Glucocorticoid Receptors and in Vitro Corticosensitivity in Human Thymoma

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ABSTRACT

The presence of a specific glucocorticoid receptor in human thymomas and the in vitro corticosensitivity regarding the inhibitory effect of DNA synthesis is described. The [3H]α-fluoro-11β,16α,17,21-tetrahydroxyprogesterone-1,4-diene-3,20-dione cyclic 16,17-acetal with acetone receptor complex was rapidly transferred into the nuclei at 37°C but not at 0°C. Using cell-free preparations and dextran-coated charcoal assay, cytosols derived from four lymphoepithelial thymomas and one pure epithelial thymoma were found to bind [3H]α-fluoro-11β,16α,17,21-tetrahydroxyprogesterone-1,4-diene-3,20-dione cyclic 16,17-acetal with acetone with a dissociation constant of 5.2 ± 1.3 × 10⁻⁹ M and 5.7 ± 10⁻⁹ M, respectively. The number of receptor sites was 291.75 ± 83 fmol/mg cytosol protein for the lymphoepithelial thymomas and 93.5 fmol/mg cytosol protein for the pure epithelial one. Cytosols from tumors contained binding components that sedimented at approximately 8 to 9S with a reproducible 4 to 5S shoulder (lymphoepithelial thymomas) and at approximately 8 to 9S and 3 to 4S (pure epithelial thymoma).

Competition studies showed high specificity for steroid with glucocorticoid activity. It is suggested that the greater number of receptor sites in thymocytes derived from lymphoepithelial thymomas as compared to those from normal and hyperplastic thymus may result from the advanced maturation step attained by these cells in the altered thymus compartment. Despite the greater number of receptor sites, the corticosensitivity of thymocytes from thymoma relative to the inhibition of DNA synthesis appears quite similar to that reported for the normal thymus.

INTRODUCTION

The inhibitory effects of glucocorticoids on lymphoid tissue have long been appreciated (5). Glucocorticoid treatment of normal and neoplastic lymphoid tissue results in an inhibition of various metabolic functions including the synthesis of proteins and nucleic acids (6, 17–19, 21, 31, 38, 41, 42), the transport of amino acids and nucleosides (19, 30), and the uptake and utilization of glucose (1, 14, 21, 22, 24, 26, 31, 43). These effects are eventually followed by cell lysis (3, 34, 37). Although the molecular mechanism of glucocorticoid hormone action on lymphoid cells still remains unclear, it has been suggested that the interaction between these hormones and cytoplasmic components, termed "glucocorticoid receptors," is essential for most, if not all, of the effects elicited by such steroids in the target cells.

The presence of a specific receptor system for glucocorticoids in the thymus cells of various mammalian species has been reported by several investigators, and some of the physicochemical properties of these receptors have also been described (13, 23, 33). In spite of the fact that the mechanism of action of these hormones has been extensively investigated in rat thymus (for review, see Ref. 24), little is known with regard to the human thymus. Recently, the existence of glucocorticoid receptors has been described in human thymus hyperplasia (28) and in normal thymus in which the effect of these steroids on RNA and DNA synthesis was also demonstrated (12).

In this paper, we describe the presence of a specific glucocorticoid receptor in human thymoma and the in vitro corticosensitivity of lymphocytes derived from neoplastic tissue regarding the effect on DNA synthesis. Although glucocorticoids have often been used in patients with thymoma to relieve the associated symptoms of myasthenia gravis, they have not been used as a form of treatment for the tumor itself (8, 11, 27). This investigation suggests that corticosteroids might be effective in actual thymoma treatment due to the established presence of the specific receptor.

MATERIALS AND METHODS

Reagents and Chemicals. [1,2,4-3H]Triamcinolone acetonide (29 Ci/mmol) and [methyl-3H]thymidine (56 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, England. A radiopurity of labeled steroid of at least 95% was confirmed by thin-layer chromatography in methylene chloride: acetone (70:30). Nonradioactive steroids and their sources were as follows. Cortisol, corticosterone, prednisolone, triamcinolone acetonide, aldosterone, 17β-estradiol, and testosterone were from Calbiochem, La Jolla, Calif. Dexamethasone was from Merck Chemical Co., Darmstadt, Germany, and R5020 was a gift of Dr. J. P. Raynaud, Roussel Uclaf, Romainville, France. RPMI Medium 1640 and fetal calf serum were obtained from Grand Island Biological Company, Grand Island, N. Y. HBSS came from Labtek Eurobio, Milan, Italy. Sucrose (RNase free) was purchased from Schwarz/Mann, Orangeburg, N. Y. Dextran T-70 was from Pharmacia Fine Chemicals, Uppsala, Sweden. Trizma Base, monothioglycerol, and activated charcoal were from Sigma Chemical Co., St. Louis, Mo. Scintillation fluor Instagel came from Packard Instruments International S. A., Zurich, Switzerland. All other chemicals were reagent grade.

Tissues and Isolation of Thymocytes. Thymus glands were
collected at the time of surgery from 5 patients who demonstrated myasthenia gravis and had been previously untreated. The diagnosis of thymoma was verified histologically and according to the degree of lymphocytic infiltration, i.e., absent, moderate, or predominant. Tumors were classified as follows: 4 of lymphoepithelial type and one of pure epithelial round-oval cell type (>90% of epithelial cells in various tumor specimens). Thymocytes were obtained by gently teasing fragments derived from lymphoepithelial thymomas in HBSS. The suspensions were washed 3 times in HBSS containing 0.5% bovine serum albumin by centrifugation at 200 × g for 10 min, and adherent cells were removed by incubation in Petri dishes. The cell pellets were resuspended in RPMI Medium 1640 supplemented with 2 mM l-glutamine and gentamicin (60 μg/ml) and diluted to a final concentration of 3 × 10^6 to 5 × 10^6 cells/ml. Cell viability (determined by the trypan blue dye exclusion test) was greater than 80%. Cell number was obtained by hemocytometer count.

Steroid Retention by Intact Thymocytes. The uptake of labeled steroid was measured in thymocytes derived from neoplastic thymus by incubating 0.2-ml aliquots of cells (5 × 10^7 cells/ml) in RPMI Medium 1640 with [3H]triamcinolone acetonide (2 × 10^-8 M) alone or in the presence of an excess of nonradioactive triamcinolone acetonide (2 × 10^-6 M) at 37° in 5% CO_2-air atmosphere. The incubations were carried out for various periods of time up to 180 min. At the end of the incubation period, cells were harvested by centrifugation at 300 × g for 5 min. The cell pellets were washed twice more with fresh medium and resuspended in 0.5 ml of the same solution to which 1 ml of absolute ethanol was added to assist in the extraction of bound steroid. Radioactivity was measured after the addition of 8 ml of scintillation fluor Instagel in a Mark II liquid scintillation counting system (Nuclear-Chicago Corp., Des Plaines, Ill.).

Nuclear and Cytoplasmic Receptor-bound Hormone. Aliquots (0.4 ml) of thymocytes (4.4 × 10^7 cells/ml) were incubated with [3H]triamcinolone acetonide (2 × 10^-6 M) alone or in the presence of nonradioactive triamcinolone acetonide (2 × 10^-6 M) in RPMI Medium 1640 at 2°. At the end of the incubation period, one-half of the cell suspensions were heated at 37° for 10 min and then rapidly cooled in an ice bath while the other half were maintained at 2°. Cells were sedimented at 400 × g for 10 min at 2°, washed twice with fresh medium, resuspended in 3.1 ml of 1.5 mM MgCl_2, and vortexed periodically over a period of 30 min. The completeness of cell lysis was monitored by phase-contrast microscopy. After centrifugation at 800 × g for 10 min, the supernatant-bound radioactivity was assayed by the dextran-coated charcoal technique according to the method of Bell and Munck (2). Nuclear pellets were washed twice more with 1.5 mM MgCl_2 and resuspended in 0.5 ml of the same solution to which 1 ml of absolute ethanol was added to assist in the extraction of nuclear-bound steroid. The radioactivity of the extracts was determined by adding 8 ml of scintillation fluor Instagel.

Steroid-induced Inhibition of Thymidine Incorporation. Thymocytes, suspended at a concentration of 3 × 10^6 cells/ml in RPMI Medium 1640 supplemented with 10% heat-inactivated fetal calf serum, were incubated for 24 hr in the absence (control cultures) or in the presence of varying concentrations of triamcinolone acetonide ranging from 10^-9 M to 10^-7 M in a humid 5% CO_2-air atmosphere. For the final hr of the incubation period, each culture received 1 μCi of [methyl-3H]thymidine (56 Ci/mmol). At the end of incubation, the cultures were terminated by centrifugation at 400 × g for 10 min and then washed twice with fresh medium. Cell count (hemocytometer) and cell viability (trypan blue dye test) were made for control and steroid-treated cultures at the end of the incubation period. The acid-insoluble residue was then extracted with successive washes of 10% trichloroacetic acid (w/v, twice), 80% ethanol (v/v), and absolute ethanol:ethyl ether (1:1; v/v). The dried residue was then dissolved in 0.3 ml of formic acid, and radioactivity was counted after addition of 10 ml of scintillation fluor Instagel. The counting efficiency for tritium was 35%.

Preparation of the Cytosol. Tissue fragments derived from neoplastic thymus glands were homogenized with three 10-sec bursts of an Ultraturrax TP 18/2 homogenizer (Janke & Kunkel K. G., Cologne, Germany) with TE buffer containing 0.25 mM sucrose and 10 mM monothioglycerol (pH 7.6). Homogenates were centrifuged at 105,000 × g for 60 min at 2°, and supernatants, referred to as cytosols, were used in binding experiments. Protein concentration was determined by the method of Lowry et al. (16) using crystalline bovine serum albumin as protein standard.

Binding Experiments. Cytoplasmic receptor measurement was carried out by a competitive assay using the dextran-coated charcoal method of Bell and Munck (2). Specific binding was calculated as the difference between total binding (uncompeted tubes) and not specific binding (competed tubes). To determine the concentration and the apparent equilibrium dissociation constant (Kd) of receptor sites, specific binding data were analyzed by the Scatchard equation (32) using a P6060 (Olivetti, Ivrea, Italy) desk top computer.

Sucrose Density Gradients. Cytosols were incubated for 5 hr at 2° with [3H]triamcinolone acetonide (3 × 10^-6 M) alone or in the presence of triamcinolone acetonide (6 × 10^-6 M). At the end of the incubation period, free and loosely bound steroid was removed by use of a dextran-coated charcoal pellet. Aliquots of 0.25 ml were applied on the top of linear 10 to 30% sucrose gradients made in TE buffer containing 10 mM monothioglycerol. Gradients were run at 48,000 rpm for 16 hr at 2° in a Beckman L2-65B ultracentrifuge with a Spinco SW 56 titanium rotor (Beckman-Spinco Instruments, Inc., Palo Alto, Calif.). Fractions of 7 drops were collected from the top of the tubes by injecting a concentrated sucrose solution at the bottom of the gradients. Radioactivity was measured after addition of 8 ml of scintillation fluor Instagel. [3H]-labeled proteins, ovalbumin, and human γ-globulin were run simultaneously to calculate sedimentation coefficients. The counting efficiency for tritium was 40%.

RESULTS

Studies on Intact Cells. The capacity of lymphocytes derived from human lymphoepithelial thymoma to take up and retain glucocorticoid was first demonstrated in suspensions of intact cells. A time course of [3H]triamcinolone acetonide uptake by isolated cells is illustrated in Chart 1. At the concentration of steroid used (2 × 10^-8 M), the specific accumulation of the steroid in the cells appeared to reach a maximum after 20 min at 37° and remained at that level for another 160 min.

Regarding nuclear and cytoplasmic receptor-bound hormone, the results of one of the experiments, as illustrated in
Chart 2, demonstrate that the hormone-receptor complex is transferred to the nucleus through a temperature-dependent process. Thus, heating cell suspensions, which were preincubated with \(^{3}H\)-triamcinolone acetonide at \(2^\circ\) for 120 min and at \(37^\circ\) for 10 min, resulted in over 90% hormone uptake by the nuclei. Conversely, in incubations carried out at \(2^\circ\), about 85% of specifically bound radioactivity was recovered in the cytoplasmic fraction.

The effect of triamcinolone acetonide on the \([^{3}H]\)thymidine incorporation into acid-insoluble material in the isolated thymocytes is shown in Chart 3. A 50% reduction of nucleoside incorporation was observed at a steroid molarity (\(-3 \text{nM}\)) corresponding to approximately the value of the apparent equilibrium dissociation constant of the receptor-steroid complex (see below). The greatest reduction of \([^{3}H]\)thymidine incorporation (70%) occurred at a steroid concentration of \(\sim 6 \text{nM}\) and was no longer modified for hormone concentration up to 100 nM. After 24-hr incubation, cell viability was greater than 80% both in the control and hormone-treated cultures even at the highest concentration of steroid used; in the same period, the number of cells did not differ. Furthermore, in both the control and treated cultures, there was a 10% reduction of cell number as compared to that observed at the beginning of culture.

**Studies on Cell-free Preparations.** Having observed that isolated thymocytes specifically bind triamcinolone acetonide, determinations of the properties of the binding components in 5 histologically different thymomas (4 lymphoepithelial and one pure epithelial) were carried out with cell-free preparations (which also gave the opportunity of comparing them). In a preliminary experiment, a time course of specific binding to 105,000 x g supernatants from tumors revealed that at \(2^\circ\) in the presence of a saturating concentration of \([^{3}H]\)triamcinolone acetonide \((3 \times 10^{-6} \text{ M})\), the equilibrium condition was reached after 5 hr of incubation and remained stable for at least 24 hr (data not shown).

Protein concentration dependence of \([^{3}H]\)triamcinolone acetonide binding to cytosols derived both from lymphoepithelial and epithelial tumors was examined next. As illustrated in Chart 4, the amount of specifically bound \([^{3}H]\)triamcinolone acetonide was linearly correlated to cytosol protein concentration up to 8 mg/ml both for lymphoepithelial and epithelial thymomas.

The affinity of the cytoplasmic component-binding triamcinolone acetonide was measured by titrating a constant volume of cytosols from lymphoepithelial and epithelial tumors with increasing concentrations of the tritiated steroid alone or in combination with unlabeled steroid at \(10^{-5} \text{ M}\). The incubation was carried out overnight at 0 to 2\(^\circ\), and then the unbound and loosely bound steroid was removed by adsorption onto dextrancoated charcoal.

An example of the direct plot of the specific binding calculated as the difference between uncompetet and competed tubes is shown in Chart 5A for one of the lymphoepithelial thymomas and for the pure epithelial one. These plots displayed a limited number of binding sites, and an analysis of the binding data according to the method of Scatchard (32) yielded a straight line indicating a single class of high-affinity binding sites with a dissociation constant (K\(_d\)) and a concentration of receptor sites (nm), respectively, of \(3.4 \times 10^{-9} \text{ M}\) and 278 fmol/mg of protein for the lymphoepithelial thymoma and 5.7
binding to cytosols derived from thymomas. Aliquots of cytosols were incubated overnight at 2° with increasing concentrations of unlabeled triamcinolone acetonide (10⁻⁸ M) alone or in the presence of triamcinolone acetonide (10⁻⁸ M). The specifically bound [³H]triamcinolone acetonide was determined by dextran-coated charcoal assay as described in 'Materials and Methods.' The straight lines were drawn by least-squares regression. r, the correlation coefficient for these lines.

× 10⁻⁸ M and 93.5 fmol/mg of protein for the epithelial one (Chart 5B). The concentration of receptor sites and the Kₐ measured in all 5 thymomas are given in Table 1.

The specificity of the binding sites has been evaluated in competitive assays by incubating the cytosols from thymomas with [³H]triamcinolone acetonide (2 × 10⁻⁸ M) alone or in the presence of increasing concentrations of various unlabeled steroids at 10⁻⁸, 10⁻⁹, and 10⁻¹⁰ molar excess. Incubations were carried out overnight at 0 to 2°, and then the unbound and loosely bound steroid was removed by absorption onto dextran-coated charcoal. For example, the results of the competition studies relative to one of the lymphoepithelial thymomas are illustrated in Chart 6A, and those relating to the pure epithelial one are shown in Chart 6B. The stereospecificity of the binding sites was quite similar in both types of tumor. Among the natural glucocorticoids, corticosterone and cortisol were equally effective in inhibiting the binding of [³H]triamcinolone acetonide to the glucocorticoid-binding sites. Dexamethasone > prednisolone acetate were in order the most potent inhibitors of the binding. The synthetic progestin R5020 and aldosterone were also effective in competing with [³H]triamcinolone acetonide. Other nonglucocorticoids such as estradiol and testosterone showed little, if any, competition but only when present at a large molar excess (1000 times).

In order to evaluate if [³H]triamcinolone acetonide was bound to a macromolecule(s), the cytosols from tumors were incubated with [³H]triamcinolone acetonide (3 × 10⁻⁸ M) and analyzed for radioactivity in 10 to 30% linear sucrose gradients made in TE buffer with 10 mM monothioglycerol. The profile of radioactivity relative to the lymphoepithelial thymomas displayed one single peak of [³H]triamcinolone acetonide-binding component in the 8 to 9S region of the gradient with a reproducible 4 to 5S shoulder (Chart 7A) while that relative to the pure epithelial thymoma displayed peaks sedimenting at approximately 8 to 9S (large component) and 3 to 4S (small component) (Chart 7B).

The addition of an excess (100 times) of unlabeled triamcinolone acetonide to the incubations greatly reduced the radioactivity in binding components of both lymphoepithelial and pure epithelial tumors (Chart 7, A and B).

**DISCUSSION**

In this study, we have demonstrated that human thymomas contain receptor macromolecules that bind triamcinolone acetonide with high affinity and specificity. The binding has been demonstrated in both lymphoepithelial and pure epithelial thymomas and satisfies all the general criteria by which steroid receptors are characterized in other target organs.

The concentration of specific binding sites appears significantly greater in the lymphoepithelial thymomas examined as compared to that observed in thymus hyperplasia (28) and in pure epithelial thymoma. Furthermore, the presence of glucocorticoid-binding component(s) in pure epithelial thymoma suggests that the epithelial cells may contain specific receptor(s) for these hormones as it seems unlikely that the very small lymphatic cell contaminants (lower than 10%) can account for the observed binding. This possibility is of great interest considering the known influences of epithelial cells on intrathymic lymphocyte maturation (4, 9, 39) and suggests that glucocorticoids can regulate thymus physiology by exerting their actions both on epithelial and lymphatic thymus components. However, we do not know at present if the presence of glucocorticoid receptors is related to any of the parameters of the neoplastic.
Control values for lymphoepithelial and pure epithelial thymomas were 210 ± 8.3 and 130 ± 14 fmol/mg of protein, respectively.

state of epithelial cells, such as growth rate or biochemical differentiation, since there is no information about the occurrence of these receptors in the epithelial component of normal human thymus as yet. Therefore, determining the presence of glucocorticoid receptors in purified epithelial cell fractions derived from both normal and neoplastic thymus seems worthy of further investigation.

The greater number of receptor sites observed in lymphoepithelial thymomas as compared to normal (Ref. 12 and Footnote 4) and hyperplastic thymus (28) could be explained by 2 different hypotheses; i.e., the lymphocytes in the tumor may or may not be thymic in origin. In other words, they may be either peripheral lymphocytes that might have infiltrated the gland because of an autoimmune process or they might be thymic in origin, and their characteristics may reflect altered intrathympic differentiation pathways due to abnormalities of the environment established by the neoplastic epithelial proliferation. The first hypothesis seems unlikely as virtually all lymphocytes derived from 4 lymphoepithelial thymomas are T-cells and form stable E-rosettes as demonstrated in the same cells by a previous work (15). On the contrary, peripheral blood T-lymphocytes lack the capacity to form E-rosettes resistant to a 37° incubation. Therefore, this property, probably due to changes in cell membranes and/or erythrocyte-receptor properties such as surface charge (40) and sialic acid content of cell membrane (7, 29), seems to distinguish between thymocytes and peripheral T-lymphocytes. So, the capacity of lymphocytes derived from lymphoepithelial thymomas to form stable E-rosettes suggests that these cells do not enter from the periphery but derive from bone marrow precursors which have migrated into the altered thymus compartment. On the other hand, as previously observed (15), the presence of receptors for IgM on a significant percentage of thymoma lymphocytes suggests

that these cells could undergo an altered maturation process, acquiring at least one of the surface markers which are normally present in peripheral T-lymphocytes and rarely expressed in normal thymus lymphocytes (10, 15, 20). This observation can also account for the higher mitogen responsiveness of thymoma lymphocytes when compared to that of normal thymus (15). Furthermore, the greater number of glucocorticoid receptor sites observed in lymphoepithelial thymomas (as compared to normal and hyperplastic thymus) could be correlated to the advanced and/or abnormal maturation step attained by these cells in the altered thymus compartment.

Finally, the presence of glucocorticoid receptor activity in thymomas suggests that the oncolytic effect of corticosteroid therapy observed in the treatment of some of these tumors (35) may be mediated via a receptor mechanism.

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