Direct Effects of Thyroid Hormones on Bone Marrow Erythroid Cells of Rats

By Luis A. Malgor, Carlos C. Blanc, Elisa Klainer, Susana E. Irizar, Pedro R. Torales, and Lilian Barrios

A stimulatory effect on bone marrow cellularity was observed in normal and nephrectomized rats continuously infused with T3 and T4. Results of bone marrow studies are expressed in absolute numbers of total nucleated erythroid cells per milligram of femoral marrow at the beginning and after 8 hr of continuous intravenous infusions. Administration of T3 and T4 to nephrectomized rats produced a marked and significant increase in total erythroid cells counted. After differential analyses of the nucleated erythroid elements, a significant increase in all erythroid cell types was also observed. Similar results were seen in a control group of rats in which both ureters have been previously ligated and in groups of nephrectomized rats receiving rabbit antiserum against erythropoietin before starting the intravenous infusions of T3 and T4. These results indicate that stimulation of marrow erythropoiesis produced by thyroid hormones in our system is not dependent on renal or extra-renal production of erythropoietin. The progressive introduction of T3 and T4 into the circulation of rats with bilateral nephrectomy or ureter-ligated normal rats, may overload the mechanism of transport of these hormones in plasma. As a consequence, a progressive increase in free active forms of T3 and T4 in plasma may occur. Our interpretation of the present findings is that thyroid hormones stimulate directly bone marrow erythropoiesis. This stimulation is clearly evident when high levels of free active forms of thyroid hormones are present in plasma.

It has been reported that thyroid hormones stimulate erythropoiesis in laboratory animals. The association of anemia with hypothyroidism in human patients has been recognized repeatedly. In addition, thyroidectomy is associated with decreased red cell production, while administration of thyroid hormones is associated with increased erythropoiesis.

Most workers have correlated the erythropoietic effects of thyroid hormones with their calorigenic properties. It has been proposed that a parallel exists between the level of oxygen consumption and the degree of erythropoiesis, and that thyroid hormones stimulate production of red cells through an increase in oxygen need. Erythropoietin (ESF) has been firmly established as the most important regulator of red cell production. It has been postulated that ESF elaboration is inversely related to the oxygen tension in a critical renal cell. Thyroid hormones may stimulate erythropoiesis through a renal hypoxic mechanism by increasing the elaboration of endogenous ESF. In this regard, Peschle et al. showed that daily administration of thyroxine to normal mice produced an increase in the ESF content of the plasma, as well as in the elaboration of
renal erythropoietin, a factor which converts a plasma ESF precursor into active ESF. Other reports have also been made suggesting that the erythropoietic effects of thyroid hormones are ESF dependent.\textsuperscript{14,15}

However, other investigators have suggested that thyroxine and triiodothyronine influence erythropoiesis through a noncalorigenic effect.\textsuperscript{16,17} Chronic administration of thyroxine in normal rats induces polycythemia, and no erythropoietic stimulant can be found in the plasma of these animals.\textsuperscript{2} Another report\textsuperscript{18} also indicates that oxygen utilization and erythropoiesis did not increase to a similar degree in triiodothyronine-treated rats. Rats given \(D\) and \(L\)-triiodothyronine showed similar increases in \(^{59}\text{Fe}\) incorporation in red cells in spite of the fact that these hormones have quite different effects on oxygen utilization. These data can be interpreted as evidence of a probable direct action of thyroid hormones on erythropoiesis.

In the present report, we describe the effects of continuous intravenous infusions of thyroid hormones on bone marrow erythropoiesis in normal and nephrectomized rats. A method\textsuperscript{19} first developed to study the in vivo effects of ESF on bone marrow erythroid cells of normal rats was used.

**MATERIALS AND METHODS**

Albino male rats, 300-350 g in weight, were used in these studies. All experiments were carried out under pentobarbital anesthesia (30 mg/kg). A small plastic cannula was placed in the jugular vein of each rat before starting the experiments. Sigma l-thyroxine (T\(_4\)) and 3,3',5-triiodo-l-thyronine (T\(_3\)) sodium salts were dissolved in 0.1 \(N\) NaOH solution, placed in 0.85% saline solution, and adjusted to pH 9.0 before administration. A group of normal rats was continuously infused intravenously through the jugular vein for 8 hr with either saline or T\(_3\) at a rate of 9.75 \(\mu\)g/hr/100 g or T\(_4\) at a rate of 30 \(\mu\)g/hr/100 g. A second group of animals was previously nephrectomized bilaterally and also infused intravenously with saline, T\(_3\), or T\(_4\) at the same rates. Two separate groups of nephrectomized rats infused with T\(_3\) or T\(_4\) received, in addition, 0.33 ml of rabbit antiserum against ESF. Antibodies were injected subcutaneously 30 min before starting the infusions. The neutralizing capacity of the anti-ESF used in the present experiments was 60 U of ESF (Standard B) per ml of antiserum. Antibodies were prepared in accordance with the scheme of immunization of Dorado et al.\textsuperscript{20} and were also tested against rat endogenous ESF. A similar capacity for the neutralization of erythropoietic activity was seen. Finally, a control group of ureter-ligated normal rats, infused intravenously with T\(_4\) at the same rate, was also used.

A Harvard infusion pump was used to administer approximately 2.0 ml of solution to each rat during the 8-hr period. Prior to starting the intravenous infusions, the left femur of each rat was surgically removed and cleaned; its two ends were cut off, and the total bone marrow tissue was carefully withdrawn. A method similar to that described by Yoffey et al.\textsuperscript{21} was used. A sample of the fresh marrow was used for the preparation of smears and stained with May-Grünwald-Giemsa. The remainder was weighed carefully into a humidity chamber. A sample of 30 mg of bone marrow was diluted with 0.5 ml of 2% acetic acid, completely transferred to a small ground glass homogenizer, and homogenized slightly. To avoid damage of the bone marrow nucleated cells, a pestle homogenizer which had completely lost its fit was used. The pestle was operated slightly up and down by hand not more than three times. Microscopic examination of samples of the marrow cell suspensions obtained showed no appreciable destruction of nucleated cells after this procedure. Bone marrow nucleated cells were counted with a hemocytometer counting chamber. Total nucleated cells per milligram of bone marrow were calculated from these hemocytometer counts in accordance with the method described by Rekers and Coulter.\textsuperscript{22} Bone marrow differential analyses were also performed, and the total nucleated erythroid cells per milligram of bone marrow was determined.

The contralateral femurs were removed at the end of experiments, and the same bone marrow studies were performed. In all cases, results of the 0 hr, initial time, were compared with results
obtained after 8 hr of intravenous infusions in the same animal. The mean and the standard error of the mean in each group of animals were determined.

The t test for paired comparisons (0 hr versus 8 hr) was used in the statistical analyses.

Hemoglobin, hematocrit, number of leukocytes, and per cent of reticulocytes in peripheral blood were also determined at the beginning, as well as at the end, of each experiment.

RESULTS

Figure 1 compares results of the nucleated bone marrow erythroid cells in all groups of normal rats continuously infused for 8 hr. Results are expressed as the per cent change in total erythroid cells, pronormoblasts, basophilic normoblasts, polychromatic normoblasts, and orthochromatic normoblasts after the 8-hr period. A slight increase in total erythroid cells counted was seen in normal rats infused with T4 (14.5 ± 8.0%). The increase observed was more evident in normal animals infused with T3 (33.9 ± 22.1%), but differences are not significant. The absolute numbers of total erythroid cells per milligram of femoral marrow was 404.653 ± 51.455 at the beginning and 498.362 ± 52.036 at the end of experiments in the group of normal animals infused with T3. As indicated in this figure, no appreciable change in total nucleated erythroid cells was observed in the control group of normal rats infused with saline. Figure 1 also shows the per cent change of the four basic nucleated erythroid cells within the femoral marrows of the normal animals. As indicated, no significant change in any erythroid cell type was noted in the group of normal rats infused with saline or thyroid hormones.

Table I shows results of the erythroid bone marrow studies in nephrectomized rats infused for 8 hr with saline, T4, or T3. Results are expressed in absolute numbers of total nucleated erythroid cells per milligram of femoral marrow at the beginning (0 hr) and after 8 hr of intravenous infusions. In order
<table>
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<tr>
<th>Continuous Intravenous Infusions</th>
<th>Total Erythroid Cells</th>
<th>Pronormoblasts</th>
<th>Basophilic Normoblasts</th>
<th>Polychromatic Normoblasts</th>
<th>Orthochromatic Normoblasts</th>
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Number in parenthesis indicates number of rats in each group. T₄, 1-thyroxine; T₃, 3,3',5-triiodo-L-thyronine; anti-ESF, rabbit antiserum against erythropoietin injected 30 min before starting the continuous intravenous infusions; p, level of significance; N.S., not significant.
to determine the particular erythroid cell types which were predominantly changing during the intravenous infusions, a differential analysis was made of the nucleated erythroid cells from bone marrow of the nephrectomized animals. Results are also expressed in absolute numbers of pronormoblasts, basophilic, polychromatic, and orthochromatic normoblasts. A control group of ureter-ligated rats infused with T₄ for 8 hr was also prepared, and the same bone marrow studies were performed. In addition, Table 1 shows the absolute numbers of nucleated erythroid elements from femoral marrows of nephrectomized rats pretreated with antibodies against ESF and infused for 8 hr with T₄ and T₃. In all cases, results of the initial preinfusion time are compared with results obtained after 8 hr of infusions in the same group of animals.

As demonstrated in Table 1, continuous intravenous infusions of T₄ and T₃ in nephrectomized rats produced a marked and significant increase in total bone marrow nucleated erythroid cells after 8 hr. The per cent increase observed in the group of nephrectomized rats infused with T₄ was 30.7 ± 6.0%.

A more pronounced increase was observed in the group of nephrectomized rats infused with T₃ (55.8 ± 19.1%). A similar, and also significant, increase in total erythroid cells counted was seen in those nephrectomized rats infused with T₄ (38.2 ± 8.5%) and T₃ (69.1% ± 19.2%) which had been previously injected with antibodies against ESF.

The group of rats in which both ureters had been previously ligated also demonstrated a marked and significant increase in total erythroid cells after 8 hr of continuous intravenous infusions with T₄.

Total nucleated erythroid cells counted in femoral marrows from nephrectomized rats infused with saline showed no appreciable difference from the initial preinfusion control.

Table 1 also shows the differential analyses of nucleated erythroid cells from bone marrows of nephrectomized or ureter-ligated rats continuously infused for 8 hr with saline, T₄, or T₃.

Nephrectomized rats infused with T₄ showed a significant increase in all four erythroid cell types. A similar significant increase in pronormoblasts, and basophilic and polychromatic normoblasts was observed in nephrectomized rats infused with T₄ and pretreated with anti-ESF, and also in the group of normal rats in which the ureters were previously ligated. In the latter two groups, a marked but not significant increase was seen in orthochromatic normoblasts.

Nephrectomized rats infused with T₃ for 8 hr showed a significant increase in pronormoblasts and basophilic normoblasts. The marked increase observed in polychromatic and orthochromatic normoblasts was not significant after the 8-hr period. However, nephrectomized rats receiving T₃ which have been previously injected with antibodies against ESF showed a significant increase in all types of nucleated bone marrow erythroid cells after 8 hr of continuous intravenous infusions.

The most pronounced change was always seen in pronormoblasts and basophilic normoblasts in nephrectomized rats infused with T₄ or T₃. The per cent change in pronormoblasts was 105.65 ± 15.00%, after the 8-hr period in the group of nephrectomized animals infused with T₄, and 199.67 ± 69.00% in the T₃-treated group of nephrectomized rats. Similar results in per cent change were
observed in nephrectomized rats infused with T₄ and T₃ which were previously injected with anti-ESF, and in the control group of ureter-ligated rats.

A moderate increase in per cent of reticulocytes in peripheral blood was seen in all groups of nephrectomized rats and in the group of normal rats with ligated ureters after the 8 hr of infusions with T₄ or T₃. This moderate increase in reticulocyte count was statistically significant when values of the preinfusion time were compared with results obtained at the end of the experiments: in the group of nephrectomized animals infused with T₄, the mean rose from 1.08 ± 0.29 to 1.85 ± 0.18% (p < 0.05), in the group of nephrectomized rats pretreated with anti-ESF and infused with T₄ the mean rose from 1.01 ± 0.23% to 1.82 ± 0.25% (p < 0.05), and in the group of nephrectomized rats infused with T₃ and pretreated with antibodies to ESF the mean rose from 1.33 ± 0.19% at 0 hr to 1.71 ± 0.20% at 8 hr (p < 0.01). The increase observed in per cent of reticulocytes in peripheral blood in the group of nephrectomized animals infused with T₃ and in the group of normal rats in which both ureters were ligated was not significant. These changes in reticulocyte count observed after the infusion period were not seen in nephrectomized rats infused with saline nor in any group of normal rats.

Hemoglobin, hematocrit, and number of leukocytes in peripheral blood were also determined at the beginning and after 8 hr of infusions in all groups of experimental animals. No significant differences were seen in hemoglobin, hematocrit, or number of leukocytes in all groups of rats when compared with the initial preinfusion findings.

DISCUSSION

The present studies demonstrate that thyroid hormones are capable of stimulating the bone marrow erythroid compartment directly in nephrectomized rats. Considerable evidence has been accumulated to indicate that thyroid hormones stimulate erythropoiesis. However, the primary site of action and the exact mechanism of T₄ and T₃ in stimulating erythropoiesis is not presently known. It has been postulated that thyroxine stimulates red cell formation by increasing oxygen consumption. A noncalorigenic effect on erythropoiesis has been demonstrated. In addition, it has been postulated that thyroid hormones exert their erythropoietic actions through a previous stimulation of kidney erythropoietin production.

In the present report, we have compared the stimulating effects of continuous infusions of thyroid hormones for 8 hr on bone marrow erythroid cells of normal and nephrectomized rats. It is important to point out that results of the bone marrow observations were obtained as absolute numbers of erythroid cells per milligram of bone marrow, which is a more direct experimental model for evaluating influences of drugs and hormones on marrow cellularity. In addition, results were obtained in the same animal before and after intravenous infusions of thyroid hormones. After differential analyses, it was possible to determine the absolute numbers of pronormoblasts, and basophilic, polychromatic, and orthochromatric normoblasts per milligram of femoral bone marrow before and after thyroid hormone administration. In previous works, we have presented our studies of the effects of intravenous infusions of ESF, dexamethasone, and testosterone on femoral marrow erythroid cellularity in the
whole animal, and results indicate that our system is sufficiently sensitive to properly respond to an erythropoietic stimulation.

This is apparently the first report of a demonstration of a direct stimulatory influence of thyroid hormones on erythropoiesis. It seems clear that administration of T4 and T3 to normal rats produced an increase in total bone marrow erythroid cells. However, this increase was not significantly different from the initial preinfusion control. Administration of T4 and T3 to nephrectomized rats produced, on the contrary, a marked and significant increase in marrow erythroid cells. As has been demonstrated for other hormones, results obtained in normal rats could be due to an augmentation in kidney ESF production, which then stimulates bone marrow erythropoiesis. In this regard, we have previously found23a4 that administration of testosterone and dexamethasone by our system produced a marked increase in femoral marrow erythropoiesis in normal rats. However, the erythropoietic effects of testosterone and dexamethasone were completely abolished in rats with bilateral nephrectomy. In addition, simultaneous administration of testosterone and dexamethasone with antisera against ESF resulted in a complete inhibition of the erythropoietic effects of these hormones in normal rats. These results demonstrate that testosterone and dexamethasone stimulate erythropoiesis indirectly, through a previous stimulation of kidney ESF production.

In the present experiments, bilateral nephrectomy did not abolish the stimulatory effects of T4 and T3 on erythropoiesis. On the contrary, the erythropoietic actions of thyroid hormones were markedly increased, and results of bone marrow determinations were significantly different when compared with initial controls. These results clearly indicate that stimulation of marrow erythropoiesis produced by T4 and T3, in our system, is not dependent on renal production of ESF. Although none of the work included in the present study is related to plasma concentration of thyroid hormones, it seems conceivable that concentrations of T4 and T3 may reach very high levels in the plasma of nephrectomized rats subjected to continuous intravenous infusions of T4 and T3. The progressive introduction of thyroid hormones into the circulation of rats with bilateral nephrectomy may also overload the normal mechanism of biotransformation of these hormones, which may contribute to the increased plasma concentrations of active forms of T4 and T3. It is well known that thyroid hormones are transported by specific plasma proteins. As a result of continuous administration by our method, combination of T4 and T3 with these proteins can rapidly reach saturation levels. As a consequence, a progressive increase in free forms of thyroid hormones in plasma may occur. This could explain the apparent discrepancy of our results with those obtained by other investigators, indicating a lack of increase in 59Fe incorporation in red cells of nephrectomized rats receiving daily single injections of triiodothyronine. Direct stimulation of bone marrow erythropoiesis may occur only when high levels of free active forms of thyroid hormones are present in plasma. It is possible that intravenous infusions of T4 and T3 in normal rats did not result in similar high levels in plasma. Results obtained in the group of normal rats in which both ureters had been previously ligated support the present interpretation. The kidneys may play a role in the metabolic transformation of thyroid hormones, or may have some influence on the erythropoietic effects of these hormones which are not
present in nephrectomized animals. However, continuous administration of $T_4$ and $T_3$ to ureter-ligated normal rats produced a similar and also significant stimulation of the bone marrow erythroid cellularity. Thus, elimination of the main route for the excretion of drugs and metabolites seems to be the most important factor in determining the effects observed.

The kidney is considered the main source of elaboration or activation of ESF. However, extrarenal mechanisms of ESF production appear to function in rats. Bilaterally nephrectomized rats consistently show significant ESF levels in plasma after exposure to acute hypoxia. Considering the high concentration levels that $T_4$ and $T_3$ can reach in plasma of nephrectomized rats after removal of the principal organ for drug excretion, one can hypothesize that thyroid hormones may stimulate production of ESF in extrarenal sites of elaboration. However, administration of antibodies against ESF did not inhibit the stimulatory effects of $T_4$ and $T_3$ on erythropoiesis. Similar results were observed in nephrectomized rats receiving only $T_4$ and $T_3$. Although rabbit antiserum against ESF was prepared from human urinary ESF, a similar neutralizing capacity was observed when antibodies were tested against rat endogenous ESF. It is also important to point out that rabbit antiserum against ESF administered to each rat was given in sufficient quantities to block the erythropoietic stimulant activity of 20 U (Standard B) of ESF. It is unlikely that extrarenal sites of ESF production in a rat produce such an amount of ESF in 8 hr. In addition, we have previously demonstrated that administration of antiserum against ESF completely blocks the effects of continuous infusions of approximately 20 U of ESF for 8 hr. Therefore, our interpretation of the erythropoietic stimulation observed after intravenous infusions of $T_4$ and $T_3$ in nephrectomized rats pretreated with anti-ESF is that thyroid hormones directly stimulate all types of erythroid cells in bone marrows.

It is interesting to note that the most pronounced action of $T_4$ and $T_3$ on bone marrow erythropoiesis, in our system, was usually seen in early erythroid cells, which may be interpreted as evidence for a stem cell effect of thyroid hormones. The increase in the number of late erythroid cells was relatively less than that seen in pronormoblasts and basophilic normoblasts. This observation suggests that thyroid hormones may act not only on the stem cell pool; they may also increase maturation of late erythroid elements. At present, we have no satisfactory explanation of the exact mechanism of action of thyroid hormones in stimulating bone marrow erythroid cells. A similar increase in all types of nucleated erythroid elements has been previously observed in femoral marrows from normal rats infused with ESF, which confirms previous observations on the effects of ESF in marrow erythroid cells in isolated perfused hind limbs of dogs.

The possible biologic significance of the present finding remains unclear. Nevertheless, the capacity of thyroid hormones to directly stimulate erythropoiesis in some conditions may be considered of potential therapeutic value.

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