Biochemical and Biophysical Characterization of Glucocorticoid Receptors in Normal Lymphoid Tissue

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A LARGE BODY of experimental data generated over the last 20 yr has established that the physiologic effects of glucocorticoids are mediated through specific cytoplasmic receptor molecules. Such receptors have been identified in all target tissues and are usually decreased in absent in cultured cell lines. However, this direct or indirect responsiveness on lack of responsiveness of the leukemic to glucocorticoid therapy would be the presence or absence of blast cell receptors. This discrepancy requires resolution if one is to begin to understand the molecular basis of glucocorticoid-induced remission in leukemia. We have therefore initiated a study comparing certain biochemical and biophysical characteristics of glucocorticoid binders in normal cells with those in leukemia cells.

In this article we report on the properties of 3H-triamcinolone acetonide binding macromolecules in normal human and animal lymphoid tissues. Using the techniques of ion exchange chromatography, isokinetic centrifugation, and affinity binding to DNA and chromatin, we have identified two forms of cytoplasmic triamcinolone acetonide binders in these tissues. Both binding components have characteristics that suggest physiologic relevance.

MATERIALS AND METHODS

Reagents

3H-triamcinolone acetonide (3H-TA) was purchased from Amer sham, Arlington Heights, Ill.; Sephadex G-25 was obtained from Pharmacia Fine Chemicals, Piscataway, N.J.; DEAE cellulose, DE-52, from Whatman, Kent, England; native calf thymus DNA cellulose from P.L. Biochemicals, Milwaukee, Wisc.; activated charcoal (Norit A) from Fisher Scientific, Medford, Mass.; 14C-carbonic anhydrase, 125I-BSA, 125I-gamma globulin, and Biofluor from New England Nuclear, Boston, Mass.

Tissues

Thymus, spleen, and lymph nodes from 3-5-day-old calves were obtained at a local slaughter house, placed on water ice immediately after death, and trimmed of fat and connective tissue. Tissues were stored as aliquots (approximately 10 g of whole tissue) at -90°C. For certain experiments, single cell suspensions were made from fresh tissue as previously described, and stored as pellets at -90°C, or used immediately in the whole cell labeling procedure described below. Fresh human thymus was obtained from a 7-yr-old child undergoing cardiac surgery and stored as whole tissue at -90°C. Peripheral blood lymphocytes were harvested on Ficoll-Hypaque from CPD anticoagulated whole blood from normal adults and stored as pellets at -90°C.

Cytosol Preparation and Labeling

The procedures used were modified from the broken cell system described by Sakaue and Thompson. All procedures were performed at 4°C. Tissues were homogenized in a Waring blender (five 15-sec bursts at low speed with 30-sec cooling intervals) with 1.5 ml of KD buffer (5 mM K2HPO4, pH 7.6, with 0.5 mM diethiothreitol) per gram tissue. Cell pellets were thawed in KD buffer (1 ml/106 cells). The crude homogenate was centrifuged at 800 g for 5 min and the supernatant was recentrifuged at 140,000 g for 30 min. Aliquots of this supernatant, containing 15-30 mg protein/ml were incubated with 3H-TA at 2 x 10-6 M for 2 hr. Unbound steroid was removed by absorption with charcoal and Sephadex G-25 filtration, as previously described. Specific binding was defined as the difference between the amount of H-TA binding in the presence and absence of a 300-fold excess of radioactive TA. At 2 x 10-6 M H-TA specific binding was 95% of total binding.

Whole Cell Labeling

Freshly prepared thymocytes (2 x 106 cells/ml phosphate-buffered saline) were incubated with 2 x 10-8 M 3H-TA for 90 min...
at 4°C. The cells were pelleted, then disrupted by rapid freezing in an acetone-dry ice bath, thawed in the presence of 2 ml KD buffer, and further processed as described above for crude homogenates in the broken cell system.

Analytical Procedures

All procedures were performed at 4°C. DEAE cellulose columns (2 x 6 cm) and DNA cellulose columns (2 x 3 cm) were equilibrated with KD buffer. All 3H-TA-labeled materials applied to these columns, if not already in KD buffer, were first filtered over Sephadex G-25 equilibrated with KD buffer. For DEAE cellulose columns, samples were applied at 0.2 ml/min. After sample application, the columns were washed with KD buffer at 0.6 ml/min until the flow-through radioactivity was cleared (usually 3–5 column volumes of buffer) and developed with 30-ml linear gradients of 0.005–0.4 M K2HPO4 in KD buffer. Fraction size was 1 ml and 200 λ aliquots were used for radioactive counting. For DNA cellulose binding, 3H-TA-labeled samples (usually 1 ml) were applied at 0.3 ml/min and allowed to sink into the column bed, following which the outflow was clamped for 30 min. The columns were then washed and developed as described above for DEAE cellulose columns. DNA cellulose binding was calculated from the difference between the radioactivity applied to the column and that eluted by the salt gradient and expressed as a percentage of the amount applied. Plain cellulose columns were prepared as described for DNA cellulose columns and used to determine background binding to cellulose alone.

Chromatin was prepared from fresh calf thymus gland by a modification of the method of Bonner et al. and stored at –20°C as pelleted aliquots containing 1.5 mg protein. For binding studies these pellets were washed in KD buffer at 4°C, centrifuged, and resuspended in the 3H-TA labeled samples (1.5 mg chromatin/0.5 ml labeled receptor complex). Paired samples were used; one was immediately centrifuged (Brinkman microfuge, 9000 g for 30 sec) to determine zero time supernatant radioactivity. The other was incubated with gentle agitation for 1 hr at 4°C, then centrifuged and an aliquot of the supernatant taken for radioactive counting. Chromatin binding was calculated from the difference between supernatant radioactivity after 0 and 1 hr incubations and expressed as a percentage of the zero time radioactivity.

Linear gradients of 10%–30% glycerol in KD buffer were used for velocity sedimentation analyses of 3H-TA-labeled complexes eluting from DEAE columns. Eluted materials were filtered over Sephadex G-25, equilibrated with KD buffer, samples (500A) were layered on 4.6 ml gradients in polyallomer tubes and spun at 48,000 rpm for 17 hr in a SW 50.1 rotor. Five-drop fractions were collected from the bottom of the gradient and used for radioactivity measurements. S values were established in relation to standard 4C markers, which were run on companion gradients and processed identically. Aliquots for radioactive determination were diluted in 10 ml of Biofluor and counted in a Beckman LS7500 counter. Salt concentrations were measured using a Radiometer CD72 electroconductivity meter.

RESULTS

DEAE Chromatography

Receptors from normal lymphoid cytosols labeled by both the intact and broken cell procedures resolved into two species on DEAE cellulose columns (Fig. 1 A and B and 2). We refer to the minor, early eluting (0.04 M salt) component as peak I and the major, late eluting (0.22 M salt) component as peak II. Radioactivity in both peaks was equivalently reduced by 95% when the incubations were done in the presence of 300-fold excess of radioinert TA. Peak I and peak II did not alter their elution positions when individually rechromatographed on DEAE.

The chromatographic pattern is altered when labeled intact cells or cytosol are heated before DEAE chromatography. With cytosol labeled in the broken...
cell system, the pattern progressively changes from peak II to peak I predominance with increasing duration of exposure to 20°C. The pattern seen after a 30-min heat exposure of calf thymus cytosol is illustrated in Fig. 1C. Here, despite a 32% loss of total receptor-bound 3H-TA (most likely due to increased receptor-complex lability at 20°C), peak I complexes increased twofold and became the dominant species (compare Figs. 1A and 1C). To define the basis for these postheating chromatographic changes, apex fractions from the peak I and peak II areas of an unheated cytosol were individually heated and reapplied to DEAE. Peak I did not change its elution position after 30 min at 20°C (Fig. 3A). In contrast, peak II did alter its chromatographic behavior after an identical heat exposure: rechromatography now showed approximately 50% of the peak II complexes eluting in the peak I area (Fig. 3B). Thus, peak II complexes acquire peak I DEAE chromatographic characteristics after heating.

The DEAE chromatographic pattern is also altered when intact thymocytes are exposed to 37°C for varying time periods after completion of the labeling procedure and before cell lysis. There was a progressive decrease in cytosolic peak II complexes with increasing heat exposure: after 5 min exposure to 37°C, peak II complexes were reduced by 50% (Fig. 1D); after 10 min, peak II was virtually absent. Over the same time period, cytosolic peak I complexes initially decreased but then remained stable. The fall in peak I complexes was maximal at 5 min (Fig. 1D). At that point, there was a 40% reduction in peak I material (compared to peak I in cytosols from cells not exposed to 37°C before lysis), which then remained stable at the 10- and 15-min heat exposure points.

DNA and Chromatin Binding

The affinity of both peak I and peak II components for DNA and chromatin were studied using complexes generated by the broken cell labeling system. As shown in Fig. 4, peak I complexes from a heated calf thymus cytosol bind to DNA cellulose columns. Under the conditions used here, we averaged 34% binding (range 30%–37%) of peak I complexes to DNA, as defined by the ratio of macromolecular bound 3H-TA eluting from the column at 0.1 M K2HPO4 as a single peak. The 3H-TA-receptor complexes in this eluate remained intact as shown by filtration over Sephadex G-25. Data shown represent actual cpm per 0.5-ml aliquots of 1-ml fractions.
background affinity (binding to DNA cellulose equivalent to plain cellulose). Exposure of peak II complexes to 20°C for 30 min (which results in the generation of peak I material on rechromatography, Fig. 3B) increases the binding to 20%. Chromatin binding of peak I and peak II parallels DNA binding. Pooled apex fractions of peak I material averaged 37% binding (range 33%-40%) to chromatin, whereas peak II complexes averaged 9% binding (range 6%-11%).

Isokinetic Sedimentation

Peak I and peak II complexes, derived from cytosols labeled by broken cell systems, were studied on low salt glycerol density gradients. Peak I material sedimented at 3.5S and peak II material sedimented at 8.5S. The sedimentation coefficient of peak II material progressively changed with heating (20°C) from 8.5S to 3.5S.

DISCUSSION

As previously shown by Sakaeo and Thompson using rat liver,8 3H-triamcinolone acetonide labeled cytoplasmic glucocorticoid receptors of normal lymphoid tissues from a variety of sources can be resolved into two components by DEAE cellulose chromatography. These components appear to have a "precursor-product" relationship. Peak I complexes elute from DEAE at 0.04 M salt, sediment at 3.5S, and have affinity for DNA and chromatin. Peak II complexes elute at 0.22 M salt, sediment at 8.5S, and do not have affinity for either DNA or chromatin. However, activation of peak II complexes by exposure to heat confers affinity for DNA and chromatin and alters the DEAE elution position on rechromatography to 0.04 M salt (peak I area) and the S value to 3.5. This acquisition by peak II complexes of peak I characteristics following activation suggests that the unactivated receptor-steroid complexes exist in the cytoplasm in an 8.5S configuration. The initial DEAE chromatograms in both the broken cell and intact cell labeling systems reflect this. The peak I complexes in the initial DEAE chromatograms are accounted for by the limited activation that occurs during the labeling process, even at 4°C, as described by Traish and colleagues for estrogen receptors.10 Activation of receptor-3H-TA complexes in intact thymocytes by heat exposure results in a progressive loss of total cytoplasmic receptors in association with a simultaneous increase in nuclear complexes, as previously shown by Atger and Milgrom in rat liver.11 The loss of cytoplasmic peak II material is not associated with a simultaneous increase in peak I complexes when studied in the intact cell system because of the rapid nuclear translocation of activated receptor-complexes at 37°C. As in the experiments of Munk and Foley,12 there is some persistent residual cytoplasmic peak I material even after 15 min at 37°C. This may represent a steady state in the translocation process, or may be due to residual peak I complexes with no nuclear affinity.

This approach to the characterization of glucocorticoid receptors provides consistent data for normal lymphoid tissues. In attempting to understand the discrepancy between receptor quantitation and hormonal responsiveness in the leukemias, our operating hypothesis is that not all of the glucocorticoid binding material that can be identified in leukemic cell cytosols represent true physiologically functional receptors. Our assumption is that the quantitative receptor measurements usually performed may identify a heterogeneous group of binding components and that only a subset of these binders function as physiologic mediators of glucocorticoid action. Using the approaches described here, we hope to distinguish such physiologically meaningful glucocorticoid binders in leukemic cell cytosols from other possible "nonreceptor", "pseudoreceptor", or "dysfunctional" intracellular glucocorticoid binding macromolecules.

REFERENCES

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