The Influence of Thyroid Hormones on In Vitro Erythropoiesis

MEDIATION BY A RECEPTOR WITH BETA ADRENERGIC PROPERTIES

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ABSTRACT The erythropoietic effect of various thyroid hormones has been studied using erythroid colony formation by canine marrow cells. Although erythropoietin was required for colony growth, physiologic levels of thyroid hormones significantly enhanced colony numbers. The order of potency of the thyroid compounds in their in vitro erythropoietic effect parallels their known calorigenic potency in vivo, suggesting that the in vitro effect is physiologically relevant. A series of studies linked the mechanism of thyroid action to adrenergic receptors on responsive cells. Propranolol, a global β-blocker, inhibited thyroid hormone-responsive erythroid colonies. When adrenergic antagonists having different blocking characteristics were added to culture, the thyroid hormone effect was blocked by those compounds having β_2-subspecificity. Velocity sedimentation analysis showed that the peak of colony-forming cells which respond to thyroid hormone and the adrenergic agonist, isoproterenol, sedimented at an identical rate (7.54 mm/h), which is slower than the major peak of colony-forming cells responding to erythropoietin alone (8.62 mm/h). These results demonstrate thyroid hormonal enhancement of in vitro erythroid colony growth which appears mediated by a receptor with β_2-adrenergic properties. The data suggest that changes in hormone-target cell interaction may occur during states of abnormal thyroid function.

INTRODUCTION

Disorders of thyroid function are generally accompanied by alterations in erythropoiesis. Although often masked by parallel changes in plasma volume, a reduced erythrocyte mass commonly occurs in patients with hypothyroidism. In the absence of complicating deficiency states, thyroid hormonal replacement restores the erythrocyte mass to normal (1). In contrast, most hyperthyroid patients have an elevated erythrocyte mass (2). Thyroid hormone increases erythrocyte production in euthyroid experimental animals as well (3). This enhanced erythropoiesis has been attributed to a direct hormonal effect on the erythroid marrow (4, 5) and to increased erythropoietin (ESF)[1] secretion (6, 7). However, since the studies were carried out in vivo, the calorigenic effect of thyroid hormones on overall metabolism must also be considered. Most recently, Golde et al. (8) have reported in vitro enhancement of murine erythroid colony growth by various thyroid hormones, suggesting a direct effect of these hormones on erythropoietic proliferation.

Erythroid colony growth is known to be modulated by several compounds, including β-adrenergic agonists, linked to the adenyl cylcase/cyclic AMP system (9). Several studies suggest that thyroid hormones may also affect cell functions by increasing the intracellular concentration of cAMP (10–12). The observation that certain clinical manifestations of hyperthy-

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[1] *Abbreviations used in this paper:* ESF, erythropoietin; IRP, International Reference Preparation; Tetrac, tetraiodothyroacetic acid; Triac, triiodothyroacetic acid; T_3, triiodothyronine; T_4, thyroxine.

roidism may result from enhanced β-adrenergic responsiveness (13) is consistent with the hypothesis that thyroid hormones interact with receptors having β-adrenergic properties.

This study examines the influence of thyroid hormones on in vitro erythropoiesis as well as the interactions of these hormones with catecholamine receptors. The results demonstrate that thyroid hormones directly enhance erythroid proliferation. As monitored by the erythroid colony-forming technique, the effect of thyroid hormones appears to be mediated by a receptor with β-adrenergic properties. However, the receptor for thyroid hormones has different characteristics than the receptor for β-agonists.

METHODS

Erythroid colony assay. The method of Stephenson et al. (14) was employed for erythroid colony growth. Canine bone marrow cells were harvested and cultured as previously described (15). The cells were dispersed in plasma clots at a final concentration of 2×10^5 trypan blue dye-excluding nucleated cells per milliliter. The source of ESF was partially purified anemic sheep plasma, step III, 3 International Reference Preparation (IRP) U/mg, (lot 3007, Connaught Medical Research Laboratory, Willowdale, Ontario, Canada). The ESF was dissolved in Hanks' balanced salt solution and added in microliter amounts to appropriate culture dishes. Erythroid colonies containing eight or more hemoglobinized cells were scored after 48 h of culture as described previously (15). The number of colonies formed was directly dependent on the number of nucleated cells plated (15).

To assess the effect of various thyroid hormone analogues on erythroid colony growth, microliter quantities of L-thyroxine (L-T_4), D-thyroxine (D-T_4), L-triiodothyronine (L-T_3), D-triiodothyronine (D-T_3), diiodo-L-thyronine, diiodo-L-tyrosine, iodo-L-tyrosine (Sigma Chemical Co., St. Louis, Mo.), triiodothyroacetic acid (Triac), or tetraiodothyroacetic acid (Tetrac) (K & K Laboratories, Inc., Plainview, N. Y.) were added in varying concentrations to appropriate cultures. L-T_4, D-T_4, Triac, and Tetrac were dissolved in 70% ethanol with 1 N NaOH, and L-T_3 and D-T_3 were dissolved in 95% ethanol with 2 N HCl. These compounds were initially dissolved in a 10 mM solution. The remaining compounds were dissolved in, and all compounds were subsequently diluted with, Hanks' balanced salt solution. In 1 ml of media, the total concentration of L-T_4 was 0.036 μM, and L-T_3 was 0.38 nM before the addition of thyroid compounds. In concurrent control cultures, the amount of ethanol (1–10 μl) with NaOH or HCl present had no effect on erythroid colony growth.

Studies of thyroid-catecholamine interrelationships. To examine the possible relationship between thyroid hormone and β-adrenergic influences, the effect of the following agents on L-T_4-enhanced canine erythroid colonies was studied: the $\beta_1\beta_2$-antagonist, propranolol (16) (Sigma Chemical Co.) and its D- and L-isomers (Ayerst Laboratories, Montreal, Canada); the relatively selective β_1-blocker, practolol (17) (Ayerst Laboratories); and the β_2-blocker, butoxamine (18) (Sigma Chemical Co.). L-Isoproterenol (Sigma Chemical Co.) was employed as the β-adrenergic agonist (19). To test the hypothesis that thyroid hormones may influence β-receptor numbers, several mixing experiments with both optimal (0.1 μM) and suboptimal concentrations (10 nM) of L-T_4 and L-isoproterenol were performed.

Cell separation by velocity sedimentation. The colony-forming cell populations responding to ESF, isoproterenol and L-T_4, were compared by the technique of velocity sedimentation at unit gravity (20) as employed in this laboratory (21). Erythrocyte contamination of the cell suspension was reduced before loading the gradient by taking only the buffy coat from the cell pellets after each Hanks' balanced salt solution wash, thereby achieving a nucleated: nonnucleated cell ratio of 1:3–4.

RESULTS

Under the described conditions, colonies of hemoglobin-synthesizing cells appeared only in cultures containing added ESF. The number of colonies formed was directly dependent on the logarithm of the concentration of ESF. In cultures of canine marrow cells, maximal colony formation was observed with ESF concentrations of 1–2 IRP U/ml (Fig. 1).

When various thyroid hormones and their analogues were added to cultures containing ESF, enhanced erythroid colony formation was consistently observed. This enhancement of colony growth depended on the concentration of thyroid hormone (Fig. 2). The peak activity and resultant potency order for the various thyroid hormones and their analogues were as follows: Triac (0.1 nm, $P < 0.05$); L-T_3 (1 nM, $P < 0.01$); D-T_3 (10 nM, $P < 0.05$); L-T_4 (0.1 μM, $P < 0.05$); D-T_4 (0.1 μM, $P < 0.02$); Tetrac (1 μM, $P < 0.05$). The diiodo and iodo derivatives did not influence colony numbers (data not shown) Because 0.1 μM L-T_4 was consistently the most effective in enhancing colony growth, it was utilized in subsequent experiments.

To delineate the thyroid hormone effect, L-T_4 was

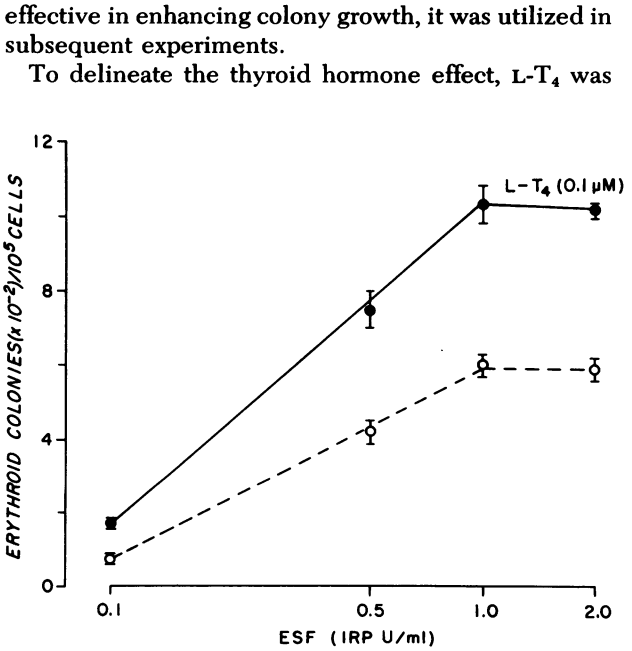

FIGURE 1 The effect of various concentrations of ESF, both alone and in combination with 0.1 μM L-T_4, on canine erythroid colony numbers. Similar results were obtained in seven additional experiments. Values represent the mean±SEM of triplicate culture dishes in this and Fig. 3–7.

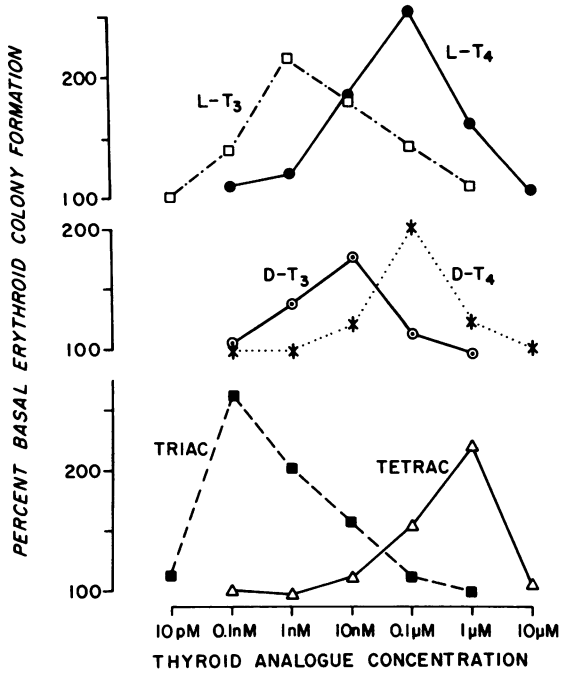

FIGURE 2 The effect of concentrations of various thyroid hormones on erythroid colony numbers. Each compound was tested in 3–10 separate experiments. Values represent the mean of individual experiments normalized as percent of control ESF-dependent colony numbers. The ESF concentration was 0.5 IRP U/ml in this and all subsequent figures unless otherwise specified.

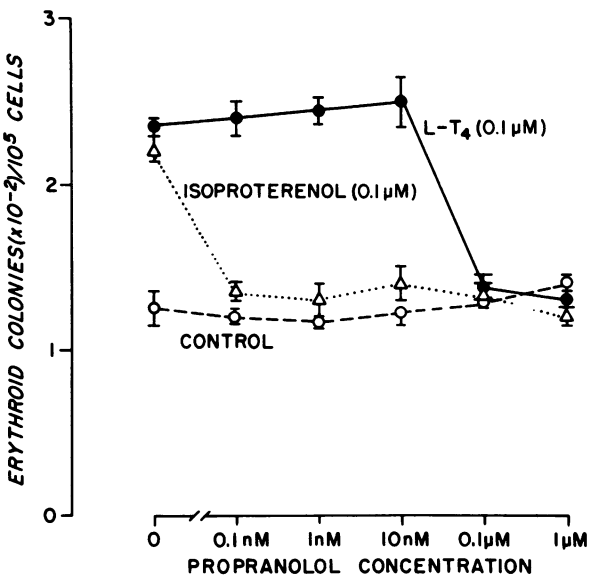

FIGURE 3 The effect of various concentrations of propranolol on L-T_4 (●) and isoproterenol (△)-enhanced colony growth. Controls (○) were cultured with either ESF alone, or with propranolol. No inhibition of ESF-dependent colonies was seen. Similar results were obtained in three additional experiments. Experiments in all subsequent figures were repeated a minimum of three times.

added to cultures containing a range of ESF concentrations. Fig. 1 shows that L-T_4 enhances erythroid colony growth over the full range of ESF concentrations and that its effect persists even at the plateau response to ESF. However, in the absence of ESF, no colonies appear despite an optimal concentration of L-T_4.

Studies of thyroid-catecholamine interrelationships. To investigate the relationship between thyroid hormones and β-adrenergic receptors, the effects of optimal concentrations of L-T_4 and isoproterenol were tested in the presence of the β-blocker, propranolol. Previous studies in culture (9) have shown that the peak erythropoietic effect of isoproterenol is at 0.1 μM, the same as L-T_4. As shown in Fig. 3, propranolol blocks L-T_4-enhanced erythroid colony growth. To determine the specificity of the inhibitory properties of propranolol, the blocking abilities of its stereospecific isomers were compared. As shown in Fig. 4, inhibition of colony numbers occurs only with the biologically active L-isomer. However, while L-propranolol blocks the erythropoietic effect of L-T_4, at least 10^3-fold less antagonist is needed to suppress isoproterenol-induced colonies (Fig. 3). Thus, whereas the in vitro erythropoietic effects of both L-T_4 and isoproterenol appear to be mediated by a receptor with β-adrenergic properties, there is a reproducible difference in receptor sensitivity to the antagonist. When L-T_4 and L-isoproterenol were tested together in various concentrations, the results failed to indicate any synergistic response, and

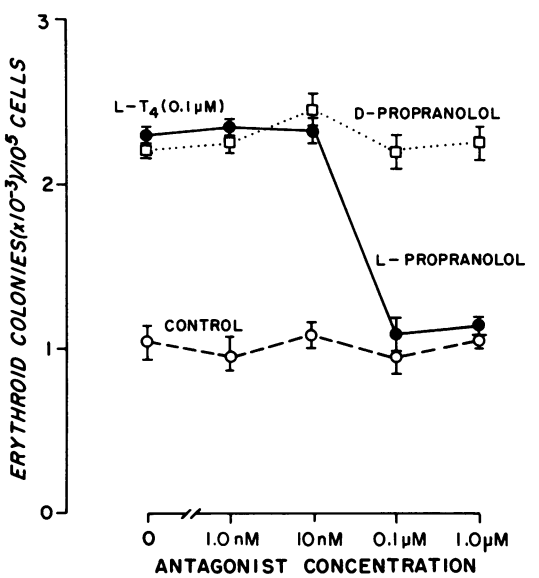

FIGURE 4 The effect of various concentrations of D- and L-propranolol on L-T_4-stimulated colony growth. Control cultures were established for D- and L-propranolol without L-T_4 and results represent the mean±SEM for both sets.

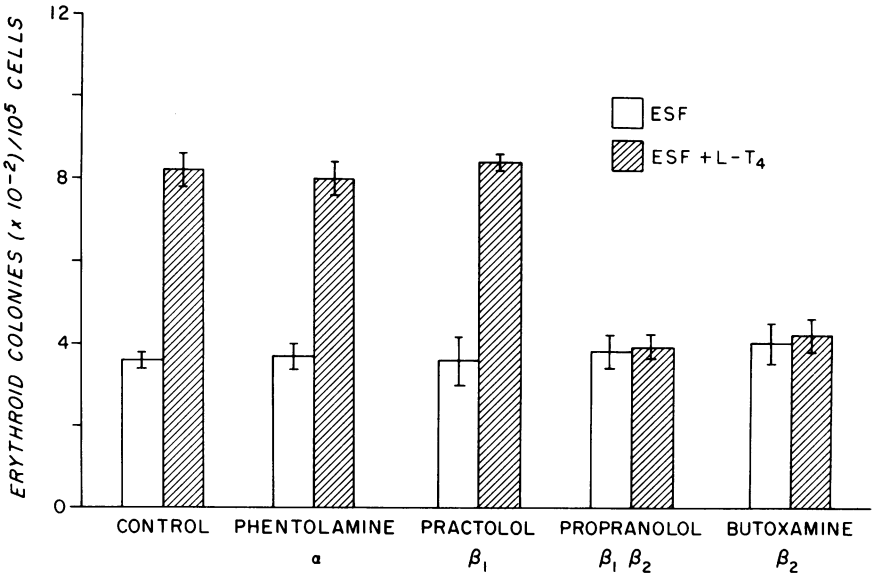

FIGURE 5 The effect of various adrenergic antagonists (0.1 μM) on erythroid colony growth both alone and in combination with 0.1 μM L-T_4. Complete dose-response curves for all antagonists were performed. Phentolamine did not inhibit colony numbers. Practolol blocked L-T_4-dependent colonies only at 10 μM and at higher concentrations reduced colony numbers below control values.

only an additive effect was seen at suboptimal concentrations of these hormones.

The adrenergic subspecificity of the thyroid receptor was further characterized by comparing the ability of various adrenergic antagonists to inhibit L-T_4-enhanced erythroid colony growth. Fig. 5 shows that L-T_4 effects are reversed by 0.1 μM concentrations of propranolol ($\beta_1\beta_2$) and butoxamine (β_2), but not practolol (β_1) or phentolamine (α), suggesting that L-T_4 influences a receptor with β_2-characteristics. As in the case of inhibition by propranolol (Fig. 3), a greater concentration of butoxamine was required to block the enhancing effect of L-T_4 than that of isoproterenol (Fig. 6).

Inasmuch as several thyroid compounds enhanced erythroid colony growth, the receptor subspecificity for all the erythropoietically active compounds was determined. In addition, the inhibitor concentrations required to block the thyroid hormone effects were compared. Fig. 6 demonstrates that the effects of both L-T_4 (0.1 μM) and L-T_3 (1 nM) were blocked by butoxamine at 0.1 μM. This same concentration of butoxamine was found to block completely the effect of all the thyroid analogues at their respective peak concentrations. The β_1-blocker, practolol, was inhibitory only at a concentration 10^2-fold higher than that required for butoxamine blockade. Thus, all the thyroid hormones examined appear to act through similar β_2-receptors by this analysis.

Cell separation by velocity sedimentation. When cells responding to ESF, L-T_4, and isoproterenol were analyzed by velocity sedimentation, the peak of ESF-dependent erythroid colony-forming cells sedimented more rapidly (8.62±0 mm/h; $n = 3$) than the peak of L-T_4-responsive cells (7.54±0 mm/h; $n = 3$, $P = 0.05$) (Fig. 7). In addition, the profiles of L-T_4 and L-isopro-

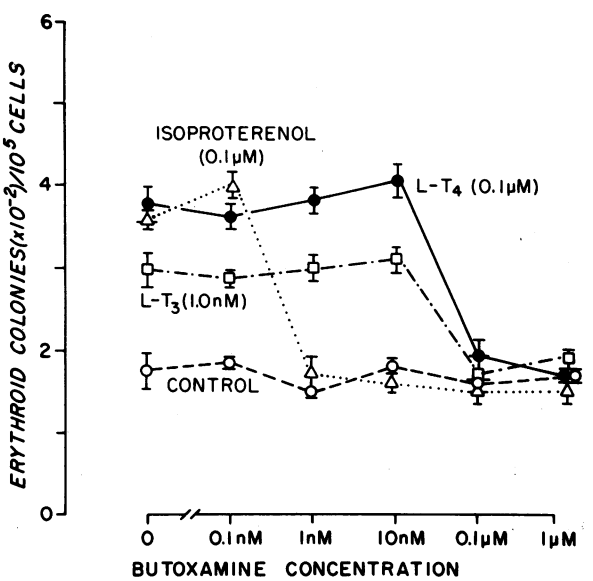

FIGURE 6 The effect of various concentrations of butoxamine on L-T_4-, L-T_3-, and isoproterenol-enhanced erythroid colonies. The concentrations chosen for the enhancing factors had previously been shown to be optimal for these compounds. Controls were cultured with ESF alone, or with butoxamine.

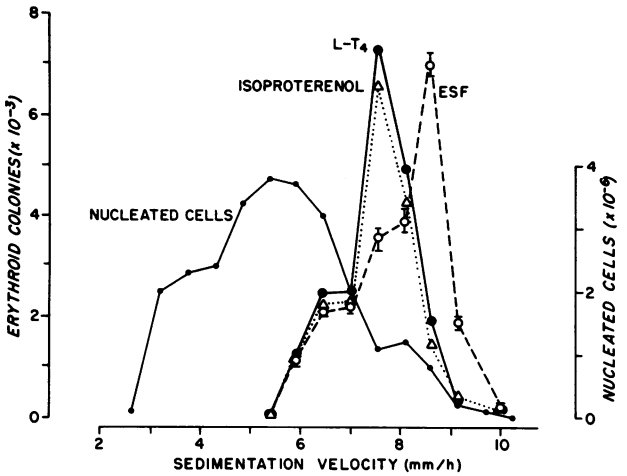

FIGURE 7 A representative velocity sedimentation profile of canine marrow cells. The profiles of L-T_4- and isoproterenol-responsive colony-forming units are derived by subtraction, and the SEM for L-T_4 (●) and isoproterenol (△) values are similar to those shown for ESF (○).

terenol-responsive cells coincided almost completely. The data suggest that these modulators affect the same cell population, one that is distinct from the majority of the ESF-responsive cells.

DISCUSSION

Although thyroid hormones have been shown to accelerate erythropoiesis in animals and man, the mechanism of this effect has not been clearly elucidated. Some investigators (6, 7, 22) have postulated that thyroid hormones exert their erythropoietic action through an elevation of ESF levels. However, others (4, 5, 23) have described augmented erythropoiesis independent of ESF activity, thus suggesting a direct effect on erythroid precursors. Most recently, Golde et al. (8) have provided direct evidence of enhanced in vitro erythropoiesis by thyroid hormones.

The observations reported here confirm a direct erythropoietic effect of thyroid hormones and characterize some of the mechanisms by which these hormones act. Thyroid hormones increase canine erythroid colony formation in vitro under conditions in which ESF levels are controlled and general metabolic changes are no longer a factor. Separation of target cells by velocity sedimentation suggests that L-T_4 affects a population that is somehow distinct from the majority of ESF-responsive progenitors. Inasmuch as the method separates cells primarily according to their size (20), such separation may reflect differences in maturation (24) and (or) stage of cell cycle (25). These observations support the contention that the erythroid progenitor cell responding to thyroid hormone may represent a subpopulation of erythroid colony-forming units; whether it is more or less differentiated than the majority of the ESF-responsive cells is not known.

The peak activities of both L-T_4 and L-T_3 were seen at physiologic concentrations (26). In addition, the order of potency of the erythropoietic effect of thyroid compounds in vitro parallels their calorigenic potency in vivo. Several other systems have been described which demonstrate a similar order of in vitro thyroid hormone activity. Samuels et al. (27) described a rat pituitary cell line responsive to concentrations of thyroid hormones that were analogous to their known in vivo activity. In nuclear binding studies, Oppenheimer et al. (28) have shown that thyroid analogue binding was closely correlated with in vivo thyromimetic activity, and confirmatory studies have been furnished by Koerner et al. (29), Samuels and Tsai (30), and Samuels et al. (31). Thus, in several systems, including the erythroid colony-forming assay, the importance of the correlations of the thyroid analogue effects and their known biologic potency is affirmed. The activity of thyroid hormones at concentrations similar to those found in vivo suggests that the erythropoietic effect of these hormones has physiologic significance (32).

Although most recent studies of thyroid action have focused on nuclear binding, Tata (33) has cautioned against equating biologic activity of thyroid hormones with such binding. Thus, thyroid hormone interactions with the cell membrane or cytosol may occur prior, or in addition, to binding to nuclear sites. In fact, binding at the cell membrane may be required to initiate hormone action, although little work has been done in this area.

Many hormones, including β-adrenergic agonists, bind initially to receptors at the cell membrane and influence cell function via adenyl cyclase-linked mechamisms (34). Such a mechanism of action has also been suggested for thyroid hormones because of the observed relationship between thyroid hormonal status and adrenergic activity (13). Evidence for such a hypothesis is provided by several observations. First, it has been proposed that thyroid hormone is capable of activating adenyl cyclase in vitro (10), although the concentrations were pharmacologic. In addition, β-adrenergic responsiveness in hypothyroid rats is reduced (11), and phosphodiesterase activity correlates inversely with thyroid hormonal status (12). Finally, augmentation of a catecholamine-sensitive adenyl cyclase system (35) and a direct increase in the number of β-receptors (36) by thyroid hormones have been reported. These studies suggest that thyroid hormonal status may affect the response of various tissues to adrenergic stimulation and intimate that thyroid hormones may interact with membrane-bound systems such as adenyl cyclase.

The results of this study demonstrate a relationship between thyroid hormones and adrenergic influences

on in vitro erythropoiesis. First, the erythropoietic effect of L-T_4 is completely inhibited by the β-blocker, propranolol. Since propranolol is known to act primarily at the cell surface (37), the data imply that this erythropoietic effect of thyroid hormones requires binding to receptors at the cell membrane. Second, inhibition of L-T_4-enhanced colony numbers occurred only with the biologically active L-isomer of propranolol. If the D- and L-isomers were equipotent antagonists, then the blocking effect of propranolol on thyroid-induced colonies might be attributed to nonspecific membrane anesthetic properties (38). Third, within the erythroid colony-forming system, the receptor subspecificity for both thyroid hormones and β-adrenergic agonists (9) appears to be of the β_2-type.

However, there is a significant difference between L-T_4- and isoproterenol-enhanced colony growth in the concentration at which β-blockade occurs. Propranolol inhibits L-T_4-enhanced colonies at a 10^3-fold greater concentration than that which inhibits isoproterenol-induced colonies. Because of this difference, however, a mechanism of propranolol blockade, independent of β_2-receptors, remains a possibility, although such a non-β-effect has not been described as having stereospecificity. Alternatively, it is possible that thyroid hormones and adrenergic compounds enhance erythroid colony growth by interaction with different β_2-receptors. Thus, there may be two distinct β_2-receptors, one responsive to β-adrenergic stimuli and the other to thyroid hormones. Additionally, thyroid hormones may somehow alter the sensitivity of erythroid colony-forming units to catecholamines or merely regulate the number of β-receptors. However, inasmuch as there is a lack of synergism when optimal or suboptimal concentrations of thyroid hormones and β-agonists are tested together, it is unlikely that the effect of thyroid hormones on erythroid colony growth is due to an increase in β-receptors on the target cells.

Thus, the action of thyroid hormone upon in vitro canine erythroid colony formation appears to be mediated by an adrenergic-like receptor with β_2-specificity. The clinical parallel between thyroid disease states and adrenergic activity appears to be reflected in the thyroid-catecholamine interactions on mammalian erythroid proliferation. These findings suggest that alterations in hormone–target-cell interaction may characterize states of disordered thyroid function.

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