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### Research Article

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# Modulation of In Vitro Erythropoiesis

## STUDIES WITH EUTHYROID AND HYPOTHYROID DOGS

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**ABSTRACT** The interactions of adrenergic agonists and thyroid hormones on the growth of erythroid colony-forming units were studied in cultures of dog marrow before and after the establishment of hypothyroidism. Erythroid colony growth in cultures from euthyroid dogs was enhanced by isoproterenol and other adrenergic agonists having  $\beta_2$ -receptor specificity. With hypothyroidism, however, this responsiveness was lost, and sensitivity to  $\alpha$ -agonists, such as phenylephrine and norepinephrine, was acquired. This alteration in receptor specificity appeared to be dependent upon thyroid hormone and was rapidly reversible. Preincubation of marrow cells from hypothyroid animals with thyroid hormone resulted in the reappearance of responsiveness to  $\beta$ -adrenergic agonists and the loss of sensitivity to  $\alpha$ -agonists. These findings are in agreement with previous suggestions that  $\beta$ -adrenergic receptor activity is modulated by thyroid hormone levels and demonstrate that the specificity of adrenergic modulations of erythropoiesis in culture may accurately reflect the thyroid status of the intact animal.

### INTRODUCTION

The growth and function of a number of cellular systems are influenced through interactions of different hormones and small molecules (1, 2). Several studies have shown that a variety of hormones, incapable of initiating a differentiative event or metabolic function by themselves, have the capacity to enhance the effect of primary regulatory hormones. As an example,

dibutyryl cyclic AMP (db-cAMP)<sup>1</sup> enhances the induction of tyrosine aminotransferase in cultured hepatoma cells, but only in the presence of, or after preconditioning of, the cells by a glucocorticoid such as dexamethasone (3).

Recent work from our laboratory, employing the in vitro growth of erythroid colony-forming cells, has shown that cAMP and related compounds enhance the growth of such colonies in marrow cell cultures from a variety of mammalian species (4). In a manner similar to other reported interactions, however, the cyclic nucleotides were incapable of initiating colony growth by themselves, and erythropoietin (ESF) was a necessary constituent of the culture medium. In addition to cAMP, a number of compounds have been shown to influence in vitro erythroid colony formation, including  $\beta$ -adrenergic agonists (5), thyroid hormones (6, 7), growth hormone (8), and various classes of steroid hormones (9). Although these observations are of interest, the relationships of in vitro modulation of erythropoiesis to the in vivo regulation of erythrocyte production is not clear. In addition, a basic question exists as to whether such interactions, defined in culture, in any way reflect the hormonal state of the intact animal.

To investigate the influence of the in vivo endocrine state on erythroid colony growth, the erythropoietic effects of thyroid hormones and adrenergic agonists have been compared in marrow cultures from euthyroid and hypothyroid dogs. These two modulators have been shown to enhance erythroid colony growth from normal dog marrow via receptors which appear to have similar properties (6). The results of these studies demonstrate that the responsiveness in culture to

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<sup>1</sup>Abbreviations used in this paper: db-cAMP, dibutyryl cyclic AMP; ESF, erythropoietin; T<sub>3</sub>, triiodothyronine; T<sub>4</sub>, thyroxine; TRIAC, triiodothyroacetic acid; TSH, thyroid-stimulating hormone.

TABLE I  
Serum T<sub>3</sub>, T<sub>4</sub>, and TSH Levels before and after <sup>131</sup>I Administration

Dog	Before <sup>131</sup> I			4 mo after <sup>131</sup> I		
	T <sub>3</sub>	T <sub>4</sub>	TSH	T <sub>3</sub>	T <sub>4</sub>	TSH
	ng/dl	μg/dl	IU/ml	ng/dl	μg/dl	IU/ml
1	65	1.7	1.9	<50	<0.5	14.6
2	60	2.0	2.3	<50	<0.5	18.9
3	70	1.8	1.7	<50	<0.5	20.6
4	70	1.9	1.6	<50	<0.5	17.1

β-adrenergic agonists is abolished in the hypothyroid state, whereas the effectiveness of thyroid hormone persists. In addition, enhanced responsiveness to α-adrenergic agonists is found in cultures from hypothyroid animals. This alteration in adrenergic receptor function on canine erythroid colony-forming cells is regulated, at least in part, by thyroid hormones.

## METHODS

**Study animals.** Four random-bred dogs were employed in the studies. The experiments were carried out in two stages: when the animals were euthyroid and then 4 mo after the establishment of chemically defined hypothyroidism. Hypothyroidism was achieved by administering 10 mCi of <sup>131</sup>I (New England Nuclear, Boston, Mass.) intravenously to the animals. The hypothyroid state was verified 3–4 mo later by determining serum triiodothyronine (T<sub>3</sub>) and thyroxine (T<sub>4</sub>) by radioimmunoassay (10, 11). Serum thyroid-stimulating hormone (TSH) levels were determined by a commercially available radioimmunoassay (Beckman solid phase HTSH; Beckman Instruments, Inc., Fullerton, Calif.).

**Erythroid colony assay.** This laboratory's modification (4) of the method of Stephenson et al. (12) was employed for erythroid colony growth. The same batches of fetal calf serum and bovine serum albumin were employed throughout the experiments. Canine bone marrow cells were aspirated, washed, and cultured in plasma clots at a final concentration of  $2 \times 10^5$  trypan blue dye-excluding nucleated cells/ml. The source of ESF was a commercially obtained preparation from anemic sheep plasma (Step III; 3 International Reference Preparation U/mg; lot 3010; Connaught Medical Research Laboratory, Willowdale, Ontario, Canada). The ESF was dissolved in Hank's balanced salt solution and added in microliter amounts to appropriate culture dishes. Erythroid colonies which contained eight or more hemoglobinized cells were scored after 48 h of culture as described previously (4).

**Hormonal effects on in vitro colony growth.** The in vitro erythropoietic effects of L-T<sub>4</sub>, L-T<sub>3</sub>, L-isoproterenol, L-norepinephrine, L-phenylephrine (all from Sigma Chemical Co., St. Louis, Mo.), and triiodothyroacetic acid (TRIAc) (K and K Laboratories, Inc., Plainview, N. Y.) were tested by adding these compounds in varying concentrations to appropriate cultures. L-T<sub>4</sub> and TRIAC were dissolved in 70% ethanol with 1 N NaOH, and L-T<sub>3</sub> was dissolved in 95% ethanol with 2 N HCl. These compounds were initially dissolved at a concentration of 10 mM. The remaining compounds were initially dissolved in alpha medium (Microbiological Associates, Walkersville, Md.), and all compounds were subsequently

diluted in Hank's balanced salt solution and tested over a range of concentrations (0.1 mM–1.0 nM). Before the addition of thyroid hormones, the concentrations of L-T<sub>4</sub> and L-T<sub>3</sub> were 0.036 μmol and 0.38 nmol, respectively, in 1 ml of medium plus serum.

To examine the interaction of thyroid hormones and β-adrenergic agonists, the following antagonists (with their relative receptor specificities) were studied: propranolol (β<sub>1</sub>β<sub>2</sub>), butoxamine (β<sub>2</sub>), phentolamine (α) (all from Sigma Chemical Co.), and practolol (β<sub>1</sub>) (Ayerst Laboratories, Montreal, Canada). In addition, the general adenylyl cyclase stimulator, cholera enterotoxin, the phosphodiesterase inhibitor, RO-20-1724 (Roche Diagnostics Div., Hoffman-LaRoche, Inc., Nutley, N. J.), and db-cAMP (Sigma Chemical Co.) were also tested.

## RESULTS

**Study animals.** The serum T<sub>3</sub>, T<sub>4</sub>, and TSH levels of the four dogs before and after administration of <sup>131</sup>I are shown in Table I. Base-line T<sub>4</sub> levels were all >1.5 μg/dl, and TSH levels were all <2.5 IU. 3–4 mo after receiving 10 mCi of <sup>131</sup>I, T<sub>4</sub> levels were all <0.5 μg/dl, and TSH levels were all >14.0 IU, confirming the hypothyroid state. In each instance, thyroid hormone values were reduced, and TSH levels rose and remained elevated after isotopic thyroid ablation.

**Hormonal effects on in vitro colony growth.** Thyroid hormone and its analogues consistently enhanced erythroid colony formation whether added to cultures of marrow cells obtained from euthyroid or hypothyroid dogs. The peak enhancement of colony growth depended upon both the concentration of thyroid hormone and the endocrine state of the animal. Whereas the optimal concentration for the L-T<sub>4</sub> effect in cultures from euthyroid animals was 0.1 μmol, the optimal concentration was 1.0 μmol in cultures from hypothyroid animals. Fig. 1 shows the data from 14 separate experiments employing all four animals. The values represent the mean of all these experiments, normalized as percentage of control ESF-dependent colony numbers. The observed 10-fold concentration difference was demonstrated for L-T<sub>3</sub> and TRIAC as well (data not shown).

To investigate in culture the correlation of reduced or absent β-adrenergic activity observed in clinical hypothyroidism, the effect of the β-adrenergic agonist, L-isoproterenol, was evaluated for its influence on colony growth. Fig. 2 summarizes the results observed in cultures from euthyroid and hypothyroid animals. Whereas isoproterenol enhanced erythroid colony growth from euthyroid dogs, with peak activity found with 0.1 μM, the response to this agonist was absent in cultures from hypothyroid animals.

β-adrenergic agonists are thought to influence cell function at least in part by activating membrane-bound adenylyl cyclase. To determine whether the observed alteration in the response to such agonists was

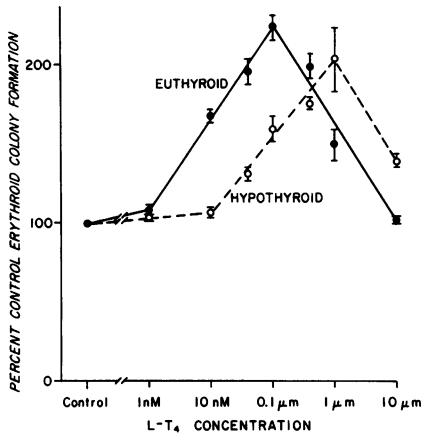


FIGURE 1 The influence of L-T<sub>4</sub> on canine erythroid colony growth from euthyroid and hypothyroid animals. The concentrations given are those which were added to the cultures in each experiment. The concentration of ESF used in these experiments and those shown in subsequent figures was 0.5 International Reference Preparation U/ml. Erythroid colony growth is shown as the percentage of control values. The data points represent the mean ( $\pm$ SEM) of a total of 14 separate experiments performed on the four dogs before and after establishment of hypothyroidism.

specific for the adrenergic receptors linked to adenylate cyclase or whether there was a more general disturbance of the adenylate cyclase-cAMP system, several other compounds were studied. Fig. 3 demonstrates that cholera enterotoxin, RO-20-1724, and db-cAMP all enhanced erythroid colony growth at the same concentrations in both endocrine states. Only the

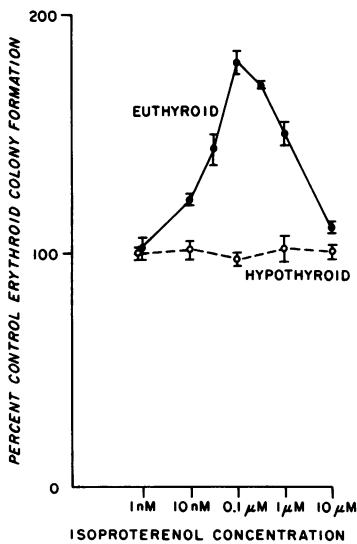


FIGURE 2 The influence of varying concentrations of isoproterenol on erythroid colony growth from euthyroid and hypothyroid animals. The data points represent the mean ( $\pm$ SEM) of eight separate experiments performed on the four dogs.

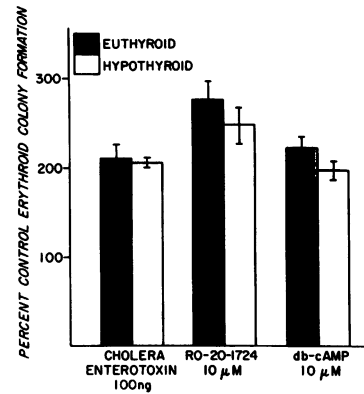


FIGURE 3 The influence of various agents known to involve the adenyl cyclase-cAMP system on erythroid colony growth from euthyroid and hypothyroid dogs. The data points represent the mean ( $\pm$ SEM) of eight separate experiments performed on the four dogs.

most effective concentrations are shown, although each compound was active in a concentration-dependent manner. These results imply that the loss of sensitivity of the erythroid colony-forming unit to isoproterenol is a result of altered  $\beta$ -agonist receptor function which is specific for the adenylate cyclase linked to the catechol receptor.

The  $\alpha\beta$ -agonist, norepinephrine, fails to enhance erythroid colony growth in marrow cells from euthyroid animals (5). As shown in Fig. 4, however, this compound was active in cultures from hypothyroid dogs. As isoproterenol was inactive in identical cultures, the adrenergic receptor functioning in the hypothyroid state appears to have  $\alpha$ -adrenergic properties. Because only  $\beta_2$ -agonists are active in cultures from normal animals, the data imply an alteration in receptors from those having  $\beta_2$ -selectivity

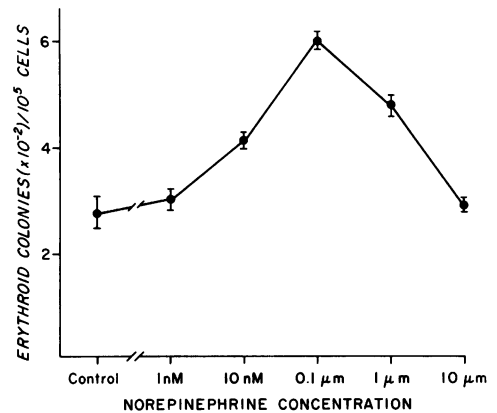


FIGURE 4 The concentration-dependent influence of norepinephrine on erythroid colony growth from hypothyroid dog marrow. This is a representative experiment and was repeated a minimum of three times on each of the four dogs.

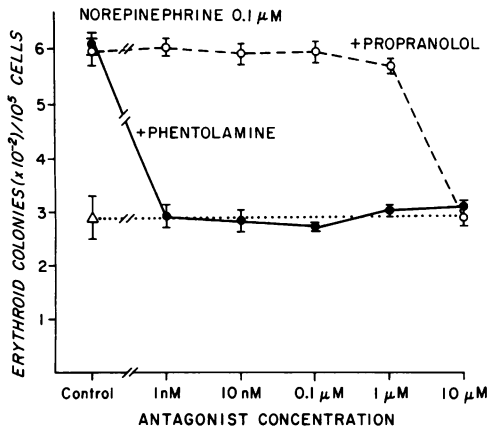


FIGURE 5 The relative effectiveness of phentolamine ( $\alpha$ ) and propranolol ( $\beta_1\beta_2$ ) blockade on norepinephrine-enhanced erythroid colony growth from hypothyroid dog marrow. This is a representative experiment that was repeated on six different occasions with similar results.

prevalent in the euthyroid state to a functional  $\alpha$ -receptor in the hypothyroid state.

To verify that the effect of norepinephrine was a result of interaction with an  $\alpha$ -receptor, cultures were established in the presence of propranolol and the  $\alpha$ -blocker, phentolamine. Only 1.0 nmol of phentolamine was required to inhibit norepinephrine-stimulated colony growth; however, 10  $\mu$ mol of propranolol was necessary for the same degree of inhibition (Fig. 5). This concentration difference again implied that the adrenergic receptor functional in the hypothyroid state

is an  $\alpha$ -receptor. A similar lack of effectiveness of the  $\beta_1$ -antagonist, practolol, and the  $\beta_2$ -blocker, butoxamine, was also observed (data not shown).

Further confirmation of the presence of an  $\alpha$ -receptor on erythroid colony-forming units from hypothyroid animals was provided by examining the effect of the  $\alpha$ -agonist, phenylephrine. Although this compound is ineffective in cultures of marrow from euthyroid animals, it enhances erythroid proliferation in cultures from hypothyroid animals, with optimal activity at 0.1  $\mu$ M, as shown in Fig. 6. Additional evidence for the specificity of the receptor was indicated by complete inhibition of the phenylephrine effect by 1.0 nmol of phentolamine.

The hypothesis that the hypothyroid state somehow induces the conversion of  $\beta$ - to  $\alpha$ -adrenergic receptors was evaluated by incubating marrow cells from hypothyroid dogs for 30 min in the presence of 0.1  $\mu$ mol of L-T<sub>4</sub>. Fig. 7 shows the results of such an experiment. The preincubation in the presence of thyroid hormone resulted in the reappearance of responsiveness to the  $\beta$ -agonist, isoproterenol, and loss of the previously observed responsiveness to the  $\alpha$ -agonist, norepinephrine. Control suspensions of marrow cells without added thyroid hormone showed the pattern of agonist enhancement expected for cultures from hypothyroid animals (Figs. 2 and 4).

## DISCUSSION

Although ESF is the primary regulator of erythropoiesis, various other hormones influence the prolifera-

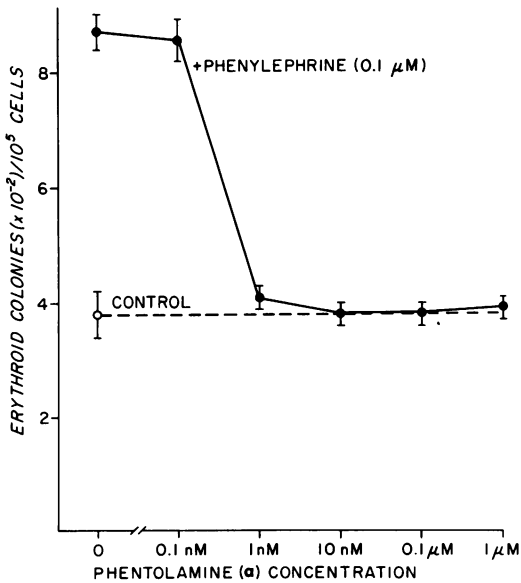


FIGURE 6 The influence of different concentrations of the  $\alpha$ -agonist, phenylephrine, on erythroid colony growth from hypothyroid dogs. This experiment was repeated on six different occasions with similar results.

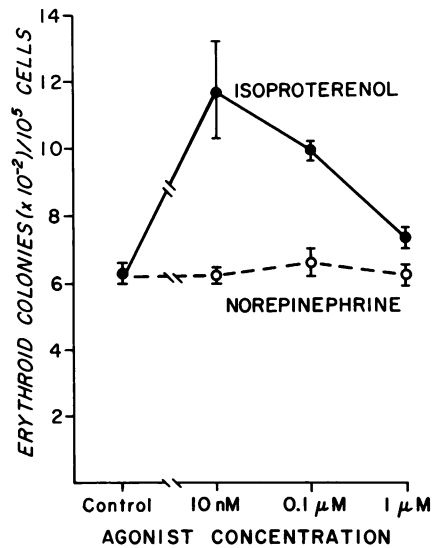


FIGURE 7 The results of preincubation for 30 min of marrow cells from hypothyroid dogs in the presence of 0.1  $\mu$ M L-T<sub>4</sub>. After this preincubation, the cells were plated for erythroid colony growth, and their responsiveness to various adrenergic agonists was tested.

tion of erythrocyte precursors (13). Analysis of the erythropoietic effects of such hormones has been difficult in the intact animal because of the variety of effects and tissues influenced. In an attempt to better define their influences, we have studied the direct effects of a number of these hormones in marrow cell culture.

In all of the studies reported to date, those hormones shown to be erythropoietically active in culture are incapable by themselves of initiating the growth of erythroid colonies; ESF is an obligate constituent of the culture medium. Thus, the concept has arisen that such hormones modulate the effect of ESF on its target cell(s). However, the relationship of the observations in culture to the activities of the hormones *in vivo* has not been established.

Previous studies in our laboratory of the influence of thyroid hormones on *in vitro* erythropoiesis have suggested that the observations in culture might have physiological significance in the intact animal. First, the thyroid hormone concentrations found to be effective were those easily achieved in the intact animal when one considers the available protein binding. Second, the order of effectiveness of the various thyroid hormones paralleled their known calorigenic effects *in vivo* (6).

In addition, several observations were made which suggested that at least part of the influence of thyroid hormones in this setting was mediated by cell receptors having the properties of receptors for adrenergic agonists (6). Thus, thyroid hormones enhanced the growth of erythroid colonies in a population of cells which, by velocity sedimentation analysis, was identical to cells whose growth was enhanced by isoproterenol. In confirmation, the thyroid hormone effect was completely abolished by the active stereoisomer of propranolol, although a higher concentration was required for blockade than was necessary to block the effect of isoproterenol.

Several lines of evidence indicate that thyroid hormone status may determine the response of an end organ or tissue to adrenergic stimulation. In hypothyroid animals,  $\beta$ -adrenergic responsiveness is reduced or absent (14). In hypothyroidism in man, clinical evaluation suggests reduced adrenergic responsiveness as well (15). Kunos and Nickerson have proposed that  $\alpha$ - and  $\beta$ -receptors in rat myocardium may be interconverted by thyroid hormone (16, 17). In heart preparations, contractile and metabolic responses to  $\beta$ -agonists are decreased in hypothyroidism (18), and recent evidence suggests that thyroid hormones regulate the number of  $\beta$ -adrenergic binding sites in catecholamine-sensitive tissue (19), including adipose tissue from rats and man (20). Other hormones, as well, may influence the level of  $\beta$ -adrenergic receptors because adrenalectomy in experimental animals

results in a marked increase in the number of  $\beta$ -receptors in liver cells and there is a partial restoration toward normal levels after administration of cortisone (21). Finally, thyroid hormone may influence receptors other than those for catecholamines, as Madsen and Sonne (22) have reported an increase in glucagon receptors in fat cells from hyperthyroid rats. Such studies provide experimental evidence that altered thyroid hormone levels influence end-organ  $\beta$ -adrenergic receptor activity.

The results of this study demonstrate that manipulation of the endocrine state of the intact dog alters modulator-receptor interaction on erythroid colony-forming units. First, the response to thyroid hormone shows different optimal concentrations in cultures from euthyroid and hypothyroid animals. This difference in optimal concentration presumably results from decreased occupancy of hormone receptors secondary to decreased levels of circulating thyroid hormones. Second,  $\beta$ -adrenergic responsiveness is absent in cultures from hypothyroid animals, whereas the responses to cAMP, cholera enterotoxin, and the phosphodiesterase inhibitor, RO-20-1724, are all intact and of similar concentration dependence in both the hypothyroid and euthyroid states. These results imply a specific effect on  $\beta$ -adrenergic receptors induced by hypothyroidism. Third, with the disappearance of  $\beta$ -adrenergic receptor function, a receptor having primarily  $\alpha$ -adrenergic properties was demonstrated in cultures from hypothyroid animals. Thus, norepinephrine and phenylephrine enhance erythroid colony growth of hypothyroid dog marrow, but not marrow from euthyroid dogs. Whether this results from the actual steric interconversion of the same receptor site or the disappearance of receptors with one specificity and the appearance of new receptors of another specificity cannot be resolved by these data. Finally, the incubation of marrow cells from hypothyroid animals with thyroid hormone restored  $\beta$ -adrenergic responsiveness and abolished the response to  $\alpha$ -agonists.

Thus, the clinical and laboratory observations of decreased  $\beta$ - and enhanced  $\alpha$ -adrenergic activity in hypothyroidism are paralleled by changes in receptor activity on erythroid colony-forming units. These results suggest that the altered responses to catecholamines and thyroid hormones *in vitro* reflect the thyroid state of the intact animal and suggest a physiologic role for such modulators in erythropoiesis.

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