelphia, Pa.) against distilled water at 4°C. The protein precipitate formed was removed by centrifugation.

The cultures were incubated in a humidified atmosphere containing 5% CO₂ at 37°C for 3 days. The numbers of colonies formed were counted in one-fourth of the area of the plate using an inverted microscope. Before counting, the cultures were flooded with a benzidine chloride solution. Benzidine-positive colonies containing eight or more cells were scored. The plates were scored at random, without the examiner knowing the contents of the individual plates. All cultures were done in duplicate or triplicate. Counting one-eighth of the area of each plate was found to give results equivalent to counting one-fourth of the area and was used in the latter part of the study where indicated in the figure legends.

The maximal numbers of colonies formed varied from time to time, probably because of differences in the fetal calf serum and the other constituents, as well as differences in the CFU-e content of the individual livers. For this reason, when comparing later results to Fig. 1, the data are normalized. A correction factor is obtained by dividing the colony number produced in response to erythropoietin (50 mU/ml) of the standard curve by that in response to 50 mU/ml on the particular day. The values found in that experiment are then multiplied by this factor. In the tables, colony counts over 1000 are given with 3 and 2 significant figures in the mean and error values, respectively.

The ex hypoxic polycythemic mice assay used in our laboratory has previously been described by Lindemann.

RESULTS

Removal of Adherent Cells From Cell Suspension

Generally between 40% and 60% of the incubated cells were found to be nonadherent after the incubation. The procedure increased the plating efficiency of the cell suspension, as shown in Table 1, regularly doubling the number of colonies obtained in the presence of erythropoietin, whereas unstimulated cultures did not show any increase. The twofold increase in the CFU-e number of the suspension after adherence is consistent with the recovery of about 50% of the cells as nonadherent. The increased yield obtained by adherence probably merely reflects an increased concentration of the CFU-e in the suspension plated, by removal of
nonerythroid cells. The procedure increased the sensitivity of the system and consequently was performed throughout the rest of the study.

**Number of Colonies Formed Versus Incubation Time, Cell Number, and Erythropoietin Concentration**

The number of colonies formed increased up to 2–3 days of incubation, whereafter it decreased. The colony count was usually higher after 3 days than after 2 days. Moreover, after 3 days the colonies appeared more fully hemoglobinized. In the cultures where no erythropoietin was added, it was thus easier to differentiate between erythroid colonies and benzidine-negative clusters/colonies. For these reasons incubation periods of 3 days were used.

**Table 1. Number of Colonies Formed From the Same Cell Suspension Before and After Removal of Adherent Cells**

<table>
<thead>
<tr>
<th>Number of Cells Plated</th>
<th>Erythropoietin Concentration</th>
<th>Colonies/Plate Before Adherence</th>
<th>Colonies/Plate After Adherence</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 × 10⁴</td>
<td>0 mU/ml</td>
<td>9 ± 9</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>5 × 10⁴</td>
<td>200 mU/ml</td>
<td>1460 ± 120</td>
<td>3270 ± 180</td>
</tr>
<tr>
<td>2 × 10⁵</td>
<td>200 mU/ml</td>
<td>1270 ± 130</td>
<td>2350 ± 100</td>
</tr>
<tr>
<td></td>
<td>0 mU/ml</td>
<td>8 ± 4</td>
<td>13 ± 3</td>
</tr>
</tbody>
</table>

Results are means ± SEM of triplicate plates in three different experiments.

**Fig. 1.** Numbers of colonies formed versus doses of erythropoietin, expressed as means ± SEM. Closed circles: with added thiol (10⁻⁴ M); open circles: without thiol. Numbers of plates per point in parentheses; 2 × 10⁴ nucleated cells plated; pooled data from six different experiments with thiol and three experiments without thiol.
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Table 2. Minimum Concentration of Step-III Erythropoietin Measured by the Assay

<table>
<thead>
<tr>
<th>Erythropoietin Concentration</th>
<th>Colonies/2 × 10^5 Nucleated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mU/ml</td>
<td>16 ± 8</td>
</tr>
<tr>
<td>0.5 mU/ml</td>
<td>61 ± 10*</td>
</tr>
<tr>
<td>0 mU/ml</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>0.5 mU/ml</td>
<td>94 ± 22*</td>
</tr>
</tbody>
</table>

Means ± SEM of triplicate plates in two different experiments.
*Significantly higher (p < 0.025) than the control.

A linear relationship between colony count and number of cells plated was found in the range of 0.5–8 × 10^5 nucleated cells/ml. A large number of colonies formed permits detection of low concentrations of erythropoietin (Fig. 1). However, too many colonies in the plates makes accurate counting difficult. A cell concentration of 2 × 10^5/ml was found to be a suitable compromise between these two considerations.

The dose–response curve for Connaught step-III erythropoietin is shown in Fig. 1. The response is linear (semilog) up to a dose of 50 mU/ml, when a plateau is reached. In accordance with other reports, the addition of α-thioglycerol to the cultures to a final concentration of 10^{-4} M approximately doubles the number of colonies formed. It was added to the cultures throughout the rest of the study. In 20 experiments the maximal stimulation (colony production by erythropoietin at 50 mU/ml) was 3710 ± 230 colonies per 2 × 10^5 nucleated cells (mean ± S.E.M.), whereas the colony count in response to 12.5 mU/ml was 50.0 ± 3.8% (mean ± S.E.M.) of that produced by 50 mU/ml.

Using triplicates, step-III erythropoietin as low as 0.5 mU/ml is detectable with statistical significance (Student’s t-test) (Table 2).

Addition of Plasma to Cultures

Three ways of adding the plasmas to be tested were examined. The volume of plasma replaced either alpha medium or fetal calf serum, or it was simply added so that all constituents were diluted. As can be seen from Table 3, no differences in colony counts were found. Substitution of the alpha medium was subsequently used.

The use of plasma or serum did not influence the assay.

Some of the plasmas with known high titers of erythropoietin in the exhypoxic polycythemic mice assay stimulated erythroid colony formation very actively, with

Table 3. Number of Colonies Formed in Response to Added Plasma: Comparison of Different Ways of Adding Plasma

<table>
<thead>
<tr>
<th>Experiment Number</th>
<th>Plasma No. and Volume Added</th>
<th>Colonies/2 × 10^5 Nucleated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Plasma Replaces Alpha Medium</td>
</tr>
<tr>
<td>1</td>
<td>5 (50 μl)</td>
<td>1680 ± 150</td>
</tr>
<tr>
<td>2</td>
<td>6 (100 μl)</td>
<td>1720</td>
</tr>
<tr>
<td>3</td>
<td>7 (100 μl)</td>
<td>2810</td>
</tr>
<tr>
<td></td>
<td>7 (200 μl)</td>
<td>1030</td>
</tr>
</tbody>
</table>

Values from plasma No. 5 are means ± SEM of triplicates; the other results are means of duplicates; 2 × 10^5 nucleated cells plated; thiol (10^{-4} M) added to the cultures.
linear (semilog) dose–response curves even up to plasma concentrations of 15%–
20% in the cultures.

However, most plasmas were clearly toxic to the cultures. When they were
added, no colonies or very few colonies were formed, and the cultures were usually
practically devoid of benzidine-positive single cells at the end of the incubation
period. The occurrence of this toxicity was not related to the use of serum or
plasma, nor was it affected by using heparin with or without preservative. It was
found in plasma both from rabbits and from man and was not related to specific
anemias. Approximately two-thirds of the plasmas studied were found to be toxic.

Inhibitory substances in serum interfere with the assay for granulocyte colony-
stimulating activity (G-CSA). Different procedures have been reported to remove
the inhibition, among them heating the sera to 55°C for 30 min, chloroform
extraction, and dialysis against distilled water for 3 days.7,13

Application of the same procedures to the plasmas found to be toxic in the
erythroid cultures was studied. After preliminary experiments, heat treatment and
chloroform extraction were found unsatisfactory. Both methods were effective in
some instances, but they failed to remove the toxicity in other plasmas. In addition,
the amounts of plasma recovered from the chloroform treatment varied greatly,
and a substantial part of the plasma was regularly lost.

Dialysis of the plasmas for 3 days, on the other hand, was found to remove the
toxicity consistently (Fig. 2). After dialysis, the toxic plasmas stimulated colony
formation as actively as nontoxic plasmas from similar conditions, whereas plasmas
that were active when untreated retained their stimulating ability. Moreover,
dose–response curves of dialyzed plasmas show acceptable parallelism with the
standard curve of erythropoietin (Fig. 3).

To further test the efficacy of dialysis in removing toxicity, a toxic plasma was
dialyzed in several aliquots, whereafter erythropoietin was added to the aliquots in
different concentrations. The stimulating effects of the erythropoietin with and

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**Fig. 2.** Numbers of colonies formed in response to added plasma. Plasmas
tested before and after dialysis for 3 days (crossed bars) in the same experiment;
filled bars: toxic plasmas; open bars: nontoxic plasmas; plasma concentrations
100 µl/ml, except for the plasma from the patient with Diamond-Blackfan ane-
mia, which is 2.5 µl/ml. Results are means of duplicate plates; 2 × 10⁵
nucleated cells plated; thiol (10⁻⁴ M) added to the cultures.
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Fig. 3. Dose–response curves for plasmas dialyzed for 3 days normalized to the standard curve of step-III erythropoietin (solid line). Results are means of duplicate plates. Dotted line: plasmas toxic before dialysis; dash line: nontoxic plasma before dialysis; 2 × 10⁶ nucleated cells plated; thiol (10⁻⁴ M) added to the cultures.

without plasma were then examined. The results are shown in Fig. 4, where it can be seen that good parallelism in the dose–response curves was achieved.

The length of the dialysis period needed for removal of toxicity was studied using four toxic plasmas and testing them untreated and after 1, 2, 3, and 3.5 days of dialysis. The stimulatory activities of all the four plasmas rose with increasing dialysis time up to 3 days, whereas at 3.5 days the activities of two plasmas decreased, and a further increase was noted in one plasma.

In Table 4 the erythropoietin concentrations of eight plasmas measured by this
Table 4. Erythropoietin Concentrations of 8 Plasmas Measured by the Method Compared with the Values Found by the Exhypoxic Polycythemic Mice Assay

<table>
<thead>
<tr>
<th>Plasma</th>
<th>In Vitro (Concentration U/ml)</th>
<th>In Vivo (Concentration U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxia, rabbit</td>
<td>0.15</td>
<td>0.16</td>
</tr>
<tr>
<td>Hypoxia, rabbit</td>
<td>0.56</td>
<td>0.39</td>
</tr>
<tr>
<td>Hypoxia, rabbit</td>
<td>0.07</td>
<td>0.10</td>
</tr>
<tr>
<td>Hypoxia, rabbit</td>
<td>0.16</td>
<td>0.20</td>
</tr>
<tr>
<td>Diamond-Blackfan anemia</td>
<td>6.7</td>
<td>6.0</td>
</tr>
<tr>
<td>Diamond-Blackfan anemia</td>
<td>6.2</td>
<td>4.8</td>
</tr>
<tr>
<td>Diamond-Blackfan anemia</td>
<td>0.8</td>
<td>0.40</td>
</tr>
<tr>
<td>Pure red cell aplasia</td>
<td>0.72</td>
<td>0.94</td>
</tr>
</tbody>
</table>

In vitro values obtained by normalizing colony count to the standard curve (Fig. 1).

method are compared with the values obtained employing the exhypoxic polycythemic mice assay. As can be seen, there is good agreement between the values obtained by the two methods ($p < 0.001$ by Kendall's test for correlation).

Table 5 shows the ability of dialyzed plasmas from 10 normal adults to stimulate CFU-e growth. Eight of the plasmas showed significantly higher colony counts than the control cultures ($p < 0.01$ by Student's $t$ test).

Plasma erythropoietin levels of newborn mice are shown in Table 6. The normal level is significantly higher than the control, and significant increases occur when the mice are exposed to hypoxia.

DISCUSSION

The drawbacks of the in vivo bioassay for erythropoietin restrict future progress in the field. In line with the advances in the measurements of other hormones, the logical solution is a radioimmunochemical method. However, the published works on this have not resulted in a practical clinical method. Moreover, the specificities of the assays described are far from established. The major increase in purity of erythropoietin recently achieved may overcome these problems.

Table 5. Erythropoiesis-Stimulating Factor in Dialyzed Plasma from 10 Normal Adults

<table>
<thead>
<tr>
<th>Colonies/2 $\times$ $10^3$ Nucleated Cells</th>
<th>Expressed As Means ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Epo. →)</td>
<td>(5) 74 ± 14</td>
</tr>
<tr>
<td>50 mU/ml of Epo.</td>
<td>(4) 3750 ± 120</td>
</tr>
<tr>
<td>Normal plasma</td>
<td>(3) 88 ± 12*</td>
</tr>
<tr>
<td>Normal plasma</td>
<td>(3) 125 ± 23*</td>
</tr>
<tr>
<td>Normal plasma</td>
<td>(3) 155 ± 11</td>
</tr>
<tr>
<td>Normal plasma</td>
<td>(3) 163 ± 19</td>
</tr>
<tr>
<td>Normal plasma</td>
<td>(2) 192 (187–198)</td>
</tr>
<tr>
<td>Normal plasma</td>
<td>(3) 245 ± 30</td>
</tr>
<tr>
<td>Normal plasma</td>
<td>(3) 325 ± 67</td>
</tr>
<tr>
<td>Normal plasma</td>
<td>(3) 405 ± 62</td>
</tr>
<tr>
<td>Normal plasma</td>
<td>(3) 525 ± 35</td>
</tr>
<tr>
<td>Normal plasma</td>
<td>(3) 630 ± 160</td>
</tr>
</tbody>
</table>

Numbers of plates per value in parentheses; plasma concentrations 100 μU/ml; 2 $\times$ $10^8$ nucleated cells plated; thiol (10$^{-5}$ M) added to the cultures; one-eighth of each plate counted.

*Not significantly different from the control. The other plasmas all have significantly higher values than the control ($p < 0.01$).
Table 6. Erythropoiesis-Stimulating Activity in Dialyzed Plasma From Newborn Mice Less Than 24 hr Old Expressed As Means ± SEM

<table>
<thead>
<tr>
<th>Condition</th>
<th>Colonies/2 × 10^5 Nucleated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Epo. -)</td>
<td>99 ± 14</td>
</tr>
<tr>
<td>50 mU/ml Epo.</td>
<td>3280 ± 220</td>
</tr>
<tr>
<td>Plasma, newborn mice; hypoxia -</td>
<td>234 ± 14*</td>
</tr>
<tr>
<td>Plasma, newborn mice; hypoxia +</td>
<td>980 ± 130†</td>
</tr>
</tbody>
</table>

Numbers of plates per value in parentheses; plasma concentrations 50 mU/ml; 2 × 10^5 nucleated cells plated; thiol (10^-4 M) added to the cultures.

*Significantly higher than the control (p < 0.001).
†Significantly higher than the preceding value (p < 0.005).

Several reports have shown that short-term suspension cultures of bone marrow or fetal liver cells in vitro can be used for quantitative estimation of erythropoietin, measuring total radioiron incorporation or incorporation into heme. However, so far they have not gained any widespread use.

Hemopoietic cells from fetal liver (13th or 14th day of gestation) or adult bone marrow have usually been employed to obtain murine colonial growth. This report shows that livers of newborn mice less than 24 hr old contain large numbers of CFU-e with high sensitivity to erythropoietin. Newborn livers are more practical to use than fetal livers, as they are easier to obtain, they are of larger size, and the age is simpler to estimate. It is also easier, in our hands, to achieve growth from newborn liver than from bone marrow.

In the mouse the change from hepatic to myeloid erythropoiesis starts shortly before birth and is completed at about the end of the first week of life. On the basis of this, the finding of substantial numbers of erythroid colony-forming cells in the newborn liver is not surprising. However, it is apparently in discordance with the work of Cole et al., who, using plasma clot cultures, were unable to induce erythropoietin-mediated colony formation from fetal livers after the 16th day of gestation.

Removal of the adherent cells prior to culture has been recommended to reduce the number of granulocytic colonies/clusters formed when cells from rabbits or man are used for erythroid growth. That the procedure applied to cells from mouse liver would double the number of colonies formed in response to erythropoietin was a surprising finding, but it may be explained as a concentrating effect on the erythroid cell population plated.

Our observation that most plasmas are toxic when tested untreated has important implications for the culture system in general. It follows that the specificity of negative effects or even zero effects observed when plasma/serum is added may be questionable and that “normal” plasma/serum run as a control does not disprove unspecific toxicity. The specificity of effects observed when, for instance, antiserum to erythropoietin is added to the cultures may thus be questionable.

Toxic substances in plasma/serum have also interfered with suspension cultures. These have been adapted for measuring their erythropoietin concentrations. There is, furthermore, a common experience that the growth-promoting effect of fetal calf serum varies greatly from batch to batch, some batches being practically without any stimulating ability. Whether this is due to inhibitory/toxic substances or lack of stimulatory factors is not established.


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The system is presently not sensitive enough to detect erythropoietin in all normal plasmas, as 2 of 10 plasmas did not stimulate colony growth significantly more than that in control cultures (Table 5). The findings of Guilbert and Iscove\textsuperscript{24} that the concentration of fetal calf serum can be reduced to 4\% when transferrin, bovine serum albumin, and selenium are added may make it possible to increase the amount of plasma added and thus measure lower plasma erythropoietin levels.

That the assay measures erythropoietin is borne out by three findings. The stimulating activities of the different plasmas tested varied according to the erythropoietin concentrations found by other methods in these conditions. Dose–response curves of plasmas run parallel to the dose–response curve of the erythropoietin preparation (Fig. 3). Finally, the measured erythropoietin concentrations of plasmas using the method are in good agreement with the values obtained using the exhypoxic polycythemic mice assay (Table 4).

The necessity of pretreating the plasmas before testing reduces the practicality of the method somewhat. However, the dialysis is an uncomplicated procedure requiring little work. The main advantage of the method described compared with the exhypoxic polycythemic mice assay is the reduction in required volume of the test material. At most, 400–500 µl of plasma are needed, and this is less than 10\% of that demanded by the in vivo method. This advantage is illustrated in Table 6, where plasma erythropoietin levels in newborn mice have been measured.

ACKNOWLEDGMENT

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In vitro assay for erythropoietin: erythroid colony formation in methyl cellulose used for the measurement of erythropoietin in plasma

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