

# Ectopic $\beta$ -Adrenergic Receptor Binding Sites

## POSSIBLE MOLECULAR BASIS OF ABERRANT CATECHOLAMINE RESPONSIVENESS OF AN ADRENOCORTICAL TUMOR ADENYLATE CYCLASE

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**ABSTRACT** The molecular basis for the aberrant catecholamine responsiveness of the adenylate cyclase of adrenocortical carcinoma 494 was explored. The adenylate cyclase of this corticosteroid-producing, transplanted, adrenal cancer of the rat was stimulated not only by adrenocorticotrophic hormone and fluoride, but also by the  $\beta$ -adrenergic agonist, isoproterenol. The adenylate cyclase of normal adrenal tissue was unresponsive to isoproterenol. Direct binding studies with the specific high affinity  $\beta$ -adrenergic ligand,  $(-)[^3\text{H}]$ dihydroalprenolol, demonstrated the presence of 0.094 pmol of specific binding sites per milligram of tumor membrane protein. By contrast, normal adrenal membranes contained too few binding sites to accurately measure and study using these techniques. The tumor binding sites had high affinity for  $(-)[^3\text{H}]$ dihydroalprenolol with an equilibrium dissociation constant of 2.1 nM. Adrenergic agonists competed for the binding sites in an order of potency,  $[(-)\text{isoproterenol} > (-)\text{epinephrine} \sim (-)\text{norepinephrine}]$ , paralleling their order of potency as  $\beta$ -adrenergic agonists. The  $\beta$ -adrenergic antagonist,  $(-)\text{propranolol}$ , competed for binding, causing half-maximal inhibition of specific binding at a concentration of 6 nM. The  $\alpha$ -adrenergic antagonist, phentolamine, and several catecholamine metabolites and precursors did not effectively compete for the binding sites at high concentrations. Binding was stereospecific, the  $(+)$ stereoisomers of  $\beta$ -adrenergic agonists and antagonists requiring 40- to 300-fold higher concentrations than the corresponding  $(-)$ stereo-

isomers to half maximally inhibit  $(-)[^3\text{H}]$ dihydroalprenolol binding. These results indicate that adrenocortical carcinoma 494 membranes contain  $\beta$ -adrenergic receptor-binding sites which are not normally present in membranes of adrenal tissue. These ectopic  $\beta$ -adrenergic receptors presumably confer on the neoplastic tissue the catecholamine sensitivity of its adenylate cyclase.

### INTRODUCTION

The biological effects of many hormones are mediated by increases in the intracellular cyclic AMP levels in the responding tissue. Cyclic AMP-mediated responses of a hormonally sensitive tissue are elicited only by those hormones which activate the membrane-bound adenylate cyclase in that tissue. In the adrenal cortex, the binding of adrenocorticotrophic hormone (ACTH) to specific receptors which are functionally coupled to adenylate cyclase (1), initiates cyclic AMP-mediated steroidogenesis. Compounds such as the endogenous catecholamines are normally ineffective in stimulating adrenocortical adenylate cyclase, and hence do not initiate steroidogenesis. Recently, Schorr et al. (2, 3) have reported that the adenylate cyclase of adrenocortical carcinoma 494 unexpectedly responds to catecholamines with a  $\beta$ -adrenergic specificity. This aberrant response to catecholamines could be explained by one of two hypotheses. One possible explanation is that normal adrenal cortical tissue is devoid of  $\beta$ -adrenergic receptors, whereas the adrenocortical neoplastic tissue possesses ectopic  $\beta$ -adrenergic receptors linked to adenylate cyclase. An alternative possibility is that both normal adrenal cortical and tumor tissue possess  $\beta$ -adrenergic receptors, but only in the tumor are the receptors functionally coupled to adenylate cyclase.

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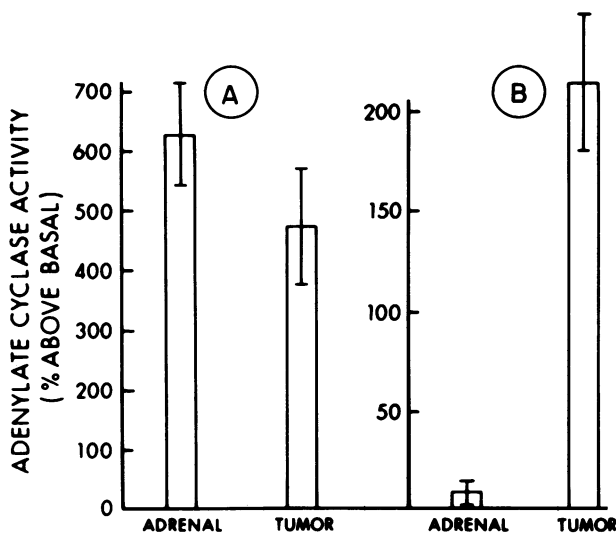


FIGURE 1 Effects of ACTH (A) and isoproterenol (B) on normal adrenal and adrenocortical carcinoma adenylate cyclase. Adenylate cyclase assays were performed on membranes prepared from normal rat adrenals and adrenocortical carcinoma 494 as described in Methods. Basal enzyme activity was 8 and 25 pmol/min per mg protein in normal adrenal and tumor membranes, respectively. Panel A represents the responses of adrenal and tumor membranes to 10 U/ml ACTH and panel B represents the response of adrenal and tumor membranes to  $10^{-4}$  M isoproterenol. Values shown represent the mean values of three to six determinations from a representative experiment.

The purpose of this study was to determine which of these hypotheses explains the appearance of the aberrant response to catecholamines in this neoplastic tissue. Direct binding techniques using the potent  $\beta$ -adrenergic antagonist,  $(-)[^3\text{H}]$ dihydroalprenolol, were used to test for the presence of  $\beta$ -adrenergic receptors in normal adrenal and adrenocortical tumor membranes. This approach is based on the previously reported use of  $(-)[^3\text{H}]$ dihydroalprenolol to identify  $\beta$ -adrenergic receptors in a variety of non-neoplastic tissues (4-8). These binding techniques provide a direct method for assessing whether or not the aberrant catecholamine responsiveness of the adenylate cyclase in this neoplastic tissue is associated with the appearance of ectopic  $\beta$ -adrenergic receptors.

## METHODS

**Pharmacological agents.**  $(-)[^3\text{H}]$ Dihydroalprenolol (sp act 33 Ci/mmol) was prepared by New England Nuclear (Boston, Mass.) by catalytic reduction of  $(-)$ alprenolol (Hassle Pharmaceuticals, Mölndal, Sweden) with tritium gas using palladium as a catalyst. The labeled material is homogeneous in six different chromatographic systems as detailed elsewhere (9) and is indistinguishable from unlabeled dihydroalprenolol. The structure of  $(-)[^3\text{H}]$ dihydroalprenolol is identical to that of unlabeled dihydroalprenolol as determined by mass spectroscopy. The labeled compound as well as

unlabeled dihydroalprenolol have been demonstrated to be potent antagonists of isoproterenol-stimulated adenylate cyclase (9).

Other compounds used in this study were:  $(-)$ isoproterenol bitartrate,  $(-)$ epinephrine bitartrate,  $(-)$ norepinephrine bitartrate,  $(\pm)$ normetanephrine and dihydroxymandelic acid (Sigma Chemical Co., St. Louis, Mo.);  $(+)$ isoproterenol bitartrate,  $(+)$ epinephrine bitartrate,  $(+)$ norepinephrine bitartrate (Winthrop Laboratories, New York);  $(-)$  and  $(+)$ propranolol hydrochloride (Ayerst Laboratories, New York); and phentolamine mesylate (Ciba Pharmaceutical Company, Summit, N. J.) and pyrocatechol (Mann Chemical Corp., Louisville, Ky.).

**Tissue preparation.** Adrenocortical carcinoma 494, which has previously been characterized morphologically (10) and metabolically (11), was maintained by transplantation in male Sprague-Dawley rats in the laboratory of Dr. R. L. Ney as previously described (12). Normal adrenals were obtained from male Sprague-Dawley rats weighing 200-250 g. For adenylate cyclase assays, normal and tumor tissues were homogenized in a buffer composed of 62.2 mM Tris-HCl, pH 7.4, and 15.5 mM theophylline as previously described (2). The crude homogenate was centrifuged at 1,000 g for 10 min and the pellet was then resuspended in buffer (discussed below) for use in the adenylate cyclase assay. This membrane preparation has previously (2) been shown to possess the highest adenylate cyclase activity of several fractions tested from normal and tumor tissue. Membranes for  $(-)[^3\text{H}]$ dihydroalprenolol binding assays were prepared by homogenization in cold (4°C) buffer containing 0.25 M sucrose, 5 mM Tris-HCl (pH 7.4), and 1 mM  $\text{MgCl}_2$ , by using a Potter-Elvehjem homogenizer (20 strokes) with a motor-driven Teflon pestle at high speed. The homogenate was centrifuged at 300 g for 10 min and the pellet was discarded. The supernate was centrifuged at 25,000 g for 10 min and the resulting pellet was washed twice in ice-cold wash buffer (50 mM Tris-HCl, pH 7.5, 10 mM  $\text{MgCl}_2$ ) by resuspension and centrifugation. The final pellet was resuspended in wash buffer for use in the binding assay.

**Adenylate cyclase and  $(-)[^3\text{H}]$ dihydroalprenolol binding assays.** Adenylate cyclase activity of normal adrenal and tumor membranes was assayed as previously described (13). Incubations were performed in a 50- $\mu\text{l}$  volume which contained 40 mM TrisHCl (pH 7.6), 3 mM  $\text{MgSO}_4$ , 5 mM theophylline, 0.1 mM cAMP, 1.5 mM ATP,  $[\alpha\text{-}^{32}\text{P}]$ ATP ( $1 - 2 \times 10^6$  cpm), 5 mM phosphoenolpyruvate, 40  $\mu\text{g}/\text{ml}$  pyruvate kinase, and 20  $\mu\text{g}/\text{ml}$  myokinase. Incubations were for 15 min at 37°C and were stopped by the addition of 1 ml of a solution containing  $[\text{H}]$ cAMP (15,000 cpm/ml), 100  $\mu\text{g}$  ATP, and 50  $\mu\text{g}$  cAMP.  $[\text{H}]$ cAMP that was formed was isolated by the method of Salomon et al., (14).

$(-)[^3\text{H}]$ Dihydroalprenolol binding was assayed by incubating 0.3-mg/ml membranes with  $(-)[^3\text{H}]$ dihydroalprenolol (0.7 nM unless otherwise specified) for 10 min at 37°C in a 2-ml volume of incubation buffer (50 mM Tris-HCl, 10 mM  $\text{MgCl}_2$ , pH 7.5). The use of significantly smaller incubation volumes (<1 ml) resulted in the complication of a significant amount of nonspecific binding of  $(-)[^3\text{H}]$ dihydroalprenolol to both normal adrenal and tumor membranes. Incubations were terminated by rapidly filtering the entire incubation mixture through a Whatman GFC glass fiber filter. Each filter was immediately washed with two 5-ml portions of cold (4°C) incubation buffer. The filters were dried by gentle heating and added to a Triton-toluene based scintillation cocktail. Radioactivity was measured in a liquid scintillation spectrophotometer at an efficiency of 40%. In each experiment nonspecific binding to membranes was determined by measuring the amount of radioactivity retained on filters when incuba-

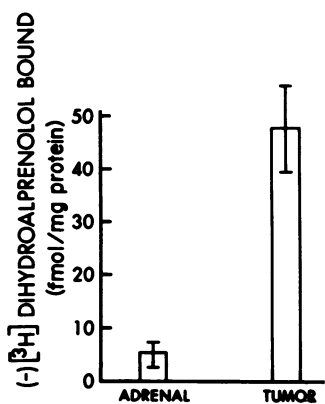


FIGURE 2 (-)[<sup>3</sup>H]Dihydroalprenolol binding to normal adrenal and adrenocortical carcinoma membranes. Membranes were incubated with 0.7 nM (-)[<sup>3</sup>H]dihydroalprenolol and binding was assayed as described in Methods. Values shown represent the mean ± SEM of triplicate determinations from four (adrenal tumor) or five (normal adrenal) separate experiments. The average specific binding was 520 cpm in the tumor and 25 cpm in the control membranes. The average nonspecific binding was 280 cpm in the tumor and 210 cpm in the control membranes and the average protein concentrations were 0.3 mg/ml in the tumor and 0.2 mg/ml in the control membranes.

tions were performed in the presence of 1 μM (-)propranolol. "Specific binding" is defined as total binding minus nonspecific binding. In membranes from adrenocortical carcinoma 494, specific binding ranged from 43 to 75% of the total binding to protein. Protein was determined by the method of Lowry et al. (15).

## RESULTS

**Adenylate cyclase responses of tumor and normal adrenal membranes.** Basal adenylate cyclase activities for normal adrenal and tumor membranes were 8 and 25 pmol/min per mg protein, respectively. The activity of the enzyme was stimulated by ACTH (10 U/ml) in both normal adrenal (620% above basal) and adrenocortical tumor (480% above basal) membranes (Fig. 1A). Similarly, 20 mM of sodium fluoride stimulated normal adrenal (5,400% above basal) and adrenocortical tumor (1,090% above basal) adenylate cyclase activity (not shown). By contrast, the β-adrenergic agonist, isoproterenol (10<sup>-4</sup>M), caused virtually no stimulation of the normal adrenal cyclase while stimulating the tumor adenylate cyclase 220% above basal activity (Fig. 1B). This confirms the results of Schorr and Ney (2) who demonstrated that the adenylate cyclase of adrenocortical carcinoma 494 was stimulated by catecholamines, whereas normal adrenal adenylate cyclase was unresponsive to β-adrenergic agonists. It has been previously demonstrated (3) that catecholamines stimulate the adrenocortical tumor, adenylate cyclase, with a typical β-adrenergic specificity.

(-)[<sup>3</sup>H]Dihydroalprenolol binding studies in tumor

and normal adrenal membranes. The binding of the β-adrenergic antagonist, (-)[<sup>3</sup>H]dihydroalprenolol, was studied in membranes prepared from both normal adrenal and tumor tissue. In the presence of 0.7 nM (-)[<sup>3</sup>H]dihydroalprenolol, 48 ± 8 fmol (-)[<sup>3</sup>H]dihydroalprenolol was specifically bound per milligram of tumor membrane protein (Fig. 2). By contrast, membranes from normal adrenal tissue bound only 5 ± 2 fmol (-)[<sup>3</sup>H]dihydroalprenolol per milligram of protein (Fig. 2), an amount of binding barely detectable by these methods. Hence, very few, if any, specific (-)[<sup>3</sup>H]dihydroalprenolol binding sites are detectable in normal adrenal membranes under the same conditions that exist when a significant amount of binding is measured in adrenocortical carcinoma 494 membranes.

**Characteristics of (-)[<sup>3</sup>H]dihydroalprenolol binding sites in tumor membranes.** Experiments were designed to determine whether the binding sites detected in adrenal tumor membranes had the characteristics expected of β-adrenergic receptors. Binding of 0.7 nM of (-)[<sup>3</sup>H]dihydroalprenolol to 0.3 mg protein/ml of tumor membranes was rapid, reaching a steady-state level in <5 min at 37°C. Binding was constant for at least 16 min of incubation at 37°C. In separate experiments, the reversibility of binding was tested by adding a large excess (10<sup>-5</sup>M) of propranolol to an equilibrated mixture of (-)[<sup>3</sup>H]dihydroalprenolol and

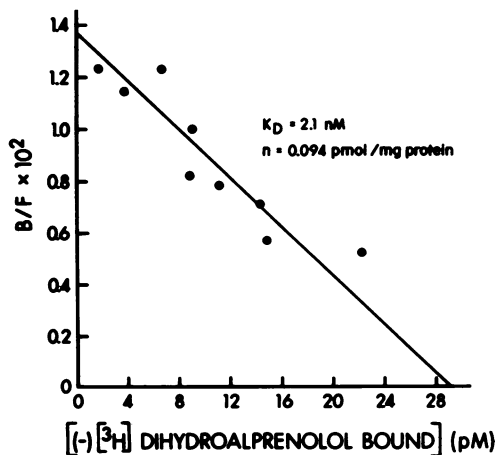


FIGURE 3 Scatchard plot of (-)[<sup>3</sup>H]dihydroalprenolol binding to rat adrenocortical carcinoma membranes. Specific binding determined at a series of (-)[<sup>3</sup>H]dihydroalprenolol concentrations from 0.138 to 4.15 nM, ranged from 100 to 1,260 cpm. Nonspecific binding ranged from 90 to 2,800 cpm. The protein concentration was 0.307 mg/ml. B/F refers to the ratio of bound-to-free dihydroalprenolol at equilibrium. Each value shown is the mean of three determinations. The line shown was drawn by linear regression analysis ( $r = 0.91$ ). The equilibrium dissociation constant ( $K_D$ ) was calculated from the negative reciprocal of the slope of the line and the number of sites ( $n$ ) was calculated from the intercept of the line with the abscissa.

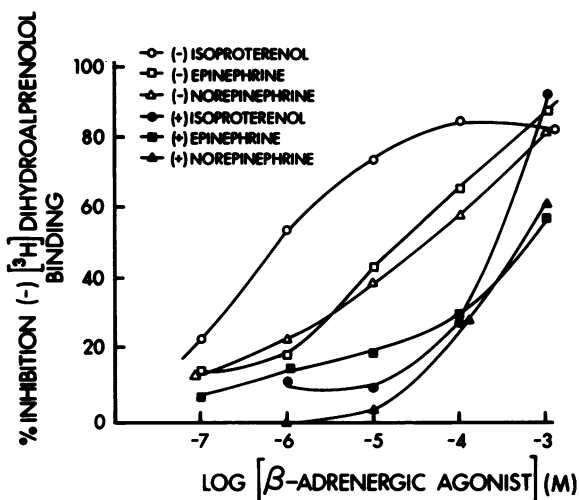


FIGURE 4 Competition for  $(-)[^3\text{H}]$ dihydroalprenolol binding sites by adrenergic agonists. The ordinate represents the percent inhibition of  $(-)[^3\text{H}]$ dihydroalprenolol binding by the specified agonists present in the incubation at the concentrations indicated on the abscissa. 100% inhibition refers to complete inhibition of specific binding. The average specific binding was 410 cpm and the average nonspecific binding was 550 cpm. The protein concentration was 0.3 mg/ml and  $(-)[^3\text{H}]$ dihydroalprenolol was present at 0.7 nM. Each value represents the mean of three determinations in two to three separate experiments.

tumor membranes. The specific binding was rapidly and totally reversible ( $t_{1/2} \sim 4$  min). Scatchard analysis (16) (Fig. 3) of the  $(-)[^3\text{H}]$ dihydroalprenolol binding sites in the tumor membranes demonstrated that the sites have a high affinity for  $(-)[^3\text{H}]$ dihydroalprenolol. The negative reciprocal of the slope of the plot provided an estimate of the equilibrium dissociation constant (2.1 nM) for the interaction of dihydroalprenolol with its binding sites on the tumor membranes. This value is consistent with the reported dissociation constant (5–9 nM) of alprenolol as a  $\beta$ -adrenergic antagonist of catecholamine-stimulated adenylate cyclase in a variety of tissues (4–6). From the intercept of the Scatchard plot (Fig. 3) with the abscissa, the number of binding sites (0.094 pmol/mg protein) on the tumor membranes was calculated. This is comparable to the number of  $(-)[^3\text{H}]$ dihydroalprenolol binding sites found previously (5–8) in non-neoplastic mammalian tissues known to possess  $\beta$ -adrenergic receptors. Data points at low bound-to-free values on the Scatchard plot (Fig. 3) were difficult to obtain because of the large amount of nonspecific binding present when high concentrations of radioligand were used.

The specificity of the binding sites for adrenergic agonists and antagonists was assessed by testing the ability of these agents to compete for  $(-)[^3\text{H}]$ dihydroalprenolol binding sites. The binding displayed a typical  $\beta$ -adrenergic specificity. Adrenergic agonists

(Fig. 4) competed for the binding sites in an order of potency,  $[(-)\text{isoproterenol} > (-)\text{epinephrine} \sim (-)\text{norepinephrine}]$ , consistent with the potency order of these compounds as  $\beta$ -adrenergic agonists. This  $\beta$ -adrenergic pattern has been previously reported for these compounds as activators of the tumor, adenylate cyclase (3). The  $\beta$ -adrenergic agonists competed for the binding sites stereospecifically (Fig. 4), the (+)-stereoisomers requiring 40–300-fold higher concentrations than the corresponding (–)stereoisomers to inhibit an equivalent amount of binding. The  $\beta$ -adrenergic antagonist, (–)-propranolol, potently competed for the binding sites (Fig. 5) causing half-maximal inhibition of binding at a concentration of 6 nM. This is consistent with the previously reported (5, 17, 18) dissociation constant (3–9 nM) of propranolol for inhibition of catecholamine-stimulated adenylate cyclase in mammalian tissue. The (+)stereoisomer of propranolol (Fig. 5) required a 20-fold higher concentration than (–)propranolol to inhibit 50% of the binding. Structurally related compounds, devoid of  $\beta$ -adrenergic activity, did not effectively compete for  $(-)[^3\text{H}]$ dihydroalprenolol-binding sites. Phentolamine, ( $\pm$ )normetanephrine, pyrocatechol, and dihydroxyphenylalanine, when present at concentrations of  $10^{-5}\text{M}$ , inhibited 10% or less of the binding. These data demonstrate that these  $(-)[^3\text{H}]$ dihydroalprenolol binding sites in adrenocortical carcinoma 494 mem-

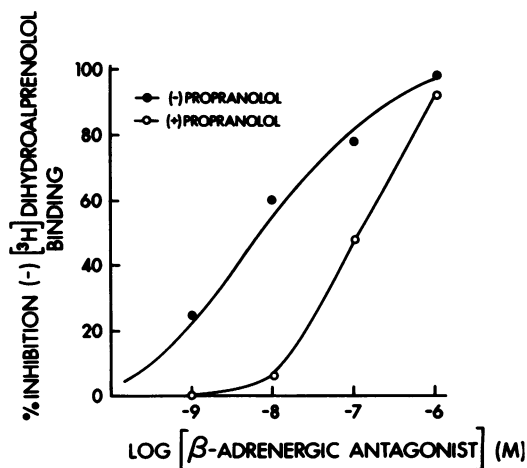


FIGURE 5 Competition for  $(-)[^3\text{H}]$ dihydroalprenolol binding sites by the stereoisomers of propranolol. The ordinate represents the percent inhibition of  $(-)[^3\text{H}]$ dihydroalprenolol binding by the specified stereoisomers of propranolol present in the incubations at the concentrations indicated on the abscissa. Each value represents the mean of two to three determinations in four separate experiments. The average specific binding in the absence of propranolol was 300 cpm and the average nonspecific binding was 260 cpm. The protein concentration was 0.3 mg/ml.  $(-)[^3\text{H}]$ Dihydroalprenolol was present at 0.7 nM.

branes have the affinity, specificity, and stereospecificity for adrenergic agonists and antagonists expected of  $\beta$ -adrenergic receptors.

## DISCUSSION

The results of these binding studies with (-)[<sup>3</sup>H]-dihydroalprenolol demonstrate that normal rat adrenal tissue, which possesses an adenylate cyclase unresponsive to catecholamines, does not possess a significant number of  $\beta$ -adrenergic receptor binding sites. By contrast, an adrenocortical carcinoma in which the adenylate cyclase responds to catecholamine stimulation, possesses (-)[<sup>3</sup>H]dihydroalprenolol-binding sites which have the characteristics expected of  $\beta$ -adrenergic receptors. The tumor binding sites have high affinity for  $\beta$ -adrenergic agonists and antagonists, and demonstrate characteristic  $\beta$ -adrenergic specificity and stereospecificity. The number of  $\beta$ -adrenergic receptor binding sites found in the tumor membranes (0.094 pmol/mg protein) is comparable to the number of sites previously found in similar membrane fractions from a variety of mammalian tissues which contain adenylate cyclase coupled  $\beta$ -adrenergic receptors (5-8). Thus, these data suggest that the aberrant catecholamine responsiveness of the tumor adenylate cyclase can be attributed to the appearance of ectopic  $\beta$ -adrenergic receptor binding sites which are not normally present in adrenal tissue.

The (-)[<sup>3</sup>H]dihydroalprenolol binding sites in the tumor membranes clearly had the specificity expected of a  $\beta$ -adrenergic receptor. It is difficult from our data to conclude whether the receptor is a  $\beta_1$  or  $\beta_2$  subtype of  $\beta$ -receptor, although the pattern seems to be closer to the  $\beta_1$  subtype. By comparison, Schorr et al. (3) previously observed a  $\beta_2$  specificity in the adenylate cyclase response of the tumor to catecholamines.

Although there have been a number of reports of ectopic hormone synthesis by neoplastic tissue (19), there have been no previous reports of directly demonstrated ectopic hormone receptor binding sites in neoplastic tissues. Our results indicate that the neoplastic adrenal membranes differ from the normal adrenal membranes in possessing  $\beta$ -adrenergic receptors which can be detected by direct binding studies. The appearance of these receptor binding sites in adrenocortical carcinoma 494 is presumably associated with "dedifferentiation" of the tumor cells which then allows expression of the ability to synthesize the  $\beta$ -receptors. This capacity is apparently lost in the highly differentiated normal adrenal cortical cell. These results are of particular interest in relation to two sets of recent observations concerning the genetic regulation of  $\beta$ -adrenergic receptors. Spiegel et al. (20) have observed that  $\beta$ -adrenergic receptors may be

present in high numbers in an immature tissue such as the rat reticulocyte and may "regress" as the tissue differentiates into a mature erythrocyte. Maguire et al. (21) have demonstrated that in hybrids of rat glioma cells grown in tissue culture, sensitivity of adenylate cyclase to catecholamine stimulation is a function of the presence or absence of  $\beta$ -receptor binding sites which is in turn genetically regulated. Our results indicate that the apparent insertion of an ectopic  $\beta$ -receptor binding site into the membranes of a neoplastic tissue can result in important functional consequences in the hormonal responsiveness of the tissue.

The concept of an ectopic receptor binding site conferring abnormal hormonal responsiveness on a tissue may have functional implications for neoplastic diseases of endocrine tissue. For example, in normal adrenocortical tissue, steroidogenesis is a cyclic AMP-mediated process which is stimulated only by ACTH. However, in an adrenocortical tumor with ectopic  $\beta$ -adrenergic receptors, cyclic AMP levels might be stimulated by endogenous levels of catecholamines, and an inappropriate steroidogenic response might be seen. Hence, an endocrine tumor which is undergoing steroidogenesis in a seemingly autonomous manner might actually be responding to an unrecognized hormone which normally does not stimulate the noncancerous tissue. If such mechanisms could be elucidated in human neoplastic diseases, therapeutic attempts at blocking the ectopic hormone receptors might be efficacious.

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