In Vitro Stimulation of Primate Hemoglobin Synthesis by L-Thyroxine

By J. E. Fuhr, N. Gengozian, and M. Overton

Bone marrow cells from adult and abortus primates (marmosets) were incubated in vitro to determine their responsiveness to L-thyroxine. Incorporation of 3H-leucine into purified globin chains was the parameter assayed to determine responsiveness. Bone marrow from spontaneously aborted animals consistently was stimulated by the presence of physiologic levels of L-thyroxine. Bone marrow cells from adult animals were unaffected by the hormone.

Red cell production is generally believed to be controlled by the hormone erythropoietin. Although thyroid hormones also affect erythropoiesis, the action of these hormones is believed to be mediated by erythropoietin. There have, however, been a number of studies, both in vivo and in vitro, which have suggested that thyroid hormone may also have a direct effect upon erythroid precursors in the bone marrow. The present study has investigated the in vitro effect of L-thyroxine upon hemoglobin synthesis by erythroid precursors obtained from fetal and adult primates (marmosets).

Materials and Methods

Bone marrow and peripheral blood were obtained from adult and abortus marmosets, New World monkeys (Saguinus fuscicollis). The normal gestation period for marmosets is 4½ mo. The majority of the responsive marrow samples were from nonviable abortuses, approximately 3½ mo of gestational age. The bone marrow was obtained primarily from femora which had been removed from the animals, wiped clean of connective tissue, and cracked to expose the marrow. The bone marrow was suspended in commercial medium, NCTC 135 (Grand Island Biological, Grand Island, N.Y.). A final single cellular suspension was obtained by gently forcing the marrow suspension through a nylon mesh filter. The cell density was adjusted to between 4 × 10⁶ and 5 × 10⁶ cells/ml of NCTC 135. L-thyroxine was purchased from Calbiochem (Los Angeles, Calif.) and dissolved in 0.01 N NaOH. Control samples were reacted in equivalent amounts of 0.01 N NaOH. Reticulocytes were incubated in a balanced salt medium as described by Grayzel et al. All reactions were carried out in sealed tubes at 37°C for the times indicated in the text.

Reactions were terminated by plunging the incubation tubes into an ice-water slurry. After the cells had been washed twice with isotonic saline, they were suspended in 2.0 ml buffer (0.01 M Tris, pH 7.4; 0.01 M KCl; 0.0015 M MgCl₂) and homogenized with five strokes of the tight pestle in a Dounce homogenizer. Stroma were removed by centrifugation at 27,000 g for 20 min. Globin was prepared by precipitation in ten volumes of 1.2% acid (HCl)-acetone. The pellet was redissolved in water, frozen, and lyophilized. Globin chains were separated on CM-cellulose columns in 8.0 M urea with a linear gradient of 0.005 M phosphate to 0.03 M phosphate, pH 6.8, as described by Clegg et al. The method of determining specific activity of the isolated globin chains has been described.

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RESULTS

The addition of L-thyroxine to short term (180 min) incubations of bone marrow obtained from a marmoset abortus stimulated the synthesis of globin (Fig. 1). Both α- and non-α-globin chain synthesis was stimulated 48% and 33% above control levels respectively by the presence of $10^{-7}$ M L-thyroxine. The levels of stimulation increased to 82% and 42% when the hormone level was increased to $10^{-6}$ M. Because of the small amount of blood which could be obtained from marmoset abortuses, serum thyroxine (T₄) levels were not determined. On the basis of total serum T₄ values obtained in adult marmosets (4–6 μg/10 dl), it was assumed that the hormone levels tested were in the normal physiologic range.

A time-course study of the effect of $10^{-6}$ M L-thyroxine indicated that increased incorporation of ³H-leucine could be detected as early as 60 min after the start of the reaction (Fig. 2). By 120 min, the level of stimulation had increased to approximately 140% of the control value. The rate of incorporation

![Fig. 1. Effect of different concentrations of L-thyroxine on globin synthesis by marmoset abortus bone marrow. Reactions contained $4 \times 10^6$ cells/ml and lasted 180 min at 37°C. The hormone and the ³H-leucine were present from the start of the incubation. After the addition of carrier hemoglobin, globin was prepared and the specific activity of the separated chains was determined as described in the text.](image)

![Fig. 2. Marmoset abortus bone marrow response to 0.001 mM L-thyroxine. The reaction contained $5 \times 10^6$ cells/ml and lasted for the times indicated. Dashed lines represent specific activity of globin prepared from cells incubated in the presence of thyroxine. Solid lines represent specific activity of globin from control incubations.](image)
was virtually linear during the course of the incubation. The effect observed would suggest that the hormone increased the rate of globin synthesis, and did not merely extend the time during which the cells incorporated in a linear fashion.

Although we have been unable to distinguish two hemoglobins in the abortus blood, or to discriminate between hemoglobins from the adult and the abortus, we felt confident that the non-α globin of the abortus was not β globin, but was fetal γ globin. Analysis of the globin chain elution profile from marmoset abortuses (broken line) was compared to the elution profile of carrier globin prepared from human cord blood (solid line). The radioactivity (broken line) eluted in two distinct peaks which corresponded to the human β and α globin (Fig. 3). On subsequent samples, specific activity was determined on the fractional volumes obtained from the beginning (ascending limb) and the ending (descending limb) fractions of the chromatograms of each globin chain (Table 1). The close agreement between the two parts of each globin chain elution fraction suggested that the specific activity was determined on virtually homogeneous polypeptides. Since the peripheral blood of adult marmosets contained less than 1% alkali-resistant hemoglobin, whereas the peripheral blood of abortuses contained more than 80% alkali-resistant hemoglobin, we believed the homogeneous non-α-globin peak represented γ globin. Nonidentity of the

Table 1. Analysis of Globin Chain Specific Activity From Different Regions of the Column Eluate*

<table>
<thead>
<tr>
<th>Protein</th>
<th>cpm/OD280</th>
</tr>
</thead>
<tbody>
<tr>
<td>β Ascending</td>
<td>52,455</td>
</tr>
<tr>
<td>β Descending</td>
<td>52,911</td>
</tr>
<tr>
<td>α Ascending</td>
<td>48,695</td>
</tr>
<tr>
<td>α Descending</td>
<td>48,681</td>
</tr>
</tbody>
</table>

*Fractional volumes from the first half (ascending) and the second half (descending) of the globin peaks were separately pooled and compared for specific activity.
adult β globin and abortus γ globin was further suggested by the apparent difference in relative absorbance. This difference was demonstrated by comparison of the globin chain profiles from adult and abortus hemoglobin (Fig. 4), in particular, the relative heights of the α and β globin and the α and γ globin peaks. In addition, human and marmoset α and β globin have been shown to lack the amino acid, isoleucine, and their synthetic rates have therefore been relatively insensitive to the isoleucine antagonist, o-methyl threonine. As shown in Table 2, however, o-methyl threonine exerted a significant influence upon globin synthesis by reticulocytes from marmoset abortuses, while it had only a

<table>
<thead>
<tr>
<th>Reticulocyte Source</th>
<th>Addition</th>
<th>β/γ</th>
<th>α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>None</td>
<td>75,434</td>
<td>101,317</td>
</tr>
<tr>
<td>Adult</td>
<td>10 mM OMT</td>
<td>64,690</td>
<td>85,755</td>
</tr>
<tr>
<td>Abortus</td>
<td>None</td>
<td>598,357</td>
<td>579,375</td>
</tr>
<tr>
<td>Abortus</td>
<td>10 mM OMT</td>
<td>333,946</td>
<td>303,370</td>
</tr>
</tbody>
</table>

*Cells were reacted for 60 min at 37°C. *H-leucine was the radioactive precursor.
*Carrier hemoglobin was added to bone marrow samples when cells were lysed at the end of the incubation. Reactions lasted 180 min at 37°C. Bone marrow reaction contained $4 \times 10^6$ cells/ml.
DISCUSSION

These results are consistent with the conclusion that under the stated conditions, L-thyroxine preferentially exerts a stimulatory influence upon the synthesis of hemoglobin by fetal primate bone marrow. It is not possible, however, to conclude whether the stimulation is due to preferential gene stimulation or to differences in membrane permeability between fetus and adult. It is also not possible to conclude that the effect is at the level of transcription because it has only been observed in bone marrow and not in reticulocytes. Permeability changes during erythroid maturation may prevent the hormone from crossing the reticulocyte membrane and thus exerting an effect at the level of translation. Krause and Sokoloff\textsuperscript{9} have reported that hemoglobin synthesis by reticulocyte cell-free systems can be stimulated by L-thyroxine when liver mitochondria are present in the reaction mixture. Fuhr and Medici (unpublished observation) also have found that mitochondria from reticulocytes are capable of replacing liver mitochondria in the reaction mixture. Our working belief that the present stimulation of fetal globin synthesis is due to a selective effect, rather than a more generalized one, is based in part on earlier unpublished observations. Bone marrow obtained, for diagnostic purposes, from a premature infant has been incubated in the presence of L-thyroxine. Analysis of the globin synthesized has revealed that the synthesis of $\gamma$ globin is stimulated more than threefold, whereas the synthesis of $\beta$ globin is increased only 10%. If the L-thyroxine effect had been mediated by an increase in heme production, or by a general increase in metabolic activity, we might have expected an approximately equal response in the production of $\beta$ and $\gamma$ globin. We are presently attempting to confirm this interpretation in the marmoset bone marrow. These observations are, however, consistent with the selective effect on fetal bone marrow demonstrated in this report.

Furthermore, although studies on the heme synthetic pathway in abortus tissue have not as yet been performed, we have established a dose response curve to exogenous hemin for adult marmoset bone marrow. Thus it is unlikely that the difference in responsiveness between adult and abortus tissue can be attributed to different levels of heme saturation in the two tissues.

Recently reported studies have revived the concept that thyroid hormone may have a direct effect upon red cell production.\textsuperscript{10,11} The present study, in particular, supports the conclusion of Golde et al.,\textsuperscript{11} who have observed potentiation of the erythropoietin effect upon the erythroid progenitor cell in colonies of fetal mouse hematopoietic cells. Concentrations of the hormone assayed in the two studies are also in the same range.

The results of Malgor et al.,\textsuperscript{10} on the other hand, indicate that adult rat bone marrow may be responsive to thyroid hormone. In that instance, however, the animals have been exposed to a continuous infusion of large amounts of thyroid hormone. Presumably, therefore, the concentrations of hormone have been significantly higher than those used in either study of fetal tissue responsiveness. This difference may explain the apparent discrepancy with the results of this study in which bone marrow from adult marmosets has been unresponsive to the hormone levels tested. In general, however, the present results are in agree-
ment with earlier reports that under the appropriate conditions, L-thyroxine can influence in vitro the metabolism of erythroid precursors in the marrow. Purification of the globin chains synthesized in vitro confirms the conclusion that the erythroid elements in the marrow respond to the hormone. The molecular level of the effect and the difference between abortus and adult tissue, however, must be examined further, since alterations in membrane permeability cannot as yet be discounted.

REFERENCES

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