Modulation of In Vitro Erythropoiesis

THE INFLUENCE OF β-ADRENERGIC AGONISTS ON ERYTHROID COLONY FORMATION

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ABSTRACT Canine marrow erythroid colony growth is enhanced by agents linked to the adenyl cyclase/cyclic AMP (cAMP) system, including cAMP, a phosphodiesterase inhibitor (RO-20-1724), cholera enterotoxin, and β-adrenergic agonists. The adrenergic effect is mediated by receptors having β_2-subspecificity. These receptors are distinct from putative receptors for erythropoietin and those acted upon by cholera enterotoxin. In addition, the population of cells most responsive to β-agonists is distinct from the majority of erythropoietin-responsive cells, perhaps representing a subpopulation of this class of cell. This demonstration of an adenyl cyclase-linked mechanism regulating mammalian erythroid colony growth provides a model for the modulation by other hormones or small molecules of in vitro and, perhaps, in vivo erythropoiesis.

INTRODUCTION

Erythropoiesis in mammals is regulated primarily by the glycoprotein hormone, erythropoietin (ESF)[1] (1). Since adenyl cyclase-linked mechanisms regulate differentiation, growth, and function in a number of tissues, such mechanisms may also influence erythropoiesis. Evidence that selected cyclic nucleotides may stimulate mammalian erythropoiesis has been provided by in vitro studies demonstrating that cyclic AMP (cAMP) increases canine hemoglobin synthesis in marrow suspension culture (2) and enhances ESF-dependent erythroid colony formation in semisolid media (3). Since the presence of ESF is essential for colony formation, and since the cells most responsive to cAMP or ESF are physically and functionally dissimilar, it has been proposed that mechanisms linked to cyclic nucleotides may modulate in vitro ESF action (4).

β-Adrenergic catecholamines are naturally occurring hormones which influence cellular metabolic events by interacting with specific cell surface receptors which are linked to membrane-bound adenyl cyclase. Thus, when the β-adrenergic hormone binds to its surface receptor, intracellular cAMP levels rise (5). The purpose of this study is to characterize the effect of adrenergic agents on in vitro erythroid proliferation. The results demonstrate that β-adrenergic agonists significantly enhance ESF-dependent canine erythroid colony growth and that the receptor has β_2-subspecificity. It is proposed that mechanisms linked to β-adrenergic receptors and adenyl cyclase(s) modulate erythropoiesis in vitro and, perhaps, in vivo.

METHODS

Bone marrow cells were aspirated in a sterile manner from the femurs or iliac crests of anesthetized normal dogs and immediately suspended in ice-cold Hanks' balanced salt solution (HBSS; Microbiological Associates, Bethesda, Md.) containing 10 U/ml preservative-free heparin and 2% fetal calf serum (Reheis Chemical Co., Div. of Armour Pharmaceutical Co., Phoenix, Ariz.). The cells were centrifuged (200 *g*; 10 min; 4°C) and, after a second washing, nucleated trypan blue dye-excluding cells were counted using a hemocytometer and aliquoted for appropriate experiments.

Erythroid colony assay. Erythroid colonies were cultured according to the technique of Stephenson et al. (6). At a total volume of 1 ml, the final concentrations of reagents in the culture dish were as follows: 30% fetal calf serum, 10% beef embryo extract (Grand Island Biological Co., Grand Island, N. Y.),

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[1] *Abbreviations used in this paper*: cAMP, cyclic AMP; ESF, erythropoietin; HBSS, Hanks' balanced salt solution; IRP, International Reference Preparation; [^{3}H]TdR, tritiated thymidine.

10% bovine serum albumin (Miles Laboratories, Inc. Kankakee, Ill.), 40% alpha medium (Flow Laboratories, Inc. Rockville, Md.), 10% bovine citrated plasma (Grand Island Biological Co.), 5 μM β-mercaptoethanol, 20 U penicillin, 20 μg streptomycin, and appropriate concentrations of ESF. The ESF used in these experiments was purchased from Connaught Laboratories (Toronto, Canada) and had an approximate specific activity of 3 International Reference Preparation (IRP) U/mg of protein and a final concentration in solution of 0.1 IRP U/μl. The canine marrow cells were suspended in a final concentration of 2×10^5/ml in 35 × 15-mm plastic culture dishes (Falcon Plastics, Oxnard, Calif.) and were initially mixed with all of the reagents with the exception of the bovine citrated plasma.

The various compounds to be tested were added to appropriate culture dishes containing the bovine citrated plasma. The cell-containing mixture was added, and the contents of the plate were rapidly mixed and allowed to clot. The cultures were then incubated at 37°C in a high humidity, 5% CO_2–95% air, tissue culture incubator (Napco Industries, Inc. Portland, Oreg.). After a 48-h incubation, the clots were fixed with 5% glutaraldehyde in phosphate-buffered saline, and the colonies were counted by using an inverted tissue culture microscope (7). To confirm the identity of hemoglobin-containing cells and thereby verify the counting results, some of the clots were removed from the dishes and stained with benzidine (8).

To examine their influence on erythroid colony formation, a number of β-adrenergic agonists and blockers were dissolved in HBSS and added to the cultures in microliter quantities over a range of concentrations. The various tested catecholamine agonists with their specificities and sources were as follows: L-phenylephrine (α[9]; Schwartz Mann Div., Becton, Dickinson and Co., Orangeberg, N. Y.), L-norepinephrine ($\alpha\beta_1$ [10]; Sigma Chemical Co., St. Louis, Mo.), L-epinephrine ($\alpha\beta_1\beta_2$ [10]; Schwarz Mann Div., Becton, Dickinson and Co.); L-isoproterenol ($\beta_1\beta_2$ [10]; Sigma Chemical Co.); albuterol (or salbutamol; β_2 [11]; Schering Corp., Bloomfield, N. J.); and metaproterenol (or orciprenaline; β_2 [12]; Boehringer-Ingelheim, Ltd., Elmsford, N. Y.). The catecholamine antagonists were phentolamine (α [13]; Ciba-Geigy Corp., Los Angeles, Calif.); D,L-propranolol $\beta_1\beta_2$; [13]; Schwarz Mann Div., Becton, Dickinson and Co.); L-propranolol and D-propranolol (Ayerst Laboratories, Montreal, Canada); practolol (β_1 [14]; Ayerst Laboratories); and butoxamine (β_2 [15]; Burroughs-Wellcome Co., Research Triangle Park, N. C.).

Other agents known to participate in the adenyl cyclase-cAMP system were also tested, including dibutyryl-cAMP (Sigma Chemical Co.), the phosphodiesterase inhibitor, RO-20-1724 ([16]; Roche Diagnostics Div., Hoffmann-LaRoche, Inc., Nutley, N. J.), and the nonspecific adenyl cyclase stimulator, cholera enterotoxin (17, 18). The cholinergic compound, carbamylcholine (Sigma Chemical Co.), was also examined. All of these agents were dissolved in HBSS with the exception of RO-20-1724, which was dissolved in ethanol. In control cultures, identical volumes of ethanol (1–10 μl/ml) had no effect in colony formation.

Tritiated thymidine [^3H]TdR suicide kinetics. The cycle characteristics of colony-forming cells responding to ESF and to isoproterenol were compared by [^{3}H]TdR suicide kinetics as described previously (19, 20). Control cell suspensions were incubated in HBSS alone, in HBSS containing nonradioactive thymidine, and in HBSS containing both radioactive and unlabeled thymidine.

Cell separation by velocity sedimentation. Physical properties of the colony-forming cell populations responding to ESF or β-agonists were compared by the technique of velocity sedimentation at unit gravity (21). Red cell contamination of the cell suspension was reduced before loading the gradient by taking only the buffy coat from the cell pellets after each HBSS wash, thereby achieving a nucleated: nonnucleated cell ratio of 1:3–4. The cell concentration was then adjusted to 2.7×10^6/ml in HBSS containing 5% fetal calf serum, and the gradient loaded, allowed to settle, and analyzed as described previously (20).

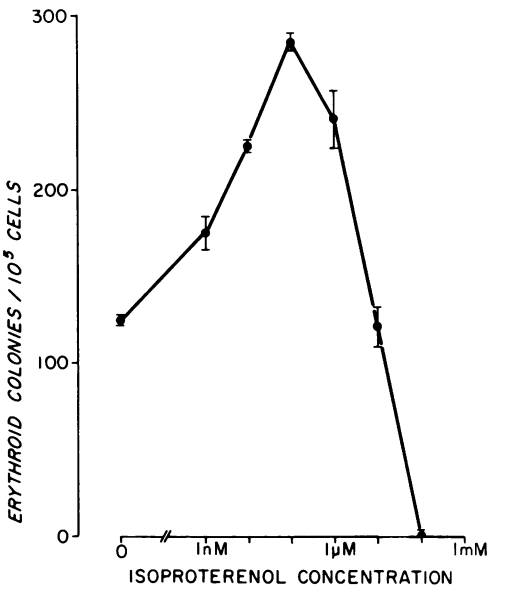

FIGURE 1 The effect of various concentrations of isoproterenol on canine ESF-dependent erythroid colony numbers. Values in this and the remaining figures represent the mean ± SEM of triplicate culture plates. The ESF concentration was 0.5 IRP U/ml in this and all subsequent figures except Fig. 8.

RESULTS

Canine marrow cells cultured under the conditions described form colonies of 8–32 hemoglobin-synthesizing cells in response to added ESF. Each colony arises from a single progenitor, the erythroid colony-forming unit, and colony numbers are related linearly to the number of nucleated cells plated (3) and to the logarithm of the concentration of added ESF up to 1.0 IRP U/ml (Fig. 8).

When the β-adrenergic agonist, isoproterenol (1 nM–1 μM), was added to cultures in combination with suboptimal concentrations of ESF (0.5 IRP U/ml), enhancement of colony numbers was observed. This enhancement was concentration dependent with the maximal effect seen at 0.1 μM (Fig. 1). At higher concentrations of isoproterenol, erythroid colony numbers fell. This decline probably resulted from a nonadrenergic toxic effect on the culture, since inhibition was not reversed by adding 0.1 μM to 0.1 mM of the β-blocker, propranolol, or the α-blocker, phentolamine.

The enhancing effect of isoproterenol on erythroid

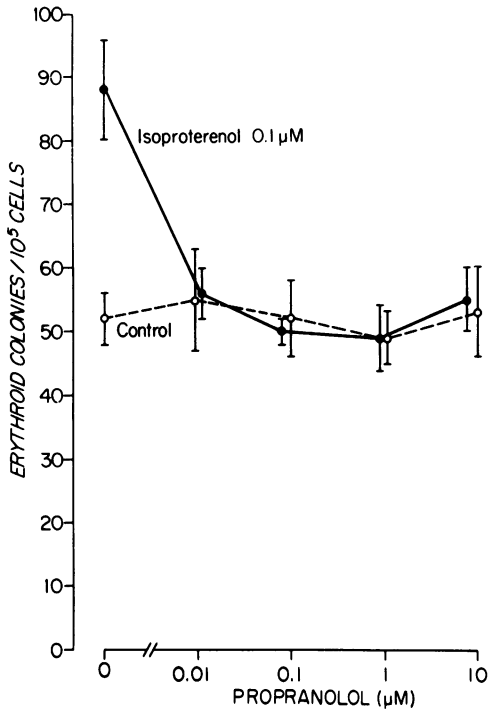

FIGURE 2 The effect of various concentrations of propranolol on erythroid colony numbers in the presence of 0.1 μM isoproterenol.

colony growth was eliminated by a 10 nM concentration of the global β-blocker, propranolol (Fig. 2), strongly suggesting that the effect of the agonist is due to interaction with a β-receptor. While propranolol inhibited the growth of isoproterenol-induced colonies, it had no effect, up to 10 μM, on the number of ESF-dependent colonies.

To clarify the specificity of the β-adrenergic effect, two types of experiments were carried out. First, the blocking effect of propranolol was tested in cultures containing other stimulators of the adenyl cyclase-cAMP mechanism (Fig. 3). Although propranolol completely blocked the effect of isoproterenol, it failed to inhibit the increased colony numbers induced by dibutyryl cAMP, RO-20-1724, or cholera enterotoxin. Thus, these other agents probably act at cytoplasmic sites or at cell surface locations which are separate from the β-receptor. In the second approach to the question of the β-specificity of the isoproterenol effect, the blocking properties of the D- and L-isomers of propranolol were compared. Fig. 4 shows that D-propranolol at 0.1 μM had no influence on colony numbers whereas 0.1 nM L-propranolol completely inhibited isoproterenol-induced colonies.

The adrenergic receptor was further characterized by adding to cultures several α- and β-adrenergic stimulators having different relative receptor selectivities (Fig. 5). The relatively selective α-adrenergic agent, phenylephrine, and the $\alpha\beta_1$-agonist, norepinephrine, did not enhance erythroid colony numbers, whereas the nonspecific adrenergic agonist, epinephrine, and the relatively selective β_2-stimulators, albuterol and metaproterenol, did. The results of these experiments suggest that the agonists influence colony growth through receptors having β_2 subspecificity. The cholinergic compound carbamylcholine had no influence on colony growth at concentrations from 0.1 nM to 0.1 mM (data not shown).

To confirm the nature of the β-receptor, various adrenergic blocking agents having a spectrum of relative selectivities were added in equimolar concentrations (0.1 μM) with isoproterenol (Fig. 6). The α-blocker, phentolamine, and the β_1-blocker, practolol, failed to inhibit significantly the isoproterenol-induced colonies, but the antagonists with β_2 properties, propranolol and butoxamine, completely inhibited the enhanced colony numbers. Again, none of these blocking agents inhibited ESF-dependent growth, so nonadrenergic or toxic effects are probably not involved. The relative nature of the subspecificity of these blockers is clearly demonstrated by a comparison of the effect of a range of concentrations of practolol (β_1) and butoxamine (β_2) on isoproterenol-induced colonies (Fig. 7). Whereas 1 nM butoxamine completely inhibited β-agonist-enhanced colony formation, 1 μM practolol was required to accomplish this inhibition. Thus, the adrenergic receptor appears to have β_2 subspecificity and is distinct from the ESF receptor.

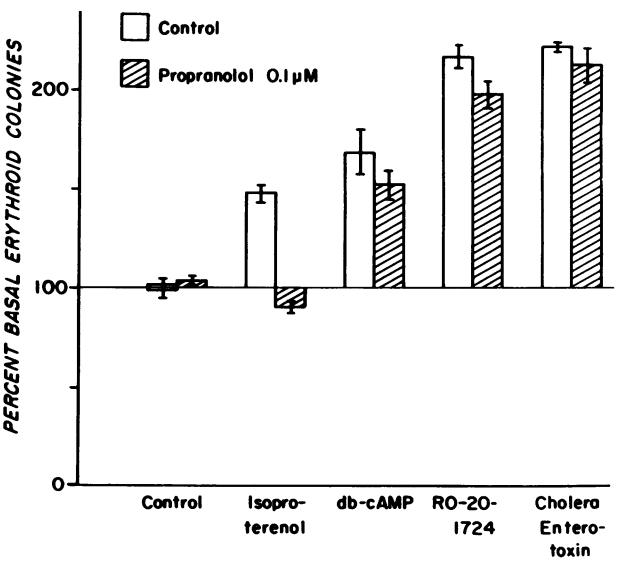

FIGURE 3 The influence of isoproterenol (0.1 μM), dibutyryl (db)-cAMP (10 μM), RO-20-1724 (10 μM), and cholera enterotoxin (100 ng/ml) on erythroid colonies in the presence or absence of 0.1 μM propranolol. Values are expressed as percent of the mean numbers of erythroid colonies formed in the absence of propranolol. The result is the same when 10 μM propranolol is used.

In further experiments designed to determine whether cells acted upon by β-agonists are functionally separable from ESF-responsive cells, 0.1 μM isoproterenol was added to cultures containing a range of concentrations of ESF (Fig. 8). The β-agonist continued to enhance colony formation even at optimal concentrations of ESF (above 1.0 IRP U/ml).

Aye (22) has shown that a soluble product from adherent cells enhances human erythroid colony growth. In the current study (data not shown) all of the enhancing factors tested remained active in the absence of adherent cells, which had previously been removed by polyester fiber filtration (22), or in the presence of buffy coat-conditioned medium (23). Thus, the influence of adenyl cyclase-linked mechanisms on erythroid colony growth does not appear to be mediated by adherent cells.

[^{3}H]TdR suicide kinetics. The proportions of cells actively synthesizing DNA in the ESF- and β-adrenergic-responsive populations were compared by exposing cells to high specific activity [^{3}H]TdR. The [^{3}H]TdR survival of the ESF-dependent colonies was 49%±2 (mean±SEM; n = 14). The survival rate for isoproterenol-induced colonies was comparable, 47%±1 (n = 2). Thus, the percentage of responsive cells in S phase was the same for ESF and isoproterenol.

Cell separation by velocity sedimentation. Marrow cells were separated by velocity sedimentation at unit gravity to compare the physical characteristics of ESF- and isoproterenol-dependent colony-forming units. Fig. 9 represents one of five similar gradients. Red cells (not shown) sedimented at 2.7 mm/h. The nucleated cell profile was broad and its peak sedimented at 6–7 mm/h. Erythroid colony-forming units sedimented more rapidly with the peak of ESF-induced colony forming units at 8.7±0.3 mm/h (n = 8) and the peak of cells responding to isoproterenol at 7.2±0.4 mm/h (n = 5; $p < 0.005$). In all experiments, the cell fractions containing most of the isoproterenol-responsive cells were consistently located in a shoulder of the profile of the ESF-responsive cells, and the peaks of agonist response were sharp.

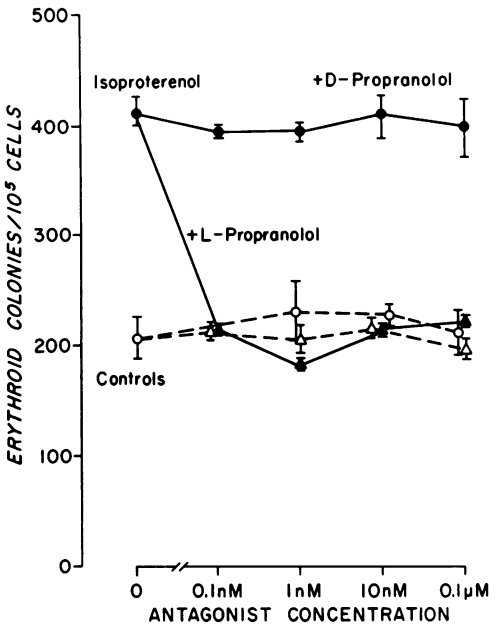

FIGURE 4 The effect of various concentrations of L-propranolol (△,▲) compared with D-propranolol (○,●) on the number of erythroid colonies induced by 0.1 μM isoproterenol (solid symbols and lines). Controls (open symbols and broken lines) contain the same concentrations of either antagonist in the absence of isoproterenol.

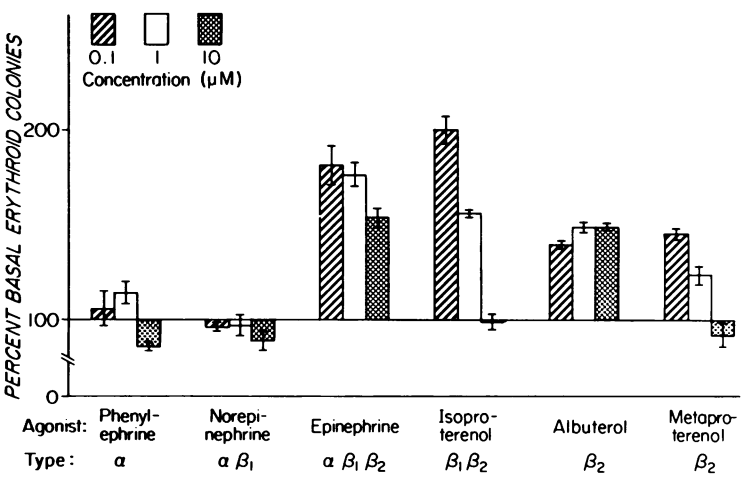

FIGURE 5 The effect of a range of concentrations of various types of catecholamines on erythroid colony growth. Values are expressed as percent of basal ESF-dependent colony numbers. All the agonists were tested from 1 nM to 10 μM but only the active concentrations from 0.1 to 10 μM are shown.

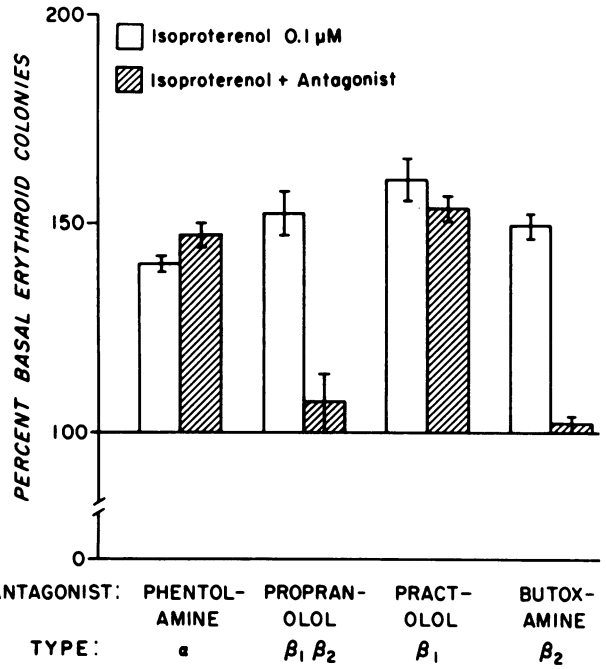

FIGURE 6 The effect of various categories of catecholamine antagonists on isoproterenol-induced erythroid colonies. Agonist and antagonists were all employed at 0.1 μM. Values are expressed as the percentage of mean ESF-dependent colony numbers. In separate experiments concentrations of phentolamine up to 10 μM also failed to inhibit β-agonist-enhanced colony numbers.

DISCUSSION

The role of adenyl cyclase-linked mechanisms in the regulation of in vitro erythropoiesis has been examined by a number of researchers; however, the results have been either inconclusive or contradictory. Some studies have demonstrated that cAMP stimulates in vitro erythropoiesis as measured by such parameters as heme synthesis or erythroblast numbers (24–27) although others have not shown such an effect (28, 29). In fetal rat liver, although stimulation of heme synthesis by ESF has been shown, no effect of ESF on cAMP levels was noted. In this same tissue, however, marked elevations in cAMP levels were induced by epinephrine (30). It is likely that the variable effects of cAMP noted in these different studies are due to variations in the species chosen as marrow source (2) and the erythropoietic parameter selected for measurement.

Recently, cyclic nucleotides have been shown to stimulate hemoglobin synthesis and enhance erythroid colony formation in cultures employing canine and human marrow cells, effects which are specific for cyclic adenosine nucleotides (2, 3). On analysis, however, a number of differences were found between cAMP and ESF in the functional and physical characteristics of the cell populations responsive to the respective agents. First, while cAMP enhanced colony growth, only ESF was capable of initiating erythroid colony formation (3). Second, the adenyl cyclase stimulator, cholera enterotoxin, continued to augment colony formation (3), and cAMP continued to induce hemoglobin synthesis in cell cultures (2) containing optimal concentrations of ESF. Third, velocity sedimentation separated the peak of cAMP-responsive cells from ESF-responsive cells (2, 3). The studies described here extend these prior observations to another class of adenyl cyclase stimulators, the β-adrenergic agonists.

Other investigators have demonstrated the presence of β-receptors on both pluripotent stem cells and mature erythroid cells. Byron has reported a functional response of mouse stem cells to adrenergic agonists (31). Adenyl cyclase-linked β-adrenergic receptors on avian and mammalian erythroid cells have been intensively studied for several years (32–34). In the current experiments the addition of β-adrenergic agonists to cultures of canine marrow cells produced significant enhancement of erythroid colony formation. Isoproterenol doubled the number of colonies formed at suboptimal concentrations of ESF, and propranolol blocked this increment, although propranolol had no ef-

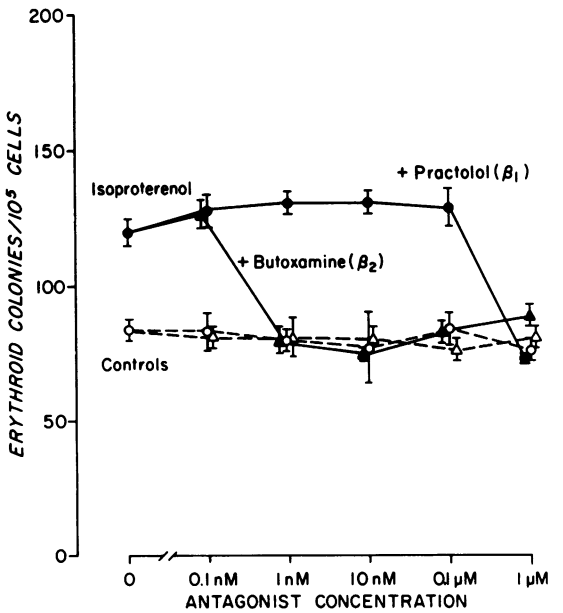

FIGURE 7 The effect of various concentrations of practolol (○,●) or butoxamine (▲,△) on erythroid colonies induced by 0.1 μM isoproterenol (solid symbols and lines). The controls (open symbols and broken lines) represent the number of ESF-dependent colonies in the presence of varying concentrations of either antagonist in the absence of isoproterenol. In separate experiments (not shown) 10 μM practolol or butoxamine did not inhibit ESF-dependent colony numbers.

fect on the number of ESF-dependent colonies. These results indicate that the isoproterenol effect is due to its β-agonist properties. Furthermore, propranolol did not block the enhancement of colony growth seen with cAMP, the phosphodiesterase inhibitor, RO-20-1724, or the adenyl cyclase stimulator, cholera enterotoxin. These observations are consistent with a cell having a variety of surface receptors, some of which are coupled to membrane-bound adenyl cyclase(s). As an agent which competitively binds to β-adrenergic surface receptors, propranolol would not be expected to interfere with those agents which act intracellularly, such as cAMP and RO-20-1724, and would interefere only with cholera enterotoxin if it were acting through a β-receptor. On the other hand, even though both β-agonists and cholera enterotoxin probably stimulate adenyl cyclases, their respective receptors must differ. Stereospecificity of β-adrenergic effects, emphasized by Lefkowitz (35), is demonstrated in the erythroid colony system. Thus, 0.1 μM D-propranolol had no effect on isoproterenol-induced colonies, but 0.1 nM L-propranolol completely suppressed the isoproterenol influence.

By manipulating agonists and antagonists with known pharmacological properties, it has been possible to determine that the subspecificity of the adrenergic receptor is β_2 in character. Thus it can be explained why only agonists with significant β_2 properties enhanced colony numbers, whereas those with α- and β_1 subspecificity were inactive. Confirmation is provided by

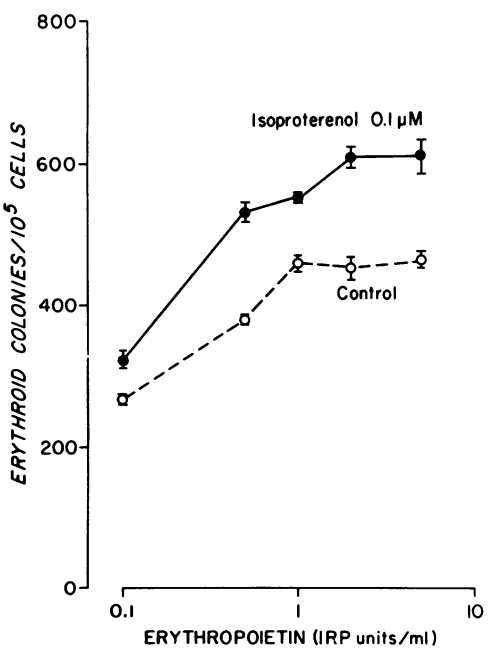

FIGURE 8 The effect of 0.1 μM isoproterenol on the number of erythroid colonies in the presence of various concentrations of ESF.

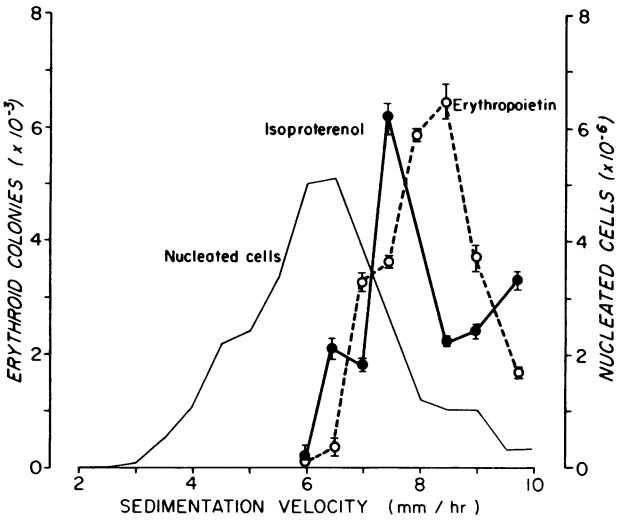

FIGURE 9 A representative velocity sedimentation gradient profile of canine marrow cells. Nucleated cells sedimented as a broad peak. Cells capable of colony formation in response to ESF (○) and isoproterenol (●) sedimented more rapidly. The profile of isoproterenol-responsive colony-forming units is derived by subtraction.

the fact that only antagonists, with known activity directed toward β_2-type receptors, inhibited adrenergic-enhanced erythroid colony growth. None of these agents suppressed ESF-dependent colony growth. Thus, the adrenergic receptor has β_2-specificity and is distinct from that proposed for ESF.

The results of a series of experiments designed to compare the cells responding to ESF or β-agonists demonstrated both functional and physical differences between the two populations. At optimal concentrations of ESF, isoproterenol continued to enhance colony numbers. If isoproterenol and ESF had been acting on the same cell population via the same intracellular mechanism, then isoproterenol would not have been expected to increase colony numbers when the ESF effect is maximal. In addition, the requirement for ESF was absolute for colony formation. Although colony growth was enhanced by isoproterenol, the agonist was incapable of initiating colony formation by itself.

The results of velocity sedimentation analysis also suggest that the β-agonist- and ESF-responsive cells are dissimilar. Unit gravity sedimentation separates cells largely on the basis of size (26), and other characteristics, including cell maturity (36–38) and stage of cell cycle (39), also affect separation probably by influencing this parameter. Consequently, cells responding to β-agonists may represent a subpopulation of erythroid colony-forming units, but whether more or less differentiated than the majority of ESF-responsive cells is not known.

The demonstration that adrenergic agonists modu-

late the in vitro effects of ESF provides a model for the action of other small molecules such as Ca^{++} (40) and prostaglandins whose effects have been linked to adenyl cyclase. The demonstrated interactions of primary and secondary regulators of in vitro erythropoiesis suggest mechanisms by which refined modulation of cellular proliferation may be achieved in vivo.

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