Mechanism of Erythropoietic Action of Thyroid Hormone.* (31509)

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The stimulating effect of thyroid hormone on erythropoiesis is well documented(1-7), but the mechanism of action has remained largely conjectural. Erythropoietin has been firmly established as the principal regulator of erythropoiesis(8), and the basic question remains as to whether thyroid hormone increases erythropoietin formation or exerts an erythropoietic effect which is distinct and independent of erythropoietin regulation. Evans and co-workers found no erythropoietin in the plasma of rats after prolonged administration of thyroid hormone(5), and equivocal results were obtained in our laboratory, in assaying the plasma of rats after 5 daily injections of 250 μg/kg of triiodothyronine (T_3). The present study was therefore designed to investigate the alternative mechanism, namely an erythropoietic effect of thyroid hormone which is unrelated to erythropoietin formation. A suppression of the latter was induced in mice by hypertransfusion and in rats by bilateral nephrectomy. The erythropoietic effect of T_3 was measured in these animals without or with additional injection of erythropoietin. The former permitted an assessment of the effects of T_3, or its intermediate, on the induction of stem-cell differentiation. Administration of erythropoietin induced a controlled number of stem-cells to differentiate into pronormoblasts, and the additional injection of T_3 provided information on its possible augmenting effect on this process and/or proliferation and maturation of erythroid cells.

Methods. Preparation of erythropoietin from anemic rabbit plasma and techniques of Fe^{59} incorporation were as described earlier (9). Virgin mice (20-23 g) were made polycythemic by i.p. injection of 0.6 ml washed isologous red cells on day 0 and 1. Groups 4 and 6 (Table I) received 5 μg T_3 daily s.c. on day 2 through 6; Groups 3 and 5 received saline instead. Groups 5 and 6 received 0.3 units erythropoietin s.c. on day 5. All groups were given 0.5 microcuries Fe^{59} i.v. on day 7 and the specific activity in circulating red cells was measured 48 hours later.

TABLE I. Effect of T3 (5 × 5 μg/day) on Fe^{59} Incorporation in Normal Mice and After Suppression of Erythropoietin Formation by Hypertransfusion Polycythemia. (8 mice each group, mean and S.E.M.)

Group	Hematocrit %	% Fe^{59} incorporation
1 Normal	46 ± 2.4	22.4 ± 1.7
2 " + T3	47 ± 1.6	35.0 ± 1.8
3 Hypertransfused	68 ± .9	.4 ± .02
4 " + T3	68 ± 1.1	.3 ± .02
5 Hypertransfused + 0.3 U. erythropoietin	66 ± 1.2	12.2 ± .98
6 Hypertransfused + 0.3 U. erythropoietin + T3	69 ± .8	13.0 ± 1.59

The time-course of Fe^{59} incorporation after a single i.p. injection of 0.5 unit erythropoietin was measured in 6 groups (6 mice each) of polycythemic mice receiving 5 μg T_3 daily on 3 days before and on 2 days after erythropoietin, and in 6 groups which received saline instead. Fe^{59} was injected i.v. in one group of T_3 and of control mice at 0, 24, 48, 72, 96, and 120 hours, respectively, after erythropoietin. The specific activity in circulating red cells was measured in each instance 24 hours later.

Experiments presented in Table IV were made on female Sprague-Dawley rats (250-300 g) placed for 14 days *ad libitum* on Protein Depletion Diet (Nutritional Biochemicals Corp.). Bilateral nephrectomies were carried out through flank incisions. Controls were subjected to flank incision only. Postoperatively, all rats received the diet (20 Cal/day) by stomach tube. The nephrectomized rats were subjected to peritoneal dialysis (30 ml Peridial) twice daily. Groups 2, 4, and 6 received from the first to the fifth p.o. day injections of 400 μg T_3 per kg. Groups 5 and

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TABLE II. Effect of T3 on Erythroid Marrow Transit Time. Fe^{59} Was Injected and Its Incorporation Measured at Time Indicated After Single i.p. Injection of Erythropoietin in Groups of Polycythemic Mice. (6 mice each group, mean and S.E.M.)

	Percent Fe^{59} incorporation during hours after erythropoietin					
	0-24	24-48	48-72	72-96	96-120	120-144
No T3	1.5 ± .4	3.6 ± .8	15.5 ± 1.6	9.0 ± 1.1	1.9 ± .8	1.4 ± .9
5 × 5 μg T3	1.9 ± .9	2.6 ± .5	16.6 ± 2.0	9.7 ± .9	1.6 ± .8	.9 ± .8

6 received 2 units erythropoietin on day 2. Oxygen consumption (12 hours fasting) was measured during 3-hour periods on day 4 in a closed system at 21°C environmental temperature. O_2 volumes are expressed at S A T. All groups were given Fe^{59} i.v. on the fifth day and their iron incorporation ascertained 24 hours later.

Results. Five daily injections of 250 μg/kg of T_3 raised the 24-hour Fe^{59} incorporation in normal mice from 22 to 35% (Table I). The same dosage had no effect upon the iron incorporation of mice whose hematocrit had been raised to 68% by transfusion of packed red cells. Serial bone marrow examinations at 18, 36, and 48 hours after T_3 injection showed no erythroid cells, indicating that T_3 did not induce the differentiation of stem-cells into pronormoblasts in these plethoric mice. The injection of 0.3 unit of erythropoietin restored an erythroid population, but the additional injection of T_3 did not increase the resulting iron incorporation, indicating that T_3 had no direct effect upon erythroid proliferation rate.

The effect of T_3 on maturation time and on emergence of newly formed red cells from the marrow was assessed by serial iron incorporation measurements after a single injection of erythropoietin in groups of polycythemic mice. The first group was injected with radio-iron at zero hour after erythropoietin, and the radio-iron present in circulating red cells was measured 24 hours later. As seen in Table 2, no significant incorporation took place because the erythroid cells, whose development was induced by erythropoietin injection, had not reached the reticulocyte stage and were not released into the circulation within this period of time. Maximal iron incorporation occurred during the 48 to 72 hour measurement, whereas incorporation returned to baseline values when Fe^{59} was injected 96 hours after injection of erythropoietin, indicating that the induced wave of erythropoiesis was terminated by that time. Administration of T_3, given daily from 4 days before to 2 days after the erythropoietin injection, did not alter the time-course of iron incorporation, indicating that the T_3 had no effect upon the speed of erythroid cellular development.

Experiments presented in Table III show that the erythropoietic effect of T_3 was restored when polycythemic mice were exposed for 6 hours at an ambient pressure of 300 mm Hg. The exposure raised Fe^{59} incorporation in T_3 injected polycythemic mice to 9.9 ± 1.1% *versus* 6.8 ± .6 in controls without T_3 ($p<0.05$).

In a second experimental series, endogenous erythropoietin was suppressed in rats by means of bilateral nephrectomy. Protein-depleted rats were used because their already depressed erythropoietin formation(10) obviated the waiting period of several days that is otherwise necessary to permit the bone marrow to adjust to the much reduced erythropoietin formation after nephrectomy. As shown in Table IV, T_3 injection raised the metabolic rate in nephrectomized rats, but the marked erythropoietic effect of T_3 seen in controls was absent. This lack of response was not due to an inability of nephrectomized animals to form red cells, because the injection of erythropoietin raised their Fe^{59} incorporation from 8.8 to 18.3%. The com-

TABLE III. Effect of 6-Hour Exposure at 300 mm Hg on Fe^{59} Incorporation of Plethoric Mice Without and With 5 μg T_3 per Day. (Mean and S.E.M.)

	No. of mice	Hematocrit (%)	Fe^{59} incorp (%)
Without T_3	10	69 ± 1.6	6.8 ± .6
With T_3	10	70 ± 1.2	9.9 ± 1.1

TABLE IV. Effect of T3 on BMR and Fe^{59} Incorporation in Protein Deprived Rats With and Without Bilateral Nephrectomy. (12 rats each group, mean and S.E.M.)

Group	BMR L. $O_2/m^2/h$	% Fe^{59} incorporation
1 Sham operated	5.36 ± .15	10.7 ± 1.3
2 " " + T3	8.19 ± .29	28.8 ± 1.9
3 Nephrectomized	5.90 ± .25	8.8 ± .8
4 " + T3	7.60 ± .70	10.5 ± 1.3
5 Nephrectomized + 2 U. erythropoietin	—	18.3 ± 1.3
6 Nephrectomized + 2 U. erythropoietin + T3	—	21.1 ± 1.8

bined administration of erythropoietin and T_3 in nephrectomized rats had no greater effect than erythropoietin alone.

Discussion. Considerable evidence has accumulated(8) to indicate that the rate of erythropoiesis, *i.e.,* the number of cells discharged from the marrow into the blood per unit of time, depends on 3 parameters, namely; 1) rate of stem-cell differentiation into pronormoblasts; 2) mean number of divisions of pronormoblasts and their succeeding stages; and 3) death rate of erythroid marrow cells (ineffective erythropoiesis). Any rate-influencing factor, irrespective of its nature or subcellular mode of action, is thus reflected in alterations of one or more of these parameters. Erythropoietin, for instance, induces differentiation of stem-cells and exerts its rate-regulation at the very beginning of the erythroid cell line. Thyroid hormone could influence any one of the above named steps, and it could exert the effect directly or through a metabolic intermediate. A direct effect of T_3 was ruled out in the polycythemic mice experiments (Table I). Differentiation of stem-cells had practically ceased in these animals and was, in contrast to erythropoietin, not restored by T_3 injections. An augmenting effect of T_3 on the number of stem-cells, which are induced to differentiate at a given level of erythropoietin, was likewise ruled out. The injection of erythropoietin restored an erythroid marrow population of controlled magnitude and permitted an assessment of possible effects of T_3 on parameter 2 and 3. If T_3 were to increase proliferation of erythroid cells or to decrease ineffective erythropoiesis, a greater response was to be excepted after combined injections of T_3 and erythropoietin. This was not the case, and data in Table II furthermore indicate that T_3 had no effect on the speed of red cell maturation as reflected in the serial measurements of iron incorporation. These measurements were carried out in view of reported accelerating effects of T_3 on protein synthesis. The appearance of Fe^{59} in the hemoglobin of circulating red cells depends on the presence of hemoglobin synthesizing marrow cells at time of Fe^{59} injection and on the emergence of these cells before the sampling of the blood. The interval between erythropoietin injection and rise in Fe^{59} incorporation thus represents an estimate of the speed with which this cohort of pronormoblasts matures into reticulocytes. Accordingly, a shortened maturation time, for instance as the result of an accelerated protein synthesis in erythroid cells, could be expected to result in an earlier rise in iron incorporation. No such effect was seen in T_3 mice, and the lack of any significant variation in the time-course of their iron incorporation is regarded as strong evidence against a direct effect of T_3 on proliferation or maturation of erythroid cells.

Gurney and Fried(11) attributed the absence of erythropoietic effect of T_3 in severely plethoric mice to a suppression of erythropoietin formation. They noted that the erythropoietic effect of T_3 varied inversely with the degree of plethora, and no effect was demonstrable in mice with hematocrits of 69% in spite of a 45% increase in their oxygen consumption. These authors interpreted the progressive loss of erythropoietic effect of T_3 with increasing plethora as the result of a shift in the oxygen demand-supply ratio in favor of the latter. The results presented in Table III support this interpretation. The exposure of plethoric mice at 300 mm Hg ambient pressure restored the erythropoietic effect of T_3, presumably by reducing the oxygen saturation so that the hypoxic effects attending the increased oxygen consumption

were no longer compensated for by overwhelming supply.

Although there is no evidence that hypertransfusion polycythemia induced variables of erythrokinetics other than those resulting from suppression of erythropoietin formation, it seemed desirable to verify the results by a second method of erythropoietin suppression, namely bilateral nephrectomy. This procedure removes in the rat the principal site of erythropoietin formation, and the renoprival rat has been shown unable to increase its erythropoietin formation in response to hypoxia or bleeding(12,13). The results fully confirmed the absence of any direct effect of T_3 on erythropoiesis. Confirmation by a second method of erythropoietin suppression also made an indirect action highly improbable because one would have to assume that both methods also suppressed a hypothetical intermediate (other than erythropoietin) responsible for the mediation of erythropoietic effect of T_3. The observations thus favor, by inference, an augmentation of erythropoietin formation as the most probable mechanism of the erythropoietic action of thyroid hormone. The failure to demonstrate unequivocally erythropoietin in the plasma of T_3 treated animals is not a compelling objection. The plasma turnover of erythropoietin is very rapid, and it has been shown in rats that the erythropoietin present in one ml of plasma represents one hundredth of their 24-hour production(9). The minimal plasma concentration which can be measured with some confidence is about 0.05 unit per ml and is equivalent to daily production in rats of 5 units. Based on increases in red cell formation observed in rats after long-term injections of various doses of erythropoietin(14), it appears that the degree of erythropoietic stimulation induced by thyroid hormone could result from a daily production of less than 5 units of erythropoietin, and the resulting increase in plasma erythropoietin might thus escape detection.

Summary. Hypertransfusion polycythemia in mice abolished the erythropoietic effect of T_3, but it was restored when polycythemic mice were exposed at 300 mm Hg ambient pressure. Combined injections of T_3 and erythropoietin had no greater erythropoietic effect in plethoric mice than erythropoietin alone. Results indicate that T_3 does not induce stem-cell differentiation, does not augment the rate of stem-cell differentiation at a given erythropoietin level, and does not increase proliferation of erythroid cells directly. Suppression of erythropoietin formation by bilateral nephrectomy in rats confirmed these results. Absence of erythropoietic effect in two experimental models with suppressed erythropoietin formation makes it improbable that erythropoietic effect of T_3 is exerted by an intermediate other than erythropoietin.

1. Larsson, S. O., Acta Med. Scand., 1957, v157, 349.

2. Tudhope, G. R., Wilson, G. M., Quart. J. Med., 1960, v29, 513.

3. Muldowny, F. P., Crooks, J., Wayne, E. J., Clin. Sci., 1957, v16, 309.

4. Waldmann, T. A., Weissman, S. M., Levin, E. H., J. Lab. Clin. Med., 1962, v59, 926.

5. Evans, E. S., Rosenberg, L. L., Simpson, M. E., Endocrinology, 1961, v68, 517.

6. Donati, R. M., Warnecke, R. A., Gallagher, N. I., Proc. Soc. Exp. Biol. and Med., 1964, v115, 405.

7. Meineke, H. A., Crafts, R. C., ibid., 1964, v117, 520.

8. Stohlman, F., New England J. Med., 1962, v267, 342.

9. Reissmann, K. R., Diederich, D. A., Ito, K., Schmaus, J. W., J. Lab. Clin. Med., 1965, v65, 967.

10. Reissman, K. R., Blood, 1964, v23, 137.

11. Gurney, C. W., Fried, W., J. Lab. Clin. Med., 1965, v65, 775.

12. Jacobson, L. O., Goldwasser, E., Fried, W., Plzak, L. F., Nature, 1957, v179, 633.

13. Reissmann, K. R., Nomura, T., Gunn, R. W., Brosius, F., Blood, 1960, v16, 1411.

14. Ito, K., Reissmann, K. R., ibid., 1966, v27, 343.

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