The Sustained Action of Erythropoietin Injected Repeatedly into Rats and Mice

By Geoffrey Keighley, Denman Hammond and Peter H. Lowy

ERYTHROPOIETIN (EP) is a substance found in the plasma of many anemic animals, in those made anemic, for example, by bleeding or phenylhydrazine. EP is also present in the plasma of animals exposed to anoxic hypoxia, at least during the first part of the exposure. EP is frequently found in the urine of anemic animals and man. It can be extracted and concentrated as a stable soluble product. If it is given to normal animals, erythropoiesis is stimulated and as a result, red cell mass, RBC, hematocrit, and hemoglobin concentration are increased; they return to normal when EP is stopped. Erythropoiesis, EP and their relation have been reviewed by Gordon and Stohlman.

Gordon and others have evaluated methods currently used for the biological assay of EP. We tested many of our first preparations by injecting them into rats and following the rise in hemoglobin concentration. The injections to be effective had to be made daily for 10 days or longer. Such an assay is slow and expensive of material. More convenient ones have been devised; for example, by measuring radioiron incorporation into red cells and by using animals made sensitive to EP by depressing erythropoiesis. Most workers now use the shorter assays because of their speed and economy, and as a rule the animals are injected only once or twice.

Experimental evidence shows that EP plays a part in the regulation of red cell production. It has been proposed that it is the sole regulator, but there are objections to this idea. Evidence against the unitary concept has been presented by Stohlman who has also reviewed this controversial point. In any case, if EP is a regulator which is constantly present and not something which appears only during great stress, as after severe hemorrhage, one of its expected properties would be the ability not only to produce but to maintain an elevated rate of erythropoiesis. A given dose of EP should maintain a corresponding high blood volume as long as it is given. This paper reports the response of rats and mice to extended courses of injections of EP.

MATERIALS AND METHODS

Erythropoietin Preparations

Plasma filtrate (PF) was derived from rabbits which had been made anemic by injections of phenylhydrazine. They were bled and most of the protein precipitated from the plasma by boiling briefly at pH 5.5. The boiled plasma was filtered and the filtrate neu-
Table 1.—Injection Schedules of Rats and Mice

<table>
<thead>
<tr>
<th>Animals</th>
<th>Group</th>
<th>Material and Dose per Day</th>
<th>Days Injected</th>
<th>Days Observed after Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats</td>
<td>1</td>
<td>PF 2 ml./100 Gm.</td>
<td>40</td>
<td>35</td>
</tr>
<tr>
<td>Rats</td>
<td>2</td>
<td>PF 2 ml./100 Gm.</td>
<td>48</td>
<td>43</td>
</tr>
<tr>
<td>Mice</td>
<td>1</td>
<td>LD 4-5 units</td>
<td>195</td>
<td>42</td>
</tr>
<tr>
<td>Mice</td>
<td>2</td>
<td>LD 8-10 units, then 4-5 units</td>
<td>58</td>
<td>0</td>
</tr>
</tbody>
</table>

*About 8 Standard A units in 2 ml. See text.

Centralized and stored at −15 C. Rabbit PF is quite stable.7 When these PF extracts were made, there were no standards. To arrive at some estimate of potency we can only compare the effect on the hemoglobin of the injected rats with the responses of other rats treated in the same way, at a later date, with standardized preparations. The comparison gives us an estimate, not at all precise, that the rats received the equivalent of 8 standard A units per 100 Gm. body weight.

A concentrated fraction was made from PF by addition of ethanol. The active fraction, precipitated between 60 and 80 per cent ethanol, was dialysed and lyophilized (LD). A similar fraction has been extensively tested for activity by several workers.9 The LD used here came from one batch. It had been assayed by red cell Fe59 uptake in fasted rats at three-dose levels along with samples of an anemic rabbit plasma kept as a laboratory standard. The standard plasma had been compared by similar assays with Erythropoietin Standard A. We estimate the LD to have a potency of between 4 and 5 Standard A units/mg. As needed, amounts of LD were dissolved in 0.9 per cent NaCl and kept frozen until just before use. Similar LD’s have been found to be stable for longer times than the length of this experiment.

Hematocrit measurements were made on blood taken from the orbital sinus directly into microhematocrit tubes. Hemoglobin was measured by the cyanmethemoglobin method of Drabkin. Platelet counts were made on tail blood by the direct chamber method of Brecher and Cronkite.10

Rats

Sprague Dawley strain female rats, about 200 Gm. mean weight, were injected subcutaneously 6 days a week with rabbit PF at a dose of 2 ml./100 Gm. The dose was adjusted as weight increased. Three groups were injected for periods of 40, 48 and 133 days respectively (table 1). Baseline hemoglobin values were determined, usually for several weeks before starting and weekly after injections began.

Mice

Two groups of Webster Swiss female mice were given subcutaneous injections 6 days a week of the LD preparation (table 1). Four mice in Group 1 received 4–5 units of LD for 195 days. Four mice in Group 2 were given 8–10 units of LD for 58 days; then the dose was reduced to 4–5 units and continued through 205 days. Hematocrit measurements were made weekly, leukocytes were counted four times, platelets were counted on day 180, and red cells were counted on day 194.

Results

Rats

The mean hemoglobin of the eight rats of Group 1 rose steadily from a pre-injection value of 14.5 Gm. per 100 ml. of blood to 19.5 Gm. at 30 days, and stayed at this level to the end of the injections at 40 days and for a week.
SUSTAINED ACTION OF ERYTHROPOIETIN

Fig. 1.—Response of rats injected with erythropoietin for 133 days. Vertical bars = mean hemoglobin ± S.D. Solid line = mean weights.

longer. The hemoglobin fell to the pre-injection level in the next 14 to 21 days and stayed at that level as long as measurements were made—another 5 weeks. The rats gained weight throughout the experiment.

The mean hemoglobin of the eight rats of Group 2 reached a plateau of 20.5 Gm. at 48 days which was maintained for 8 days after the injections stopped, then fell to normal in about 20 days and stayed there for the following 6 weeks. These rats also gained weight, but one died in the post-injection interval.

The eight rats of Group 3 reached a mean hemoglobin of about 22 Gm. in 49 days and maintained this level with small fluctuations to the end of the injections, an additional 84 days (fig. 1). The rats gained weight throughout the experiment. When the injections were stopped they were sacrificed and autopsied. No gross abnormalities were seen.

Mice

One mouse was removed from each group at the end of 3 weeks because it lost weight or appeared ill. The remaining three mice in Group 1 experienced slow continuous rise in hematocrit until the mean reached 60 at 40 days (fig. 2). At this time one mouse died, for unknown reasons (its hematocrit was 62 and weight was satisfactory). The other two maintained weight and their hematocrit remained elevated; the mean ranged from 54.8 to 62.0. After the injection of LD was stopped at day 195, the hematocrit of both mice returned to normal levels in 19 days. One mouse lost weight and died after one of the weekly bleedings, 19 days after injections were stopped. The other continued to be healthy as long as observed, 6 weeks after injections.

The three surviving mice in Group 2, receiving the larger dose of LD (fig. 2), experienced a fairly rapid rise in hematocrit to day 43 when the mean
Fig. 2.—Mean hematocrits of mice injected with erythropoietin. Open circles = group 1, 4–5 units per day to day 195. Solid circles = group 2, 8–10 units to day 58, then 4–5 units to day 205.

value was 68.5. It stayed at that level until day 58, when the dose was reduced to 4–5 units, the same as that given the mice in Group 1. The mean hematocrit remained at the high level until day 70 and then slowly fell to 66.1 at day 85, and remained in the range 55.3 to 63.2 for the rest of the experiment, to day 205. One mouse developed a lump on one side of its neck which was first noticed on day 177; the animal was killed and autopsied on day 184. All was normal except for the tumor, which was removed and sectioned. It was found to be a thyroid carcinoma. At the end of the experiment the two remaining mice were killed and autopsied. Both appeared to have infections at the end of their tails, and moderately enlarged spleens; no other abnormalities were seen.

Cell counts made throughout the last half of the experiment are given in table 2, together with mean values of cell counts made by the same observers on normal control Swiss Webster mice. The platelet counts, all the differential counts and the WBC on days 160 and 200 are within the normal range which we find for mice of this kind, and none of the differences between the experimental and control means is significant (t test). The experimental WBC on days 100 and 150 are significantly lower than those of the controls. The values of the red cell count on day 195 increased about the same amount as the increase in hematocrit. On the same day the average mean corpuscular volume of five mice was 43.5 ± 1.6 and the average mean corpuscular hemoglobin was 13.3 ± 0.4, not significantly different from the corresponding means of controls; 45.0 ± 1.9 and 15.5 ± 1.2. (These last figures are not in table 2).

**DISCUSSION**

Exogenous EP induced and maintained an elevated rate of erythropoiesis in rats and mice. The response was proportional to dose. The polycythemia was maintained as long as EP was given, in rats for up to 133 days, more than twice as long as a normal rat red cell life span, and in mice for 195 days,
about 5 times as long as the life span of mouse red cells. In some models for the regulation of erythropoiesis, the action proposed for EP is to differentiate stem cells into erythroid precursors, a view supported by the work of Erslev, of Alpen, and of Jacobson and his associates. Lajtha in this connection has pointed out the importance of knowing if the response to stimulation is maintained when the stimulation is continued for a long time. In the results reported here with rats and mice, there is no evidence that EP in the doses used exhausted the stem cell pool or depleted any tissue or organ of cells or substances needed for the production of red cells, or that the animals became adapted to repeated dosage and gave reduced responses. When the injections were stopped, the polycythemia persisted for a few days and then subsided to normal levels of hematocrit and hemoglobin. Neither the lengthy stimulus nor the polycythemia it produced abolished or modified any regulatory signal or other component of the erythropoietic control mechanism. If the setting of the “erythrostat” can be changed, it is not easy to do it this way.

There is no information about what may happen to the leukocytes and platelets during the first 5 or 10 days of the administration of EP. Through the last half of the experiment the data are not only against a lasting stimulatory effect on WBC but suggest rather the possibility of depression. Stohlman and Brecher have reported macrocytosis after acute treatment with erythropoietin. Gurney, Wackman and Filmanowicz reported no significant difference in mean corpuscular volume and mean corpuscular hemoglobin concentration between mice with high hematocrits after 27 days of EP and saline-injected controls. We found no difference in mean corpuscular volume and mean corpuscular hemoglobin between normal controls and mice after injections of EP for 194 days.

In an earlier paper we gave the results of injecting normal rats with PF for 4 weeks. We have some unpublished data on male and female mice injected with PF for 20 days or more. Two of the females were mated with injected males; they became pregnant and delivered normal litters. The hemoglobin and hematocrit curves of the rats at the end of 4 weeks appeared to be still rising. Recently Gurney, Wackman and Filmanowicz, in a paper on the effects of single and multiple doses in polycythemic mice and multiple doses in normal mice, found in the normal mice steadily increasing hemoglobin and hematocrits with 27 days of injections. Their curves appear not to have reached a plateau. From the course of the present experiments, 20–30 days is not long enough for moderate doses to produce a maximum effect. The size of the dose might affect the rate at which the maximum is reached, though the results from the two dose rates in mice (fig. 2) do not suggest this possibility. It may take 40 days with mice and 50 days with rats to reach a steady state (fig. 1 and 2).

One mouse in this series developed a thyroid carcinoma after 177 days of injections. For some weeks before its hematocrit had been lower than those of its partners, its weight was unchanged. According to the breeders, these mice have a 1 per cent incidence of mammary tumors at 1 year.
### Table 2.—Serial Blood Cell Counts of Experimental Mice during the Injection of Erythropoietin Compared to Cell Counts of Control Mice

<table>
<thead>
<tr>
<th></th>
<th>WBC 10^6</th>
<th></th>
<th>N. Segment</th>
<th></th>
<th>Lymph.</th>
<th></th>
<th>Mono.</th>
<th></th>
<th>Eosin.</th>
<th></th>
<th>Plat. 10^9</th>
<th></th>
<th>RBC 10^9</th>
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<tbody>
<tr>
<td>Day</td>
<td>100</td>
<td>150</td>
<td>160</td>
<td>200</td>
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<td>160</td>
<td>200</td>
<td>160</td>
<td>200</td>
<td>130</td>
<td>194</td>
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<tr>
<td>6.7</td>
<td>13.3</td>
<td>18.5</td>
<td>26.8</td>
<td>33.0</td>
<td>50.0</td>
<td>64.0</td>
<td>39.0</td>
<td>3.0</td>
<td>4.0</td>
<td>—</td>
<td>7.0</td>
<td>1.37</td>
<td>13.8</td>
</tr>
<tr>
<td>4.5</td>
<td>9.3</td>
<td>19.7</td>
<td>9.8</td>
<td>40.0</td>
<td>34.0</td>
<td>52.0</td>
<td>55.0</td>
<td>5.0</td>
<td>5.0</td>
<td>3.0</td>
<td>6.0</td>
<td>1.56</td>
<td>12.1</td>
</tr>
<tr>
<td>E</td>
<td>13.6</td>
<td>14.3</td>
<td>17.6</td>
<td>13.7</td>
<td>27.0</td>
<td>32.0</td>
<td>61.0</td>
<td>64.0</td>
<td>12.0</td>
<td>2.0</td>
<td>—</td>
<td>2.0</td>
<td>.99</td>
</tr>
<tr>
<td>9.8</td>
<td>10.2</td>
<td>9.3</td>
<td>11.2</td>
<td>15.0</td>
<td>27.0</td>
<td>78.0</td>
<td>62.0</td>
<td>6.0</td>
<td>1.0</td>
<td>1.0</td>
<td>10.0</td>
<td>1.15</td>
<td>15.6</td>
</tr>
<tr>
<td>7.4</td>
<td>11.7</td>
<td>8.5</td>
<td>10.1</td>
<td>30.0</td>
<td>19.0</td>
<td>58.0</td>
<td>72.0</td>
<td>11.0</td>
<td>8.0</td>
<td>1.0</td>
<td>1.0</td>
<td>—</td>
<td>11.3</td>
</tr>
<tr>
<td>EM</td>
<td>8.4</td>
<td>11.8</td>
<td>14.7</td>
<td>14.3</td>
<td>29.0</td>
<td>32.4</td>
<td>62.6</td>
<td>58.4</td>
<td>7.4</td>
<td>4.0</td>
<td>1.7</td>
<td>5.2</td>
<td>1.27</td>
</tr>
<tr>
<td>±3.5</td>
<td>±2.1</td>
<td>±5.4</td>
<td>±7.1</td>
<td>±9.5</td>
<td>±11.4</td>
<td>±9.7</td>
<td>±12.4</td>
<td>±3.9</td>
<td>±2.7</td>
<td>±1.2</td>
<td>±3.7</td>
<td>±25</td>
<td>±17</td>
</tr>
<tr>
<td>CM</td>
<td>16.1 ± 2.1</td>
<td>32.0 ± 5.8</td>
<td>60.7 ± 4.0</td>
<td>5.0 ± 1.8</td>
<td>3.0 ± 2.1</td>
<td>1.22</td>
<td>±17</td>
<td>±1.2</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

E = cell counts of single experimental mice on day shown; EM = mean cell counts of experimental mice ± S.D.; CM = mean cell counts of 4-6 control mice ± S.D.
Leaders, Dixon, Osborne and Long have published data which they believe established EP as a stimulant of tumor growth. Their experiment is different from the one described here and the evidence is indirect, and from an acute experiment. In the present studies one tumor occurred in five mice injected with exogenous EP for 200 days. On these few numbers we cannot draw any conclusions about the possibility of a causal relationship.

Erythropoietin is a substance which increases the red cell mass in normal animals. We have pointed out that the best preparations of EP may contain only a small portion of active material. One might speculate that as EP is extracted and concentrated it could acquire tumor-stimulating properties, but the chemical and biological similarities of EP from different sources do not sustain such speculations. Further, if impure samples of EP are thought to have effects other than those of stimulating erythropoiesis, before these effects are attributed to EP and before the problems are confused by renaming EP we should be certain that further purification will not separate the different actions.

There is a well established body of data from humans which bears on both our finding and that of Leaders et al. Sufferers from congenital hypoplastic anemia, a pure red cell anemia which starts in infancy, have been intensively examined. We reported results of studies of plasma and urine erythropoietin in a group of 20 such patients. Some of them have had serial determinations of plasma and urine EP for 4 years. Except for several days following transfusion, there is increased EP in their plasma and urine. The amount increases with increasing severity of anemia and can reach very high levels. In spite of the large amounts of endogenous EP which can be assumed to have been present for up to 20 years in such patients, they have leukocyte and platelet counts within the normal range. We are aware of no evidence that the incidence of tumors is greater in these patients than in the general population. Some of them have lived into adulthood. The same observations also apply to patients with thalassemia major, a lifelong chronic anemia in which Winkert and Gordon and we have found increased levels of EP in plasma and urine. In these patients there is no evidence that high levels of endogenous EP promote tumor growth, and whatever the answers to these questions the explanations must be consistent with the situation in tumor-free subjects who chronically maintain within themselves a high titer of EP. We have begun more experiments with larger numbers of mice which should in time provide some pertinent data.

**Summary**

Rats and mice were given exogenous erythropoietin for periods from 40 to over 200 days. Increased erythropoiesis was maintained as long as erythropoietin was given; when it was stopped, erythropoiesis returned to its normal level; when the dose was diminished, erythropoiesis fell accordingly.

In mice there was no long-term increase of platelets or change in the differential leukocyte counts and leukocyte counts were, during the middle period of the experiment, decreased.
One mouse developed a tumor. It is pointed out that humans with certain chronic anemias have lived for years with greatly elevated concentrations of erythropoietin in their blood and urine, yet they have normal leukocyte and platelet counts and no tumors.

**SUMMARIO IN INTERLINGUA**

Rattos e muses esseva subjicite a injectiones de erythropoietina exogene durante periodos de inter 40 e plus que 200 dies. Un augmentate erythropoiese se manteneva in co-duration con le administration de erythropoietina. Quando le administration de erythropoietina esseva arrestate, le erythropoiese retornava a su nivello normal. Quando le dosage de erythropoietina esseva reducite, le erythropoiese declinava correspondentemente.

In muses il non occurreva un perdurative augmento in le numeration del plachettas o alterationes del numeration differential de leucocytos. Durante le periodo intermedie del experimento, le numeration del leucocytos esseva reducite.

Un del muses disveloppava un tumor. Es signalate que humanos con certe anemias chronic ha vivite durante multe annos con grandemente elevate concentrationes de erythropoietina in lor sanguine e lor urina, e nonobstante illes ha normal numerationes plachettal e leucocytic e nulle tumor.

**ACKNOWLEDGMENT**

We thank Benjamin H. Landing, M.D., Pathologist at the Children's Hospital of Los Angeles, for his examinations of tissue sections.

**REFERENCES**

SUSTAINED ACTION OF ERYTHROPOIETIN


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