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Reticulocyte measurements as a bioassay for erythropoietin

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Abstract: The possibility of using reticulocyte counts in peripheral blood to assay erythropoietic activity of recombinant human erythropoietin (r-HuEPO) was evaluated in normal mice. Mice were injected subcutaneously with r-HuEPO on days 0, 1 and 2 and bled on day 4 for reticulocyte count determinations, using an automated counting system with thiazole orange fluorescent staining and flow cytometric analysis. Reticulocyte counts increased in a dose-dependent fashion upon administration of r-HuEPO. The reticulocyte count was unaffected by asialylated EPO as well as other substances tested (interleukin-1, interleukin-3, dexamethasone, human chorionic gonadotropin). These data demonstrate the usefulness of employing reticulocyte counts as a rapid, specific and reproducible assay for *in vivo* erythropoietic activity of r-HuEPO.

Keywords: *Reticulocyte; erythropoietin; flow cytometry; bioassay.*

Introduction

Erythropoietin (EPO) is a glycoprotein hormone secreted primarily by the kidney and is the primary endogenous regulator of red blood cell formation [1]. Reduction of EPO production due to the loss of viable kidney tissue in cases of renal failure as well as the anaemia from other causes (arthritis, AIDS, cancer) has led to the use of EPO for treating these and other conditions [2–5]. With the recent production of EPO by recombinant technology [6], the *in vivo* bioassay of recombinant human EPO (r-HuEPO) has become a necessity for evalution of potency for production batches. *In vitro* assays of erythropoietin are capable of demonstrating erythropoietic activity, however, EPO activity in these assays is not dependent on glycosylation of the molecule. In the absence of proper glycosylation, *in vivo* activity of r-HuEPO is limited due to rapid uptake of the hormone by the liver [7].

The method most widely used for the bioassay of erythropoietin consists of the induction of polycythemia in mice by hypobaric exposure followed by the measurement of radioactive iron incorporation into newly formed red blood cells subsequent to EPO administration [8]. This assay takes three weeks to obtain results, involves the use of radioactivity and requires controlled hypobaric chambers for quality assurance. All of these issues make it difficult to use this assay for batch testing of commercially prepared erythropoietin products.

The appearance of reticulocytes in the peripheral circulation has long been identified as an early marker for erythropoietic activity [9, 10]. With the advent of newer counting techniques, the use of reticulocyte measurements may prove to be a useful alternative for the rapid and reproducible *in vivo* bioassay of erythropoietin. The current studies were undertaken to develop a bioassay for r-HuEPO which could provide a quick and reproducible method to evaluate the *in vivo* biological activity of commercially prepared product, as well as to provide a method for further research into the erythropoietic activity of various growth factors.

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Methods

Animals

Female BDF1 mice (Charles River) weighing 18–20 g were used for these studies. All procedures used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the R.W. Johnson Pharmaceutical Research Institute (PRI).

Reagents

EPO was supplied by Ortho Biotech Inc. as the recombinant human hormone, derived from Chinese Hamster Ovary (CHO) cell expression, at a concentration of 2000 units ml^{-1}. Vehicle consisted of Dulbecco's phosphate-buffered saline (D-PBS; Gibco, Grand Island, NY) with 10% polyethylene glycol (PEG 8000; Fisher Scientific, Fair Lawn, NJ) and 0.25% human serum albumin (PBS-HSA; Armour Pharmaceutical Corp., Kankakee, IL). Asialylated r-HuEPO was prepared enzymatically by treatment with agarose-bound neuraminidase according to the method of Spivak and Hogans [11]. Briefly, the agarose-bound neuraminidase was washed and then incubated with EPO in a conical tube for 30 min in a shaking water bath at 37°C. The suspension was centrifuged and the supernatant, containing the asialylated EPO, was removed. The agarose was washed with D-PBS three times. The supernatant and washes were pooled, passed through a fritted disk to clear any remaining agarose from the solution and then passed through a sterile filter to avoid contamination. Immunoreactivity of asialylated r-HuEPO was verified with the RIA procedure previously reported [12] and confirmed to have reactivity within the limits of non-treated r-HuEPO.

EPO dosage regimen determinations

Initial studies consisted of investigating the duration of EPO treatment necessary to increase reticulocyte numbers sufficiently for a quick and reliable assay. Each group was comprised of 10 mice which were dosed subcutaneously (s.c.) with 20 units $mouse^{-1}$ (approximately 1000 units kg^{-1} day^{-1}) on day −3 (Schedule A); days −3 and 0 (Schedule B); days −3, 0 and 1 (Schedule C); day 0 only (Schedule D) or days 0 and 1 (Schedule E). Beginning on day 0, prior to dosing, two mice day^{-1} from each group were anaesthetized with CO_2 inhalation and were bled by cardiac puncture into EDTA coated syringes. The percentage of reticulocytes in peripheral blood was determined by thiazole orange staining and fluorescent analysis on a Spectrum III cytofluorograph (Ortho Diagnostics Instruments, Cambridge, MA) equipped with special software (described below).

Dose–response effect of r-HuEPO on reticulocyte count

Experiments were conducted to determine the dose–response relationship of r-HuEPO on reticulocyte count at the chosen dosage regimen. Groups of 10 mice were dosed subcutaneously (s.c.) with r-HuEPO (0.04, 0.1, 0.2, 0.4, 4, 40 or 400 units $mouse^{-1}$ total dose), vehicle, or asialylated EPO (0.4, 4, or 40 units $mouse^{-1}$ total dose). The total dose was equally divided over 2 days dosing (day 0 and day 1) with bleeding by cardiac puncture under CO_2 anaesthesia on day 4.

Optimization of the dose–response curve was achieved by equally dividing the same total dose over 3 days (days 0, 1 and 2). In addition, 10 mg $mouse^{-1}$ iron dextran (Sigma, St Louis, MO) was administered intraperitoneally (i.p.) on day 2 to prevent the possibility of iron deficiency as a rate limiting step in the production of red blood cells. Haematocrits were determined for each mouse at the time of bleeding and the reticulocyte count was corrected for any deviation in the haematocrit as follows:

$$\%\ \text{retic}_{(\text{corrected})} = \%\ \text{retic}_{(\text{observed})} \times \text{Hct}_{(\text{indiv.})}/\text{normal Hct.} \quad (1)$$

Manual reticulocyte counts

Equal volumes of blood and New Methylene Blue solution (Anderson Laboratories, Fort Worth, TX) were mixed in a test tube and allowed to incubate at room temperature for 15 min. Wedge smears were prepared on microscope slides, allowed to dry and stored in the dark. Reticulocytes were counted by microscopy under 1000× magnification with the aid of a Miller disc (Klarmann Rulings, Manchester, NH) inserted in the eyepiece. Etched on the disk are a large square and a small square, 1/9 the area of the larger. Reticulocytes were counted in the large square and a minimum of 333 red blood cells were counted in the

smaller square. Percentage reticulocyte values were calculated using the following formula:

$$\frac{(\text{Reticulocytes})(100)}{(\text{Red blood cells})(9)} = \%\ \text{Reticulocytes}. \quad (2)$$

Polycythemic mouse bioassay

To compare the reticulocyte assay to the exhypoxic polycythemic mouse assay, mice were made polycythemic by 2 weeks exposure in a hypobaric chamber (Thermodynamics, Camarrillo, CA) at 304 mmHg. Following 2 weeks in the chamber (days 1–15), mice were returned to normal atmospheric conditions for 2 days (days 16–17) and then injected with 1 dose of EPO (s.c.) on day 18. On day 20, ^{59}Fe was injected i.p. and the mice were bled by cardiac puncture 48 h later. Blood samples were analysed for total radioactivity with a Packard gamma counter and net percentage iron incorporation was determined by subtracting the background counts in treated mice. Haematocrits were also determined on each blood sample to exclude those mice with haematocrits below 58%. Groups of 10 control mice and 15 mice at each dose level of EPO (0.05, 0.15 and 0.45 units mouse^{-1} total dose) were used to construct a dose–response curve.

Automated reticulocyte count

Five microlitres of blood were added to 1 ml Retic-COUNT thiazole orange solution (Becton Dickinson Immunocytometry Systems, San Jose, CA) and mixed. Several unstained controls were also prepared in PBS to determine background fluorescence. Thiazole orange treated samples were allowed to incubate for 30 min at room temperature in the dark (60- or 120-min incubations showed no difference in the reticulocyte counts). Measurements were made in an Ortho Spectrum III argon laser flow cytometer using a modification of the Spectrum III experimental software package (Becton Dickinson, San Jose, CA) or in a Becton–Dickinson FACScan flow cytometer (San Jose, CA) utilizing the Retic-COUNT software package. Software modifications on the Spectrum III consisted of adjusting the gains to allow differentiation of the red cells based on size and the detection of the reticulocytes from the red cell cluster by reticulum staining with thiazole orange. Analysis with the Retic-COUNT software package in the FACScan consists of isolating the red cell cluster by forward and side scatter and isolating the reticulocytes by fluorescence staining of the reticulum. Each sample is counted as 10 000 events with the percentage reticulocytes determined from the red cell population.

Effect of other agents on reticulocyte count

To investigate the potential ability of other classes of compounds to affect reticulocyte formation, several other agents were tested to see if they altered the reticulocyte count. Interleukin-1 (IL-1) and Interleukin-3 (IL-3) were obtained from Genzyme (Boston, MA) as the recombinant human products. Other agents employed included dexamethasone (Organon, West Orange, NJ), human chorionic gonadatropin (HCG; Sigma, St Louis, MO), and pregnant mare serum (Sigma). These agents were all administered subcutaneously and separately from the EPO, at doses detailed in the results and which have been previously reported to have *in vivo* activity in their respective bioassays. Additionally, murine IL-3 (Genzyme, Boston, MA) was administered intraperitoneally on days 0, 1, 2, 3 and 4 with and without subcutaneous r-HuEPO administration on days 5 and 6. These mice were then bled on day 9 in accordance with the established protocol for r-HuEPO administration.

Statistical analysis of reticulocyte and exhypoxic polycythemic assays

Statistical analysis of the reticulocyte assay followed the parallel line method as described by Finney [13]. Briefly, a series of models were fit. The first part fits an unrestricted model:

$$Y_{k(ij)} = a_i + b_i X_{ij} + e_{k(ij)}, \quad (3)$$

where $Y_{k(ij)}$ = log (base 2) of percentage of reticulocytes of kth animal in the jth dose group of ith sample, a_i = intercept of the ith test sample, b_i = slope of the ith test sample, x_{ij} = log (base 2) of the jth dose in the ith sample and $e_{k(ij)}$ = random error term of the kth animal in the jth dose group of the ith sample. An analysis of variance was calculated on the basis of the unrestricted model to test for parallelism (slope by test sample interaction). If interaction is significant at a 10% level, pairwise tests are examined to indicate the offending sample(s). The model was refitted and the above procedure repeated until

parallelism was achieved. At this point, a restricted model was fit, estimating a common slope for all test compounds and standard. Relative potencies were estimated as:

$$r_i = (a_i - a_s)/b, \quad (4)$$

where r_i = relative potency of the ith test sample, a_i = estimated intercept of the ith test sample, a_s = estimated intercept of the standard and b = estimated common slope. Fiducial limits were calculated using Fieller's Theorem, as discussed in Finney [13].

The analysis for the exhypoxic mouse bioassay followed the same parallel line model described except that the statistical and biological outliers were removed from the data set prior to analysis. A biological outlier was defined as an animal with a low haematocrit value or one whose response to EPO fell below the average response for the vehicle control group. Statistical outliers were identified using Dixon's test for outliers [14].

Results

Effect of dosage regimen on reticulocyte count

Figure 1 shows the comparison of the various regimens at the 20 unit mouse^{-1} day^{-1} dose of r-HuEPO on each of the last three bleed days. The 20 unit mouse^{-1} day^{-1} dose produces increased levels of reticulocytes over baseline (vehicle treated) of 1.7 ± 0.97% on all three days. These data demonstrate that with either Schedule C (dosing on days −3, 0 and 1) or Schedule E (dosing on days 0 and 1) a significant increase in reticulocyte count can be achieved by days 3 or 4. The fact that dosing on days 0 and 1 produces an increased reticulocyte count on days 3 or 4 suggests that a complete assay could be run in the course of a single week. Subsequent experiments were designed to further investigate this possibility.

Dose–response relationship of r-HuEPO in 1-week assay

Figure 2 shows the dose–response relationship with r-HuEPO and asialylated r-HuEPO (administered on days 0 and 1) when mice were bled on day 4. It is important to note that the asialylated r-HuEPO did not produce an increase in reticulocyte count even at total doses of 40 units mouse^{-1}, demonstrating the lack of biologic activity *in vivo* with asialylated r-HuEPO. In addition, the lack of effect with asialylated r-HuEPO also suggests a specificity of the assay for intact r-HuEPO.

Figure 3 shows the optimized assay with EPO dosing divided over 3 days with iron dextran administration on day 2, and the haematocrit correction. As shown in Table 1, the precision statistics denote generally less precision with the reticulocyte assay than the

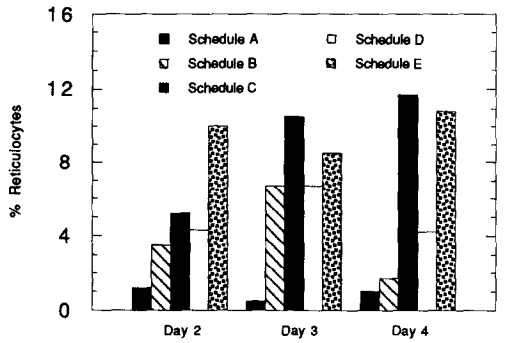

Figure 1
Effect of dosage regimen with r-HuEPO on reticulocyte count in normal mice. r-HuEPO was administered subcutaneously to groups of 10 mice at a dose of 20 units mouse^{-1} per injection in one of several dosage regimes. Two mice per day were bled by cardiac puncture and reticulocyte counts were determined by flow cytometry. Schedule describes the dosing regimen and total dose of EPO administered. Values represent the mean reticulocyte count of the two mice bled per day. Schedule A = day −3 (20 units); Schedule B = day −3 and 0 (40 units); Schedule C = days −3, 0 and 1 (60 units); Schedule D = day 0 (20 units); Schedule E = days 0 and 1 (40 units).

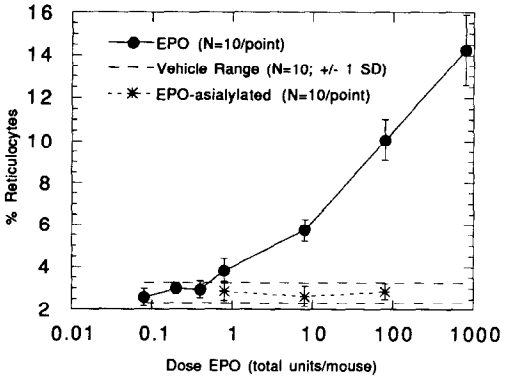

Figure 2
Dose–response effect of r-HuEPO (administered on days 0 and 1) on the reticulocyte count in normal, female, BDF1 mice. Groups of 10 mice were treated with vehicle or various doses of r-HuEPO subcutaneously. Mice were bled on day 4 by cardiac puncture and reticulocyte counts were determined by flow cytometry. Asialylated EPO (EPO-asialylated) was prepared as described in the methods and administered over 2 days in separate groups of 10 animals. Vehicle effects are shown as the range of values determined ±1 standard deviation of the mean. Per cent reticulocyte values represent the mean ± standard deviation.

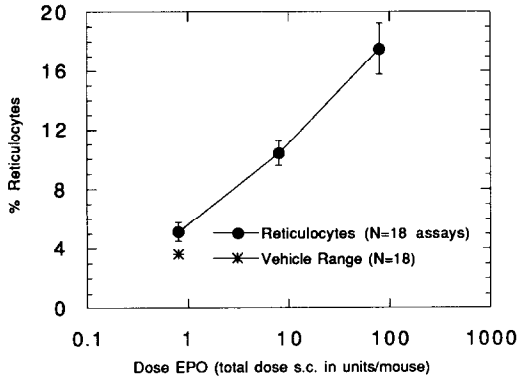

Figure 3
Optimized (3 day r-Hu-EPO dosing with iron dextran) reticulocyte assay. Groups of 10 mice dose^{-1} were treated with increasing doses of r-HuEPO or vehicle subcutaneously. Mean values of responses for each of 18 assays were used to determine the mean ± standard deviation values presented in the graph.

Table 1
Statistical comparison of the exhypoxic polycythemic mouse (five assays) and the reticulocyte assay (18 assays) for determination of potency of a single r-HuEPO lot

Parameter	Exhypoxic	Reticulocyte
G value	0.004–0.010	0.0002–0.010
Lambda	0.48–0.67	0.65–1.20
Root MSE	0.37–0.55	0.18–0.33
Slope	0.72–1.03	0.20–0.29

Slope denotes the estimated slope from the parallel line model.

Root MSE denotes the estimated standard deviation of the response (% reticulocytes) from the same regression model.

G value = t^2 var(slope)/slope^2, where t is the 95% t-deviate whose degree of freedom is the same as due to error. *G* valuc is an index of regression significance.

exhypoxic mouse bioassay. With a total of 30 animals per preparation (10 animals per dose group), the precision of the reticulocyte assay was estimated to be between 18 and 35%. With the same number of animals, the precision for the exhypoxic assay would be between 13 and 19%.

To determine if there is a circadian dependency to the EPO effect on reticulocyte count, we administererd r-HuEPO at several different times throughout the day. Figure 4 illustrates the effect of r-HuEPO administration at 8:30 a.m., 12:30 or 4:00 p.m. Each time a vehicle control group was included. In this study, no difference was observed in the erythropoietic effect of r-HuEPO given at various times of the day.

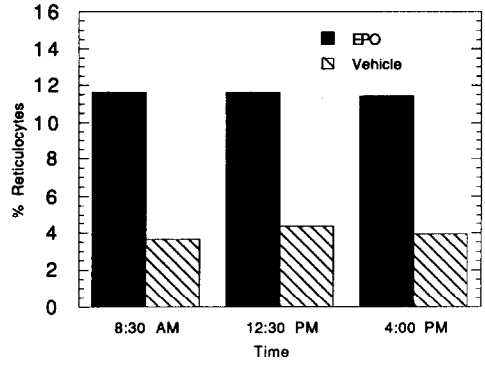

Figure 4
Influence of administration time on the reticulocyte response to r-HuEPO. R-HuEPO (2000 units kg^{-1} total dose) or vehicle was administered subcutaneously on days 0 and 1 at either 8:30 a.m., 12:30 a.m. or 4:00 p.m. Mice were bled on day 4 by cardiac puncture and reticulocyte counts were determined by flow cytometry. Per cent reticulocyte values are the mean ± standard deviation of 10 animals per group.

Correlation of manual and automated reticulocyte counts

As a means of interpreting the results obtained with the automated reticulocyte counting programs, we compared the results obtained by two procedures: manual counting versus FACScan determinations, in mice treated with vehicle, r-HuEPO (40 units mouse^{-1} s.c. total dose) or made polycythemic by 2 weeks exposure at 304 mmHg in a hypobaric chamber. Figure 5 illustrates the

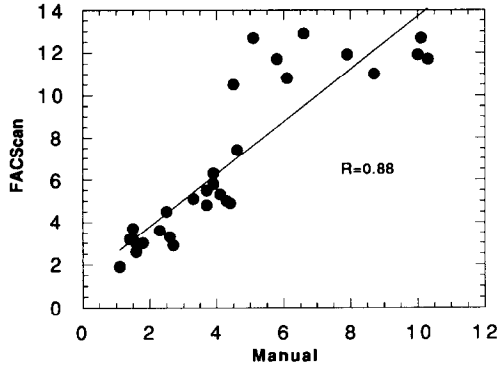

Figure 5
Correlation of manual and flow cytometric reticulocyte determinations. Mice were treated with either vehicle or r-HuEPO (40 units mouse^{-1} total dose divided over 2 days) subcutaneously on days 0 and 1, or were polycythemic from a 2 week exposure in a hypobaric chamber at 304 mmHg. On day 4 after EPO administration or 3 days after hypobaric exposure, mice were bled by cardiac puncture and the reticulocyte count determined by either flow cytometry or manual counting by the Clinical Pathology group at the R.W. Johnson Pharmaceutical Research Institute. Values represent the individual per cent reticulocyte determinations with the correlation coefficient determined by the General Linear Models procedure.

correlation of the two methods. The per cent reticulocytes obtained by manual counting showed significant correlation ($r = 0.88$) with the automated procedure, although there is less correlation at reticulocyte values above 5%. This may be due to a greater sensitivity of the thiazole orange as compared to new methylene blue, inclusion by flow cytometry of more mature reticulocytes and the imprecision of manual counts [15].

Specificity of the reticulocyte assay for r-HuEPO

Figure 6 shows the ineffectiveness of various substances on the reticulocyte count in normal mice. At the doses tested and with the same regimen as that used for r-HuEPO administration, none of the compounds tested caused any increase in reticulocyte numbers. In addition, human IL-3 given daily (4.8×10^3 units mouse^{-1} day^{-1}) either 7 days prior to r-HuEPO or 7 days prior then concurrent with 2 days r-HuEPO treatment (9 days treatment) did not affect the r-HuEPO activity in either an additive or inhibitory way (data not shown). Murine IL-3 at 140 units mouse^{-1} day^{-1} for 5 days or 800 units mouse^{-1} day^{-1} for 5 days also had no effect on reticulocyte counts (Fig. 7).

Discussion

With commercial production of a recombinant human EPO for therapeutic use, standardization of different batches becomes an important consideration. Batch to batch equivalency must be assured for reproducible performance in the clinic. To this end, a test system must be in place to determine the *in vivo* biological activity of each preparation of product. The generally accepted bioassay to date has been the exhypoxic polycythemic mouse assay [8]. This is an assay which has been used for many years to evaluate EPO levels from various body fluids and also to characterize and standardize EPO preparations worldwide [16]. However, the polycythemic mouse assay is labour intensive, which makes it a difficult assay to use for the routine evaluation of commercial batches of recombinant EPO. The current study is an attempt to provide a faster, more reproducible bioassay which could be used for such purposes.

One of the last events in the differentiation and maturation of red blood cells is the production of the reticulocyte [6]. The reticulocyte is the last cell in the generation of erythrocytes to have any nuclear material. It is this remaining nuclear reticulum that makes the reticulocyte distinct and easily recognizable microscopically. The mechanism of action of EPO is such that it stimulates the production of red blood cells and speeds up intramarrow transit time [1, 17, 18]. This effect causes more reticulocytes to migrate into the circulation which allows for the determination of erythropoietic activity based on reticulocyte counts in the peripheral blood [10]. Until recently, the need to determine reticulocytes manually precluded the use of these determinations for rapid analysis due to the extremely labour

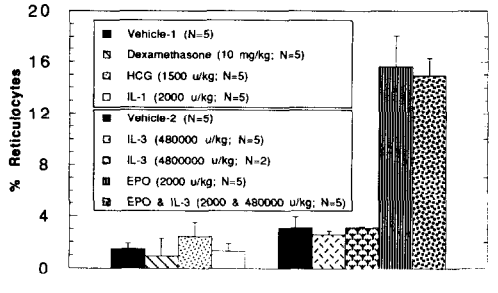

Figure 6
Effect of various substances on the reticulocyte count in female BDF1 mice. Substances were injected subcutaneously at the doses indicated (total dose administered was equally divided over 2 days, assuming a body weight of 20 g) on days 0 and 1. Mice were bled by cardiac puncture on day 4 and reticulocyte counts were determined by flow cytometry. Per cent reticulocyte values represent the mean ± standard deviation for 10 mice per group. IL-3 tested was human recombinant IL-3 supplied by Genzyme.

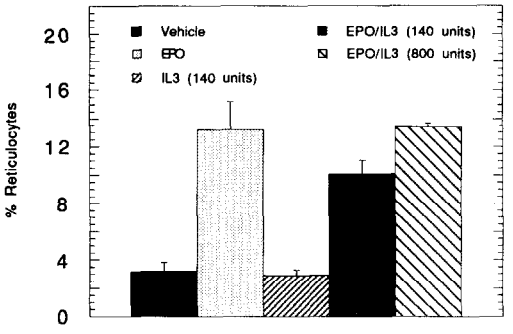

Figure 7
Effect of murine recombinant interleukin-3 (IL-3) on reticulocyte count alone or in combination with r-HuEPO. IL-3 was administered as described in the methods on days 0, 1, 2, 3 and 4 at 140 units day^{-1} with or without r-HuEPO (20 units day^{-1}) on days 5 and 6 or 800 units day^{-1} IL-3 with r-HuEPO (20 units day^{-1}) on days 7 and 8 to groups of five mice. Per cent reticulocyte values (determined by flow cytometry) represent the mean ± standard deviation.

intensive nature of the microscopic reading of slides. With the advent of new technology using flow cytometry and fluorescent staining, the determination of reticulocytes as a bioassay for EPO is now possible. Data from the current study demonstrate that reticulocyte measurements with automated programs using the Ortho Spectrum III cytofluorograph or the Becton–Dickinson FACScan are both sensitive and reproducible. Other instruments and techniques are available which can perform automated reticulocyte determinations with comparable results. These are currently under evaluation by other laboratories [19, 20] for use in the bioassay of erythropoietin. Utilization of the automated system for reticulocyte determinations allows an estimate of EPO potency to be made more quickly than with the polycythemic mouse assay. This makes it possible to test many more batches in less time. In addition to the commercial applications of the reticulocyte assay, it can also be used as a reliable experimental tool for further research on EPO and other haematopoietic growth factors.

As an alternative to the polycythemic mouse assay, we have described the development of an assay using the increase in reticulocyte numbers present in peripheral blood after EPO administration to normal mice. We have been able to demonstrate a dose related increase in reticulocyte number following administration of r-HuEPO. The reticulocyte increase is evident with two or three doses of r-HuEPO and demonstrable within 2–3 days after the last dose. Using this assay, various preparations of r-HuEPO can be tested for *in vivo* biological activity in the course of 1 week, as compared to 3–4 weeks for the polycythemic mouse assay. The reticulocyte assay is not as sensitive as the polycythemic mouse assay since it requires more EPO to demonstrate an effect. For detecting low levels of EPO in body fluids, the radioimmunoassay (RIA) is usually more appropriate than *in vivo* bioassays. However, when higher levels of EPO can be utilized, such as in testing of commercially prepared batches of recombinant human EPO, the reticulocyte assay may be preferred to the polycythemic mouse bioassay due to the shorter turnaround time. To ascertain biological activity of EPO, the *in vivo* bioassay is preferred over *in vitro* assays since it discriminates biological activity with regard to glycosylation, a property not associated with the available *in vitro* assays.

The reticulocyte assay appears to be specific for EPO since other substances tested have shown no effect on the reticulocyte count using the current dosing regimen. It is of interest to note the ineffectiveness of human or murine IL-3 on the reticulocyte count since it is an erythroid cell line stimulant. Human recombinant IL-3 has been shown to increase reticulocyte counts in primates [21, 22]. The lack of effect in the current study may be due to inability of the human product to crossreact in the mouse. However, the inactivity of the murine product suggests that some other mechanism may be responsible for this observation.

Conclusions

An EPO bioassay in normal mice has been developed. The method is accurate, easy to perform, and does not require the use of radioactivity. In addition, the turnaround time for this assay is 1 week; considerably shorter than the 3–4 weeks needed for the polycythemic mouse assay. This assay is suitable for research in the EPO area, however, the precision of the relative potency estimates from the retic assay using the current protocol is generally more variable than the precision obtained from the exhypoxic mouse bioassay.

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