Glucocorticoid-binding Components in an Irradiation-induced Thymoma of the C57BL/6J Mouse

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SUMMARY

Preliminary experiments on thymocyte suspensions derived from normal thymus and γ-irradiation-induced thymomas of the C57BL/6J mouse indicated the presence of macromolecular species capable of binding triamcinolone acetonide-3H specifically with high affinity. Retention of labeled steroid by isolated tumor cells varied considerably among thymomas. Using cell-free preparations and a dextran-coated charcoal procedure, tumor cytosols bound triamcinolone acetonide with a dissociation constant of 3 × 10⁻⁹ M; the number of binding sites was 354 fmoles/mg cytosol protein. Cytosols contained binding components that sedimented at 7 to 8 S on low-salt gradients and at 4 to 5 S on gradients containing 0.4 M KCl. These cytoplasmic components bound principally with hormones with glucocorticoid activity as indicated by the competition of triamcinolone acetonide-3H binding in the presence of 5 × 10⁻⁵ M cortisol, dexamethasone, and corticosterone as well as by progesterone and aldosterone. Neither estradiol-17β, estriol, testosterone, nor dihydrotestosterone at 5 × 10⁻⁵ M inhibited specific binding of triamcinolone acetonide-3H. Generally, the binding properties of isolated thymocytes were similar to those determined on cell-free preparations.

INTRODUCTION

Proteins binding glucocorticoids specifically have been described in both rat and mouse thymocytes (8, 15, 20), as well as in cultured lymphoma cells (1) and mouse lymphosarcoma (11). Also, it has been shown that many lymphoid tissues undergo regression and dissolution accompanied by cell death following glucocorticoid treatment in vivo (4, 5) and in vitro (7). Presumably, this occurs as a result of inhibition of macromolecular synthesis (14, 17, 21) and glucose uptake (14, 17).

Recently, it has been shown that glucocorticoid-resistant cells of lymphoid origin cultured in vitro bind much less glucocorticoid than do sensitive lines (1, 11, 18). Although the molecular mechanism of action of glucocorticoid hormones on lymphoid cells is unclear, it has been suggested that interaction of these hormones with cytoplasmic components termed “glucocorticoid-binding proteins” or “glucocorticoid receptors” is essential for the cytolytic effect. This paper presents preliminary characterization of triamcinolone acetonide-3H binding proteins found in an irradiation-induced thymoma of the mouse, which regresses in response to glucocorticoid therapy (9, 10).

MATERIALS AND METHODS

Reagents and Chemicals. Sucrose (RNase-free) and dextran were obtained from Schwarz/Mann, Orangeburg, N. Y., as was triamcinolone acetonide-1,2,4,3H (10.7 Ci/mmol). Corticosterone was purchased from Steraloids, Pawling, N. Y., while dexamethasone, triamcinolone acetonide, and Trizma base were from Sigma Chemical Co., St. Louis, Mo. All other steroids were obtained from Calbiochem, La Jolla, Calif. HBSS, used in the isolation of thymocytes, came from Grand Island Biological Co., Grand Island, N. Y.; charcoal (Norit A) was bought from Matheson, Coleman & Bell, Norwood, Ohio. Scintillation fluor (Omnifluor) was obtained from New England Nuclear, Boston, Mass., while Amersham/Searle Corp., Arlington Heights, Ill. supplied the NCS solubilizer. Irradiation Procedure. Male inbred C57BL/6J mice, 4 weeks old (The Jackson Laboratory, Bar Harbor, Maine) received 4 weekly doses of 200 rads γ-irradiation from a Theratron 80 Co teletherapy unit (Atomic Energy of Canada, Ltd., Ottawa, Ontario, Canada) at a dose rate of 160 rads/min as measured by a Baldwin-Farmer dosimeter (Nuclear Enterprises, Ltd., Reading, England). During irradiation, mice were kept in separate rubber-stoppered, ventilated polycarbonate tubes in radial position on a horizontal Lucite platform that was rotated in the irradiation beam at 5 to 6 rpm. A Lucite sheet, 0.25 inch thick, was located between the tubes and the irradiation source. Animals were sacrificed 21 to 26 weeks after irradiation. Histological evaluation of the primary thymic tumors samples showed them all to be of lymphoid cell origin; none were of epithelial, reticulum cell, or histiocytic cell type.

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origin. Sixty % of the tumors examined were lymphoblastic type and displayed varying degrees of cytolysis with macrophage ingestion of cell products. The other 40% of the tumors were composed of large and/or medium size lymphocytes.

**Thymocyte Isolation.** Mice were sacrificed by cervical dislocation and the thymus glands (normal or neoplastic) were removed and placed in cold HBSS to which 0.5% bovine serum albumin had been added. The thymus glands were cleaned of blood and connective tissue and minced into 2 to 5 portions with scissors. These were transferred to a Dounce homogenizer containing 10 ml cold HBSS and were homogenized with 4 passes with a loose-fitting pestle. The resulting suspension was filtered through a double layer of cotton gauze into a 10-ml syringe equipped with a Millipore Swinnny adapter containing a double set of metal filtering screens (Millipore Corp., Bedford, Mass.). The eluant cell suspension was collected into conical test tubes, washed with fresh HBSS, and centrifuged in an International PR-2 centrifuge, using a No. 269 horizontal head rotor (600 rpm, 10 min, 3°), twice before resuspension and dilution to a final concentration of 5 X 10^7 cells/ml as determined by hemacytometer count.

**Steroid Retention by Intact Thymocytes.** Uptake of labeled steroid was measured using 0.5-ml aliquots of freshly prepared thymocyte suspension in disposable round-bottomed test tubes (10 x 75 mm) containing either triamcinolone acetonide-3H alone (8 x 10^-9 M) or in combination with cortisolone (10^-5 M). The test tubes were incubated at 25° in a water bath and agitated intermittently to maintain a uniform suspension. After incubation, tubes were centrifuged in the International PR-2 centrifuge (600 rpm, 10 min, 3°) and the media aspirated. Cells were washed twice by resuspension in 1 ml cold HBSS followed by centrifugation and aspiration of the media. NCS (0.5 to 1 ml) was then added, and the pellet was digested overnight. Samples were counted in 10 ml of a toluene-based scintillation cocktail (4 g Omnifluor per liter toluene).

**Preparation of Cytosols.** Tissues were obtained as above and homogenized in 10 mM Tris-HCl:1.5 mM EDTA:250 mM sucrose:10 mM thio glycol water buffer (pH 7.6) (1:3 (w/v) for gradients; 1:5 (w/v) for DCC procedure) in a Dall homogenizer. Homogenate was then centrifuged at 105,000 X g for 30 min in a Beckman Model L2-65B ultracentrifuge at 2°. Supernatants were collected and stored briefly on ice. Protein was determined by the method of Lowry et al. (13).

**DCC Assay.** The DCC assay used was a modification of an earlier procedure by Korneman (12) and has been described in detail elsewhere (6). It was designed primarily to rid samples of unbound labeled steroid by adsorption to the charcoal. Briefly, 0.2-ml aliquots of cytosol were incubated with trichloracetonone acetone-3H (~2 X 10^-8 M) in the presence or absence of unlabeled steroid at a concentration of 10^-6 M. Following incubation, 1 ml of 10 mM Tris-HCl:1.5 mM EDTA:250 mM sucrose buffer (pH 7.6) containing 0.5% Norit A and 0.05% dextran was added and incubated an additional 15 min at 0°. Following centrifugation at 1500 rpm for 5 min, 0.6 ml liquid was removed and counted in 10 ml Bray's fluor (2).

**Sucrose Gradient Assay.** Tumor cytosols were incubated for 5 hr at 0° with ~2 X 10^-8 M triamcinolone acetonide-3H in the presence or absence of unlabeled steroid at 3 X 10^-8 M. Following incubation, 0.6-ml aliquots were transferred to vials containing a DCC pellet previously prepared by centrifuging 1 ml of the charcoal suspension and removing the buffer by aspiration. The vials were then agitated on a Vortex Genie (Scientific Industries, Inc., Springfield, Mass.) and incubated an additional 15 min at 0°. This procedure permitted removal of unbound steroid while precluding dilution of the sample. After centrifugation, 0.2 ml of each supernatant was layered onto linear gradients of 5 to 20% sucrose in 10 mM Tris HCl:1.5 mM EDTA (pH 7.6) or 10 mM Tris HCl:1.5 mM EDTA:0.4 M KCI (pH 7.6) buffers. These gradients were then centrifuged for 15 hr at 308,000 X g in a Beckman L2-65B ultracentrifuge using a Spino SW-56 titanium rotor (2°). Each gradient was collected into 36 fractions from the bottom of the tube; fractions were counted in 2 ml 99% ethanol and 10 ml toluene scintillation cocktail.

**Calculations.** Samples were counted in a Mark II liquid scintillation counting system (Nuclear-Chicago Corp., Des Plaines, Ill.); counting efficiency of 38%, calculated by standardization of individual samples with a 133Ba standard, was determined by a computer program previously described (3). Binding capacity was expressed as femoles (10^-15 mole) triamcinolone acetonide-3H bound either on a per mg cytosol protein or per cell basis. Conversion of radioactivity (dpm) to number of binding sites per cell was accomplished by determining the amount of triamcinolone acetonide-3H bound per reaction containing 2.5 X 10^7 cells and applying Avogadro's number.

**RESULTS AND DISCUSSION**

**Uptake of Labeled Steroid by Isolated Thymocytes.** The capacity of the irradiation-induced thymoma to take up and retain glucocorticoid specifically was first demonstrated using cell suspensions isolated from individual tumors where possible. For small tumors, tissues were "pooled" for the preparation of a cell suspension. Thymus glands from control, nonirradiated mice were pooled in the same manner. A time course of association of triamcinolone acetonide-3H with isolated thymocytes is shown in Chart 1. Corticosterone, a steroid known to inhibit glucocorticoid binding in a thymocyte system (15), was used to demonstrate the specificity of the ligand-cell interaction in addition to providing a means of quantitating specific retention. Specific retention was defined as the difference between total (triamcinolone acetonide-3H alone) and nonspecific retention (triamcinolone acetonide-3H plus unlabeled hormone in excess) expressed on a per cell basis. Accumulation of the ligand in normal thymocytes from control animals appeared to reach a maximum in 60 min at 25° and remained at that level for at least another 60 min. Because of limiting amounts of thymoma tissue, determinations of uptake in isolated tumor cells were performed only at 60 and 120 min (Chart 1). It appears that, on a per cell basis, some thymomas retained larger amounts of the glucocorticoid, although the relative amount varied considerably among tumors.

Kaplan et al. (9, 10) have shown that the level of adrenal corticosteroids influences the development of lymphoid tumors in mice exposed to total-body irradiation. These
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Chart 1. Time course of specific glucocorticoid-retention in thymocytes derived from normal and neoplastic tissue. Cell suspensions were added to glass vials containing triamcinolone acetonide-3H (8 X 10^-9 M) in the presence or absence of cortexolone (10^-5 M) and incubated for various times at 25°. Specific retention was determined as the difference between total binding and binding in the presence of cortexolone. Each point represents the average of 2 determinations. * pool of thymus glands from 20 nonirradiated mice (~30 mg/gland); •, single tumor (630 mg); ○, pool of thymus glands from 8 irradiated mice with small tumors (~50 mg/gland); ◦, single tumor (250 mg); △, single tumor (360 mg).

workers have also shown that injections of cortisone, either concurrently with or 6 weeks after total-body irradiation, significantly inhibited the development of lymphoid tumors in this strain of mouse. Demonstration of the capacity to retain triamcinolone acetonide-3H by this irradiation-induced thymoma of the C57BL mouse is compatible with its sensitivity to glucocorticoid therapy (9, 10).

Characteristics of Binding by Cell-free Preparations. For determination of the properties of the specific components binding triamcinolone acetonide-3H in these thymomas, cell-free preparations of tumors were used. A representative time course of triamcinolone acetonide-3H binding to cytosols of tumors is shown in Chart 2. As observed for whole cells, binding to the cytoplasmic fraction was complete by 60 min at 25° (Chart 2A). At 0°, the reaction was slower, reaching a comparable level 3 hr later. However, a greater level of binding was seen at 5 to 22 hr upon further incubation at 0°, suggesting that binding was temperature sensitive. Routinely, reactions were incubated for 16 to 22 hr at 0°.

The affinity of the cytoplasmic component binding triamcinolone acetonide was measured by titrating a constant volume of cytosol from a mouse tumor with increasing concentrations of the tritiated steroid alone or in combination with unlabeled steroid at 10^-5 M (Chart 3A). These mixtures were incubated for 18 hr at 0° and the unbound steroid was removed by adsorption onto DCC. The difference in the titration curves, defined as specific binding, demonstrated that the available binding sites were saturated at ~60 nM. With the method of Scatchard (19), an analysis of the data from Chart 3A yielded a straight line (Chart 3B) indicating a single type of binding site. The slope of the line gave a dissociation constant for the triamcinolone acetonide:receptor complex of 3 X 10^-9
Steroid specificity of triamcinolone acetonide-3H binding sites in cytosol
sucrose: 10 mM thioglycerol buffer, (pH 7.6). Binding was measured in the presence of 36 nM triamcinolone acetonide-3H after 16 hr at 0° using the DCC procedure described in the text.

To characterize further the glucocorticoid-binding components, tumor cytosols were examined on sucrose gradients following the formation of triamcinolone acetonide: receptor complexes. Binding components separated on low-salt gradients (Chart 4A) as a large peak sedimenting at 7 to 8 S followed by a smaller peak sedimenting at ~4 S. Cytosol incubated similarly but also containing unlabeled steroid reduced the amount of bound triamcinolone acetonide-3H to near background levels, demonstrating the specific nature of the binding in the peaks. These data differ from those of Kaiser et al. (8), who reported only the 7 to 8 S component in the cytosol of mouse thymocytes but observed both the 7 to 8 S and 4 S species in thymocytes of the rat. The significance of this distribution is unknown. Chart 4B demonstrates the effect of a high-salt concentration on the sedimentation profile of these cytoplasmic receptors. When cytosols, prepared in low-salt buffers, were separated on sucrose gradients containing 0.4 M KCl, only the 4 S form of the hormone-receptor complex was observed.

In this study we have shown that the irradiation-induced thymoma of the C57BL/6J mouse contains components in the cytoplasm that bind triamcinolone acetonide-3H specifically and with high affinity. The affinity constants and ligand specificities of these glucocorticoid-binding components are similar to those of receptors found in rat and mouse thymocytes (8, 16, 20) as well as in cultured lymphoma cells (1) and mouse lymphosarcomas (11). These results suggest that glucocorticoid-binding components are not aberrant in thymic lymphomas compared to normal thymocytes. It is probable that these binding components are involved in the prevention of thymic lymphoma development in the presence of corticosteroids (9, 10).

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