Studies on Erythropoiesis

V. The Effect of Cobalt on the Production of Erythropoietin

By EUGENE GOLDWASSER, LEON O. JACOBSON, WALTER FRIED and LOUIS F. FLYAK

FOR NEARLY three decades it has been known that cobalt increases erythropoiesis in experimental animals. Cobaltous chloride given orally or parenterally for a period of several weeks induces and maintains a polycythemia in man and experimental animals. The precise mechanism by which cobalt exerts this action has not yet been clarified completely. Early studies indicated that cobalt acts directly by producing an anoxic condition in the marrow, while later work suggested an indirect anoxic effect on the marrow caused by the formation of cobalt-amino acid complexes. However, attempts to produce a depression in respiration of the marrow by the use of cobalt, either indirectly or directly, have failed. A recent study has shown that cobalt, at a concentration that does not affect respiration, depresses the in vitro synthesis of hemoglobin from glycine in short-term experiments. This seeming paradox has not yet been resolved.

Although the appreciable toxicity of cobalt limits its clinical applicability, it has been used to correct certain anemias that are refractory to all the usual therapeutic measures, except transfusion or elimination of the underlying disease.

In a preliminary paper, we presented data indicating that cobalt exerts its effect by stimulating the production of erythropoietin. This paper gives details and further evidence of this finding. As in previous reports, the term erythropoietin is used to designate the factor found in plasma of anemic animals that accelerates erythropoiesis when such plasma is injected into assay animals.

Materials and Methods

Assay Animals

Starved or hypophysectomized* rats were used to assay the various experimental plasma preparations for erythropoietin activity as described in previous publications. The assay method measures the per cent of injected Fe that is incorporated into red cells under standardized conditions. A minimum of 5 rats was used in each assay group.

Preparation of "Cobalt Plasma"

Aliquots of a stock solution of cobaltous chloride (0.025 M) were injected subcutaneously into rats and rabbits according to the schedules described for each separate experiment. After the indicated intervals, the treated animals were bled by cardiac puncture, the plasma was separated and, if not used immediately, was stored at -18C. When Co was used, it was mixed with aliquots of the stock solution before injection. Plasma derived from animals

A preliminary report of some of these findings has been published. From the Argonne Cancer Research Hospital, USAEC, and the Departments of Biochemistry and Medicine, The University of Chicago, Chicago, Ill. Submitted May 6, 1957; accepted for publication Sept. 20, 1957. * Obtained from Hormone Assay Laboratories, Chicago, Ill.
TABLE 1.—Effect of Cobaltous Ion on the Incorporation of Fe59 into the Red Blood Cells of Hypophysectomized Rats

<table>
<thead>
<tr>
<th>Condition</th>
<th>% of Fe59 Incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Plasma</td>
<td>3.8 ± 2.0*</td>
</tr>
<tr>
<td>2.1 μM Co**</td>
<td>9.5 ± 4.9</td>
</tr>
</tbody>
</table>

* Standard deviation of the mean.

TABLE 2.—Effect of Cobalt Plasma on the Incorporation of Fe59 into the Red Blood Cells of Hypophysectomized and Normal Rats

<table>
<thead>
<tr>
<th>Condition</th>
<th>% of Fe59 Incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>2.6 ± 0.6</td>
</tr>
<tr>
<td>Cobalt plasma</td>
<td>19.4 ± 7.4</td>
</tr>
<tr>
<td>Hypophysectomized</td>
<td>26.7 ± 9.2</td>
</tr>
<tr>
<td>Normal</td>
<td>35.6 ± 1.1</td>
</tr>
</tbody>
</table>

Injected previously with cobalt is termed "cobalt plasma." Exactly the same methods were used for the other metal ions that were examined, the amounts used being limited by the toxic action of each metal.

Effect of Cobalt on the Incorporation of Fe59

The direct effect of cobaltous ion on the incorporation of Fe59 into red cells was studied by injecting 1 ml. of a cobalt solution containing 1.05 μmoles each day for 2 days into hypophysectomized rats, then determining the incorporation of Fe59 as in the standard assay. The results shown in table 1 reveal that 2.1 μmoles of cobaltous ion stimulate the incorporation of iron into red cells. This finding necessitated the use of cobalt-containing controls as described below.

Effect of Cobalt Plasma on the Incorporation of Fe59

Cobalt plasma was produced by injecting 6 ml. of the stock solution of cobalt into 400-Gm. donor rats which were sampled 18 hours later. This cobalt plasma was assayed in hypophysectomized and normal rats with the results shown in table 2. There was an unmistakable stimulation of iron incorporation by the cobalt plasma. By mixing Co59 with the stock solution of CoCl2 and measuring the amount of isotope remaining in the plasma after 24 hours, we were able to show that about 0.4 per cent of the original amount of cobalt that had been injected was present in the 4 ml. of plasma that was used for assay. As might be inferred from the data in table 1, this amount of cobalt (0.6 μmoles) is too small to elicit a response of the magnitude seen in table 2, and the observed erythropoietic effect was therefore not attributable to free cobaltous ion. Similar controls will be described in later experiments.

The relationship between the erythropoietic activity of cobalt plasma and the amount of cobalt injected into donors was studied with the results shown in table 3. Three hundred-Gm. donor rats were given single injections of cobalt solution, with the exception of the highest dose which, because of its toxicity, was given in a divided dose, 2 hours apart. The plasma was drawn after 24 hours and assayed in hypophysectomized rats. The data from this experiment reveal a rough proportionality between the amount of cobalt given to donor animals and the response of recipient animals to the resulting cobalt plasma.

Time Course of Erythropoietin Production

The time course of the appearance of erythropoietic activity in this plasma of donor animals was determined. Each donor animal was given 75 μmoles of Co** in a single injection, and enough animals were bled at each time interval to yield a minimum of 20 ml. of
Table 3.—Effect of Amount of Co** Injected into Donor Rats on Erythropoietic Activity of Cobalt Plasma

(Assayed in hypophysectomized rats)

<table>
<thead>
<tr>
<th>µM Co** Injected</th>
<th>% of Fe** Incorporated</th>
<th>Difference from Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.6 ± 0.6</td>
<td>--</td>
</tr>
<tr>
<td>37.5</td>
<td>8.0 ± 3.3</td>
<td>5.4</td>
</tr>
<tr>
<td>75.0</td>
<td>10.6 ± 6.1</td>
<td>8.0</td>
</tr>
<tr>
<td>150.0</td>
<td>15.9 ± 3.1</td>
<td>13.3</td>
</tr>
</tbody>
</table>

Fig. 1.—Time course of erythropoietin formation after cobalt stimulation. Each point represents the average of values obtained from a group of 5 assay rats.

plasma. The cobalt plasmas were then assayed in starved rats with the results shown in figure 1. After a lag period of about 4 hours, there was a sharp rise in the content of erythropoietin in the cobalt plasma. The maximum titer was reached at about 12 hours. A curve derived from these data could be indicative of the action of either of two mechanisms. A storage site of the hormone may be stimulated for a short time to produce the factor, at an accelerated rate, which has a short half-life in the plasma. This explanation seems less likely in view of the lag period and, more importantly, the known effect of cobalt in producing a true polycythemia. Further evidence that cobalt exerts its effect by stimulating synthesis is provided by additional data showing that 72 hours after an initial stimulus with cobalt, the erythropoietin titer of the plasma had returned to control levels. If, however, the animals were given another injection of cobalt and the plasma was sampled 6 hours later, the titer rose to about the same level as it had 6 hours after the first injection of cobalt.

That the effects of cobalt plasma as seen in figure 1 were not caused by residual amounts of cobalt in the plasma can be seen by examining the curve marked cobalt control. The amount of cobalt present in the plasma was determined by means of a tracer quantity of Co** as described above. An amount of unlabeled cobalt, calculated from the Co** content to be in the plasma at each time interval, was then mixed with normal plasma. This plasma-plus-cobalt was used as a control for cobalt plasma containing the same amount of the ion. The stimulated incorporations of Fe** seen were, therefore, not due to cobaltous chloride.
Table 4.—Effect of Metal Ions on Formation of Plasma Erythropoietin

<table>
<thead>
<tr>
<th>Metal Salt</th>
<th>Amount Injected into Donor Rats (μM)</th>
<th>% of Fe&lt;sup&gt;59&lt;/sup&gt; Incorporated by Recipient Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>MnSO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>186</td>
<td>4.5 ± 0.9</td>
</tr>
<tr>
<td>NiCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>114</td>
<td>3.4 ± 0.5</td>
</tr>
<tr>
<td>Fe&lt;sup&gt;+++&lt;/sup&gt; Ammonium Citrate</td>
<td>317</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>FeSO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>57</td>
<td>3.8 ± 1.0</td>
</tr>
<tr>
<td>Co(NH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;6&lt;/sub&gt;Cl&lt;sub&gt;3&lt;/sub&gt;</td>
<td>57</td>
<td>8.7 ± 2.0</td>
</tr>
<tr>
<td>CoCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>57</td>
<td>11.9 ± 3.2</td>
</tr>
<tr>
<td>Saline</td>
<td>—</td>
<td>4.6 ± 0.6</td>
</tr>
</tbody>
</table>

The possibility remains that some form of cobalt other than free cobaltous ion may be responsible for the erythropoietic effect. Crafts has shown that microgram quantities of vitamin B<sub>12</sub> have no effect on erythropoiesis in hypophysectomized animals, and we have found that as much as 1.0 mg. of B<sub>12</sub> does not stimulate incorporation of Fe<sup>59</sup>. When erythropoietin has been completely characterized, it will be possible to determine whether the material in cobalt plasma is identical with that in plasma from anemic animals. Evidence has been accumulated showing that some of the gross properties of the active principles in the two types of plasma are similar. The erythropoietic activity contained in both anemic and cobalt plasmas is, in large part, retained after precipitation of the major portion of the plasma proteins by either heat denaturation or by precipitation with perchloric acid. In addition, extracts of both types of plasma retain activity after prolonged dialysis and lyophilization.

**Effect of Bleeding on Erythropoietin Production**

A single massive bleeding acts in the same manner as cobalt, as a stimulus to increase plasma erythropoietin. In this experiment, 8 ml. of blood was removed from each donor animal (300 Gm.) by cardiac puncture, with a resulting hematocrit of 27. At the indicated intervals, each group of rats was then bled as a source of plasma, and the plasma assayed in starved rats (fig. 1). While the observed stimulus is considerably less than that seen after repeated bleedings or after cobalt, it does follow a time course similar to that seen with cobalt plasma, although the peak of erythropoietin titer is reached sooner.

**Effect of Other Metal Ions on Erythropoietin Production**

The effect of other heavy metal ions, as well as that of a cobaltic complex ion, was studied to determine whether the stimulation of erythropoietin synthesis was specific for cobaltous ion or, perhaps, due to general heavy metal toxicity. The metal ions were chosen because of their chemical or toxicologic similarity to cobalt. The data in table 4 indicate that other toxic heavy metals have no effect in stimulating the production of erythropoietin. Surprisingly, cobaltic hexamine chloride did have some effect, which may possibly be due to reduction of the complex ion, but evidence of this is not yet available.

**DISCUSSION**

These data show that the erythropoietic effect produced by cobaltous ion is probably caused by an increase in circulating plasma erythropoietin. The mechanism by which cobalt increases the production of erythropoietin is as obscure as the mechanism by which bleeding or phenylhydrazine does the same thing. We would suggest that production of erythropoietin is stimulated by a relative anoxia, and that cobalt may act by producing an anoxic state in the kidney rather than in the bone marrow.

These findings are of interest from the clinical point of view. If, as our studies indicate, the erythropoietic effect of cobalt is based upon an increase in plasma
erythropoietin, then the clinical usefulness of the hormone is obvious. There are a number of disease conditions and experimental states in which the associated anemia is reported to be influenced beneficially by the administration of cobalt. Cobalt will favorably influence the anemia of hypophysectomy,\(^1\) protein deficiency,\(^1\) chronic renal disease,\(^1\) chronic inflammation,\(^1\) and cancer.\(^1\) We have demonstrated that plasma made rich in erythropoietin by the use of cobalt is as effective in producing an erythropoietic response in hypophysectomized rats, starved rats, normal rats, and transfusion-induced polycythemic mice\(^2\) as is plasma from animals that have been bled or that have been given phenylhydrazine.

The rapidity of the response to cobalt (and to phlebotomy) is important in the search for the site of production of erythropoietin. From our experience and that of others, it is already known that removal of the pituitary, thyroid, adrenals, or gonads, does not diminish the production of erythropoietin in response to bleeding or phenylhydrazine.\(^1\) Extracts of various organs do not yield materials with erythropoietic activity either.\(^1\) Because of the rapid death that follows hepatectomy and nephrectomy, we were unable to use the usual bleeding or phenylhydrazine schedule to study erythropoietin production in these two experimental states. In preliminary experiments, we have used the rapid response to cobalt to show that removal of 85 to 90 per cent of the liver does not interfere with erythropoietin production. Removal of both kidneys, however, suppresses or eliminates the production of erythropoietin after the administration of cobalt or after acute hemorrhage.\(^2\)

**SUMMARY**

It has been shown that plasma from animals that have been injected with cobaltous chloride rapidly develops a high titer of erythropoietin. The gross properties of the active material appear to be the same from cobalt-treated as from phenylhydrazine-treated animals. Other metal ions and a complex ion have been studied as stimulants for erythropoietin formation; none was as effective as cobaltous ion with the exception of cobaltic hexamine.

**SUMMARIO IN INTERLINGUA**

Es monstrate que le plasma de animales subjicite a injectiones de chloruro cobaltose disveloppa rapidemente un alte titro de erythropoietinsa. Grossiermente le proprietates del substanzia active pare esser le mesnse in le caso de animales tractate con cobalt que in le caso de animales tractate con phenylhydrazina.

Altere iones metallic e un ion complexe esseva studiate como stimulantes del formation de erythropoietina. Nulle, con le exception de hexamina cobaltic, habeva le mesme grado de efficacia como le ion cobaltose.

**REFERENCES**

2 Schultze, M. O.: Metallic elements and blood formation. Physiol. Rev. 29: 37, 1940.
STUDIES ON ERYTHROPOIESIS. V

Studies on Erythropoiesis: V. The Effect of Cobalt on the Production of Erythropoietin

EUGENE GOLDWASSER, LEON O. JACOBSON, WALTER FRIED and LOUIS F. PLZAK