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THE PRESENCE OF A DEHYDROEPIANDROSTERONE-SPECIFIC RECEPTOR BINDING COMPLEX IN MURINE T CELLS

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Summary—We have investigated the ability of dehydroepiandrosterone (DHEA) to alter the production of interleukin-2 (IL-2) and to bind to a specific binding complex in antiCD3ε activated T cells. Binding activity correlated with the presence of a specific DHEA binding complex in the cytosol and nuclei of DHEA-responsive T-cell hybridomas, as well as in $CD4^+$ and $CD8^+$ cells isolated from peripheral lymph nodes of normal mice. Scatchard analysis determined that intact lymphocytes and cytosolic fractions contained high affinity binding for [^{3}H]DHEA (approx. 2.6 nM) with 1000–7000 binding sites existing per cell. Five of the T-cell hybridomas tested both responded to DHEA treatment with increased production of IL-2 and also contained specific high affinity [^{3}H]DHEA binding. Four additional T-cell hybridomas were found to contain no specific [^{3}H]DHEA binding and were also unresponsive to DHEA influences on IL-2 production. Sucrose density gradients demonstrated a 3–4*s* [^{3}H]DHEA binding complex in high salt and a 7–8*s* binding complex in low salt. Specific binding was inhibited by preincubation of the cytosol fractions with either trypsin or chymotrypsin, or by heating to 60°C for 1 h (<15% of control). [^{3}H]DHEA binding was unaffected by preincubation of the cytosol fractions with ribonuclease, deoxyribonuclease, or phospholipase A. The DHEA–protein complexes bound to DNA-cellulose with the amount of binding being slightly increased by preincubation at 25°C as compared to 4°C. As expected, [^{3}H]DHEA binding was inhibited by the addition of unlabeled DHEA, but was also modestly inhibited by dihydrotestosterone and cortisol. Binding of DHEA was unaffected by progesterone, dexamethasone, estradiol, androsterone, DHEAS, and β-etiocholanolone at all concentrations tested. DHEA was incapable of inhibiting the binding of [^{3}H]DHT to the androgen receptor or [^{3}H]dexamethasone to the glucocorticoid receptor. Collectively, these findings suggest that murine T cells contain a specific DHEA receptor. We believe that DHEA is a steroid hormone that is directly involved in the regulation of IL-2 production by both normal and some T-cell hybridomas.

INTRODUCTION

Dehydroepiandrosterone (DHEA), a steroid with weak androgenic activity, serves as a precursor for both potent androgens and estrogens and is an abundant adrenal cortical steroid in both adult males and females [1, 2]. Many observations have been made which indicate the existence of biologic responses to DHEA which cannot be explained as being due to one of its metabolites. These include an augmentation of T-cell interleukin-2 (IL-2) production [3], anticarcinogenic effects [4], antiobesity effects [5], and antidiabetic effects [6]. These hormone-specific effects suggest that DHEA may function through a specific intracellular hormone receptor that is independent of other known intracellular steroid hormone receptors (glucocorticoid, androgen, estrogen, mineralocorticoid, progesterone, and Vitamin D_3) [7, 8].

Our laboratory [3] has recently documented a unique effect of DHEA on enhancing the production of IL-2 by activated murine T cells. This effect could not be duplicated by any of the known precursors or metabolites of DHEA. Direct exposure of heterogeneous populations of murine T cells, T-cell clones, or T-cell hybridoma cell lines to DHEA (10^{-7}–10^{-10} M) facilitated an enhanced synthesis and secretion of IL-2 without affecting interleukin-4 (IL-4) production subsequent to activation. DHEA treatment of T cells *in vitro* or *in vivo* was also able

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to reverse the suppression of IL-2 production caused by exposure to glucocorticoids. These findings suggested that DHEA may mediate these effects on T cells via a receptor-mediated mechanism and that this steroid hormone may represent an important natural regulator of IL-2 production *in vivo*.

Establishing the presence of a specific steroid receptor and steroid hormone action is better facilitated in cells that demonstrate some definable response to the ligand [7, 8]. The binding of steroid hormones to their respective nuclear receptors leads to activation, and is generally followed by an increase in transcription and translation of specific proteins under steroid hormone control [7, 8].

Kalimi and Regelson [9] have recently reported that cytosolic fractions of rat liver were capable of binding to [^{3}H]DHEA with high affinity. This binding was mediated by a macromolecular complex with characteristics similar to other steroid hormone receptors. No biologic response for the putative receptor was analyzed by these investigators.

We have performed DHEA binding studies on a panel of murine T-cell hybridomas where individual cell lines were either responsive or unresponsive to DHEA. The results of these studies indicate that a correlation exists between a DHEA-mediated function and the presence of a high affinity receptor for this steroid hormone, suggesting that DHEA functions similar to other known steroid hormones.

MATERIALS AND METHODS

Mice

Male and female C3H/HeN MTV$^-$ strain mice were bred and housed in the University of Utah Vivarium from breeding stock originally purchased from the National Cancer Institute. The animal facility at the University of Utah guarantees strict compliance with regulations established by the Animal Welfare Act.

Reagents

[1,2,6,7-^{3}H]DHEA (100 Ci/mmol) was obtained from Dupont New England Nuclear (Boston, MA). Its purity was over 96% as determined by thin-layer chromatography on precoated silica sheets (Eastman Kodak, Rochester, NY) developed in a benzene–ethyl acetate (60:40, v/v) system. Bovine pancreas deoxyribonuclease type I, bovine pancreas ribonuclease type A, trypsin, chymotrypsin, and phospholipase A were obtained from Sigma Chemical (St Louis, MO). Unlabeled steroids were obtained from Steraloids, Inc. (Wilton, NH) or Sigma. All other chemicals employed in this study were of analytical grade.

Antibodies

The monoclonal antibody reagents used in the present study were prepared from either ascites or culture supernatants. The cell line producing the hamster antimurine CD3ϵ monoclonal antibody, 145.2 C [10], was obtained from J. Bluestone (University of Chicago, Chicago, IL). The antiIL-2 hybridoma (S4B6) was obtained as a gift from T. Mosmann, DNAX (Palo Alto, CA). Hybridomas producing antibody specific for the murine IL-4 (11B11) [11], antiCD4 (GK1.5) [12], or antiCD8 (53 – 6.72) [13] were purchased from the American Type Culture Collection (Rockville, MD).

Bioactive compounds

Murine recombinant IL-2 and IL-4 were derived from culture supernatants of X63Ag8-653 cells transfected with multiple copies of a single murine interleukin gene [14].

Preparation of T-cell hybridomas

The method of Kappler *et al.* [15], was used to construct a panel of antigen-specific T-cell hybridomas. Before use in the present study, established cell lines were recloned and retested for their capacity to produce both IL-2 and IL-4 following activation. The hybridomas have the capacity to be stimulated to produce IL-2 and IL-4 when activated by either specific antigen (hen eggwhite lysozyme) in the presence of I-A^b-bearing accessory cells, antiCD3ϵ in the presence of accessory cells, or immobilized antiCD3ϵ.

Culture conditions

Routinely, hybridoma cells were cultured at a density of 5×10^5 cells/ml/well in a 24-well Cluster culture plate (Costar, Cambridge, MA) with 2×10^5 accessory cells (B-cell lymphoma, LB15.13) and antiCD3ϵ (1 μg/ml) to induce lymphokine secretion. Culture medium consisted of serum-free RPMI 1640 supplemented

with 1% Nutridoma-SR (Boehringer-Mannheim, Germany), antibiotics, 2 mM L-glutamine and 50 μM 2-mercaptoethanol. For *in vitro* treatment of T cells with DHEA, cells were resuspended in 2×10^6 cells/ml in either 1% Nutridoma-supplemented RPMI 1640 or balanced salt solution with 10^{-7} M DHEA. The mixture was incubated for 45 min at 37°C, followed by extensive washing to remove any non-bound reagent, thereby eliminating any possible actions of steroid hormones in the performance of indicator cell lines used in the IL-2 and IL-4 bioassays. Hybridoma cells were cultured in serum-free RPMI culture media with antiCD3ϵ to induce lymphokine secretion for a period of 24 h at 38°C in a 10% CO_2, humidified incubator. This has been established as the optimal time to reach maximal accumulation of lymphokines made by T-cell hybridomas in culture supernatants.

T-cell subset enrichment

Single cell suspensions of lymph nodes were prepared in balanced salt solution, washed 2 times, counted, and resuspended in a 1:50 dilution of Ig-enriched antiCD4 (GK1.5) or a 1:10 dilution of antiCD8 (53–6.72) containing culture supernatant. Following a 45 min incubation on ice, the cells were washed 2 times in cold balanced salt solution and then resuspended to 1×10^{-7} cells/ml in PBS/5% fetal calf serum. For panning, 2×10^6 ml of the anti CD4- or 5×10^6 antiCD8-treated lymph node cells were dispensed into Petri plates previously coated with goat antirat Ig, according to the method of Wysoki and Sato [16]. After a 70-min incubation at 4°C, non-adherent cells were carefully dislodged and resuspended. Cells retrieved from the antiCD4 treatment were 85–90% enriched for $CD8^+$ cells, whereas the antiCD8 treatment yielded 90–95% enrichment of $CD4^+$ cells by FACS analysis. Fractions of unseparated lymph node T cells, $CD4^+$, and $CD8^+$ cells were pulsed with 10^{-7} M DHEA at a concentration of 1×10^7 cells/ml at 37°C for 60 min, followed by extensive washing in balanced salt solution (BSS). Unfractionated lymph node T cells, as well as $CD4^+$ and $CD8^+$ T cells, were resuspended to 1×10^7 cells/ml in serum-free RPMI 1640 culture media and then incubated in culture plates with 1 μg/ml antiCD3ϵ for a period of 24–27 h at 38°C in a 10% CO_2, humidified incubator.

Lymphokine bioassays of IL-2 and IL-4

HT-2 cells have proven to be a suitable indicator cell line for detection of IL-2 and IL-4 in a bioassay. Our laboratory has modified the MTT-based colorimetric bioassay of Hansen [17] for use in an IL-2/IL-4 bioassay using HT-2 as an indicator cell line. To distinguish IL-2 from IL-4 activity, each test supernatant is titrated in duplicate into Nutridoma supplemented media (serum-free) against 4×10^3 HT-2, and 1 μg/ml of either monoclonal antiIL-4 or antiIL-2. During the final 4 h of a 24-h incubation, 5 μg of 3-[4,5-dimethylthiazole-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) is added to each culture and following a second 4-h incubation, 100 μl of a 20% SDS/50% dimethylformamide solution was added to dissolve formazan crystals. A spectrophotometric reading at 570–650 nm is performed on a V_{max} 96-well microtest plate spectrophotometer (Molecular Devices, Menlo Park, CA). Optical density readings for each titration were then fitted to a second-degree polynomial curve using the X–Y graphing program, "Passage," to apply a unit value for the interleukin under evaluation (both a mean and a standard derivation for each unit value were determined). One unit of activity is equivalent to the o.d. of a half-maximal response of HT-2 to a standard recombinant IL-2 (0.02–0.03 ng), or IL-4 (0.2–0.4 ng). Finally, we have established that the behavior of HT-2 in the IL-2/IL-4 bioassay was not altered in the presence (intentionally added to the bioassay) of the steroids whose effects have been studied in this report.

Cell preparation

Culture medium containing cells to be tested for the presence of receptors was centrifuged at 200 *g* for 10 min at 4°C. The pellet was washed twice with a BSS, D-glucose 0.1%, CaCl2 50 μM, MgCl1 980 μM, KCl 5.4 μM, and Tris–Cl 145 μM, pH 7.6, and then diluted 1:10 with 0.14 M NaCl. The cells were resuspended in minimal essential medium and counted using a hemacytometer. Cell concentrations were adjusted to approx. 100×10^6 cells/ml.

Intact cell binding studies

Approximately 1×10^6 cells were added to tubes containing 1–20 nM [^{3}H]DHEA with or without a 500-fold excess of unlabeled DHEA. Assays in 0.5 ml were performed in triplicate and incubated at 24°C for 60 min. The cells were

then centrifuged and washed 3 times in ice-cold BSS. Over 90% of the cells were viable as determined by trypan blue exclusion. After the last wash, the cells contained 1–2% of the initial radioactivity. Bound steroid was extracted twice with 2 ml of methanol at room temperature. The specific binding was evaluated as the difference between the cell-associated radioactivity in the tubes containing radioactivity alone and in those with an equal concentration of labeled DHEA plus a 500-fold excess of unlabeled steroid. In preliminary studies, binding was constant for 1 h in whole cell assays. Radioactivity was counted in a liquid scintillation system and then corrected for quenching. The intraassay coefficient of variation for the sites per cell in the T cells was 19%.

Cytosol and nuclear preparation

The cells were homogenized for 3 min in a glass–glass homogenizer, using 10 ml of 0.01 mM Tris–HCl buffer pH 7.4, containing 0.25 M sucrose and 1 mM $MgCl_2$. The homogenate was filtered through double gauze and centrifuged at 1200 *g* for 10 min at 4°C as described previously by Grimaldo and Meikle [18]. The cytosolic fraction containing 10 mM sodium molybdate (Mallincrodt Chemical, Paris, KY) was prepared by ultracentrifugation at 104,000 *g* for 60 min at 4°C. The crude nuclear fraction was washed twice with 5 ml of buffer, resuspended in Tris-buffer, and then prepared for buoyant density separation by layering over a 2.2 M sucrose buffer. This preparation was centrifuged at 40,000 *g* for 60 min. Following a second buoyant density separation the nuclear fraction was resuspended in assay medium for analysis of receptors.

Assay procedure for cytosol and nuclei

Glass tubes (13 × 100 mm) were used for the assays. One hundred microliters of either cytosol or nuclei in 0.5 ml TEDGM buffer containing 50 mM Tris–Cl, 1.5 mM EDTA, 0.5 mM dithiothreitol (DTT), 10% glycerol v/v and 10 mM sodium molybdate were incubated for 1 h at 24°C. [^{3}H]DHEA was added (1–20 nM) with or without a 500-fold excess of unlabeled steroid. The tubes were placed in an ice bath for 10 min and then 500 μl of hydroxyapatite (Sigma) suspension was added for 15 min with vortexing every 5 min. The tubes were then centrifuged at 5000 *g* for 5 min. The pellets were then washed 3 times by removing the supernatant and resuspended with 1.5 ml aliquots of Tris–EDTA buffer and centrifuged at 5000 *g* for 5 min. The pellet was extracted with 1 ml of ethanol by incubating for 30 min at 24°C, followed by vortexing and centrifugation at 5000 *g* for 10 min. The ethanol extract was counted as described above. The assays were conducted in triplicate.

DNA was measured by the method of Burton [19] using calf thymus DNA as the standard. Protein was measured by the technique of Lowry [20].

Binding to DNA-cellulose

Cytosol was preincubated with [^{3}H]DHEA for 4 h at 4°C in the presence and absence of 10 mM sodium molybdate. Portions were further incubated for 55 min at 24°C. Two hundred microliters cytosol aliquots were mixed with 100 μl of DNA-cellulose (1.4 mg native calf thymus DNA/ml cellulose) and incubated for 60 min at 4°C with stirring. The samples were centrifuged at 5000 *g* for 10 min, washed three times with 1 ml of Tris buffer, extracted with 1 ml ethanol and counted in scintillation fluid as described above.

Sucrose density gradients

For preparation of the cytosol, the cells were resuspended with buffer A (Tris 10 mM, sucrose, 0.25 M, pH 7.6 at 4°C) in an ice bath and homogenized with a glass homogenizer (8–12 strokes). The cytosol fraction was prepared by centrifugation of the homogenate at 104,000 *g* for 60 min at 3°C. The protein content was determined using the method of Lowry [20]. The cytosolic fraction was incubated for 4 h in buffer A containing either 30 or 400 mM KCl, with 10–20 nM [^{3}H]DHEA with or without a 500-fold excess of unlabeled DHEA. The specific macromolecular binding was determined on PD 10 columns (Pharmacia, Inc., Piscataway, NJ) and/or the charcoal dextran technique.

Linear sucrose gradients from 15–30% were prepared in 10 mM Tris buffer, pH 7.6 containing either 30 (low salt) or 400 mM KCl (high salt), using a gradient former. [^{14}C]bovine carbonic anhydrase (2.9*s*, Dupont New England Nuclear), and [^{14}C]γ-globulin (7.1*s*, Dupont New England Nuclear) were referenced standards for estimating sedimentation coefficients [21, 22]. Supernatant samples (0.3 ml) were applied to either high (400 mM KCl) or low (30 mM KCl) salt gradients. Samples were centrifuged at 3°C for 18 h in

a Spinco rotor at 350,00 *g*. An Isco density gradient fractionator apparatus was used to collect 4-drop samples beginning from the top. The radioactivity was determined in a scintillation counter system and counts were corrected for quenching.

RESULTS

DHEA responsive T-cell hybridomas possess a high affinity DHEA binding complex

To demonstrate that brief exposure of T cells to 10^{-7}M DHEA has a similar biologic effect as continuous exposure at much lower concentrations (10^{-9} M DHEA), the T-cell hybridoma Hd11.2 was either pulsed for 60 min with 10^{-7} M DHEA prior to activation *in vitro* with immobilized antiCD3ϵ or co-incubated with varying concentrations of DHEA (10^{-10}–10^{-7} M) during the *in vitro* activation period (Fig. 1). Non-pulsed T cells were treated in parallel with pulsed cells, without DHEA, and control these T cells were activated in the absence of DHEA. After 24 h, culture supernatants were harvested and assayed for the presence of IL-2 using a quantitative bioassay, as described in Materials and Methods. The results of this experiment show the dose-dependence of DHEA for augmentation of T-cell production of IL-2 following activation. In addition, these results demonstrate that a pulse with 10^{-7} M DHEA provides a response equivalent to continuous exposure at 10^{-9} M DHEA. Although exposure to 0.1 μM DHEA exceeds the normal blood values, it is possible that this concentration of DHEA could be attained within tissue microenvironments where DHEA-sulfatase activity is present. We chose to employ pulse conditions (10^{-7} M DHEA) in experiments presented in this manuscript since the results obtained were similar to those employing continuous steroid exposure and to rule out any possibility that the physical presence of DHEA might influence our ability to accurately measure IL-2 levels.

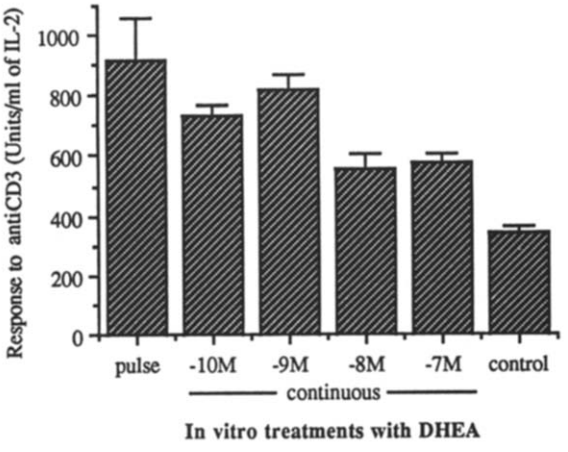

Fig. 1. The effect of single-pulse vs continuous exposure to the effects of DHEA on T-cell production of IL-2. A single clone of T-cell hybridoma (Hd11.2) was separated into two fractions, to which 10^{-7} M DHEA was added to one of them. Both fractions were incubated for 60 min at 37°C, followed by 3 washes in BSS to remove unbound steroid. After washing, 5×10^5 T cells from the DHEA-pulsed group were plated in serum-free RPMI culture media into culture wells precoated with 100 ng/ml antiCD3ϵ. The sham-pulsed cells, plated similarly, were exposed to DHEA on a continuous basis for the full 24-h activation period. The dose of DHEA in these cultures ranged from 10^{-10} to 10^{-7} M. Culture supernatants were evaluated for the amount of IL-2 by a quantitative bioassay.

Scatchard plot analysis of [^{3}H]DHEA binding was performed on 9 independent T-cell hybridomas to question the existence of high affinity binding of [^{3}H]DHEA. The results of this study are presented in Table 1. The influence of DHEA on the production of IL-2 and IL-4 by these same cell lines was also measured to correlate [^{3}H]DHEA binding characteristics with any alterations to lymphokine production (Table 1). Five of the cell lines tested had both high affinity of [^{3}H]DHEA [with dissociation constants (K_d) between 2.6–5.5 nM and 1000–7244 binding sites per cell when incubated for 1 h at 25°C], and also exhibited an increase in IL-2 production (160–420% of control) when preincubated with DHEA prior to activation. As previously reported, no change in the production of IL-4 was noted [3]. A representative Scatchard plot of the Hd11.2 hybridoma T-cell line which had a K_d of 3.0 nM and 1560 binding sites per cell is shown in Fig. 2. This cell line was also found to have both nuclear and cytosolic [^{3}H]DHEA binding complexes (data not shown). Four of the hybridomas tested exhibited neither high affinity [^{3}H]DHEA binding by Scatchard analysis nor were able to alter the amount of IL-2 produced following preincubation with DHEA. These results suggest that DHEA-mediated augmentation of T-cell IL-2 production is restricted to T-cell lines containing high affinity [^{3}H]DHEA binding sites.

The characteristics of DHEA binding

Binding specificity for [^{3}H]DHEA was assessed with a T-cell line which demonstrated high affinity binding to DHEA (Fig. 2). Cells were incubated for 1 h at 25°C with various steroid hormones being added at the initiation

Table 1. The biologic response of T-cell hybridoms to DHEA correlates with the presence of intracellular DHEA binding sites

Hybridomas	Lymphokine production IL-2 DHEA treatment −/+	IL-4 −/+	DHEA binding complexes Sites/cell	K_d (nM)	Biologic response	DHEA binding
11.20	367/**589**	122/118	1000–1560	2.6–3.8	+	+
30.17	349/**644**	208/183	2500–3100	4.3	+	+
302.11	178/**488**	151/123	1431	2.8	+	+
302.13	184/192	299/275			−	−
302.20	86/98	245/232			−	−
312.13	198/**462**	62/77	7244	3.2	+	+
312.20	203/**378**	44/45	6655	5.5	+	+
312.50	56/66	141/134			−	−
312.60	206/216	342/363			−	−

In parallel, groups of T-hybridoma cells were pulsed with 10^{-7} M DHEA in serum-free media or media alone for 45 min at 37°C. The cells were washed extensively to remove extracellular steroid and then 5×10^5 of each of the T-hybridoma groups were cultured in serum-free media with accessory cells and an optimal concentration of antiCD3ε to stimulate lymphokine production. Twenty-four hours after culture initiation, culture supernatants were harvested, clarified, and assayed for IL-2 and IL-4 using a quantitative bioassay.

of culture (Fig. 3). Moderate ability to displace [^{3}H]DHEA from its specific binding sites was observed with added dihydrotestosterone (DHT) or cortisol, whereas dexamethasone, β-etiocholanolone, testosterone, and androsterone had very weak to no ability to displace [^{3}H]DHEA from its binding sites. Progesterone, estradiol-17β, and DHEAS (the sulfated precursor of DHEA), were also incapable of competing for DHEA binding sites at any concentration tested (data not shown). This latter finding suggests that the cells used in our studies do not contain the sulfatase required to convert DHEAS to DHEA. Collectively, these results indicate that [^{3}H]DHEA binding sites are highly specific for unlabeled DHEA and not for other known classes of steroid hormones.

Two distinct classes of steroid hormones, glucocorticoids (cortisol) and androgens (DHT) exhibited some ability to displace [^{3}H]DHEA from its binding sites. The ability of DHEA to compete for these two classes of steroid hormone binding sites was therefore evaluated in the Hd11.2 T-cell line. High affinity binding was observed for [^{3}H]dexamethasone (K_d = 3.25 nM) with 13,742 binding sites being observed per cell (Fig. 4). The addition of DHEA had no effect on [^{3}H]dexamethasone binding, whereas corticosterone and RU486 (an antiglucocorticoid) were potent inhibitors (Fig. 5). A moderate inhibitory response was also observed with cortisol, and a very weak inhibitory response was observed with progesterone. DHEA also failed

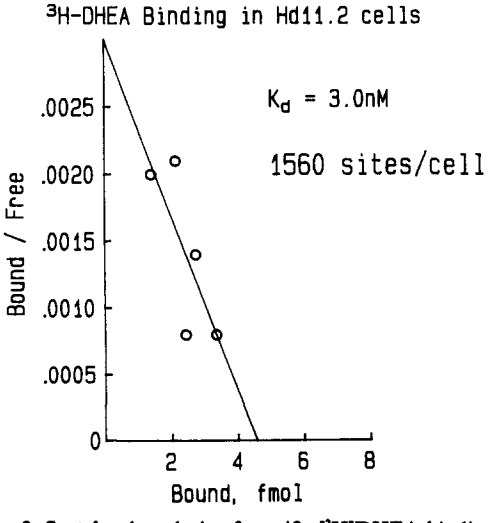

Fig. 2. Scatchard analysis of specific [^{3}H]DHEA binding in Hd11.2 cells. Binding studies were performed in triplicate with [^{3}H]DHEA (2–20 nM) with and without a 500-fold excess of unlabeled DHEA as described in Materials and Methods. There were 1.75×10^6 cells per 0.5 ml, and the non-specific binding was between 65–75% of the total binding.

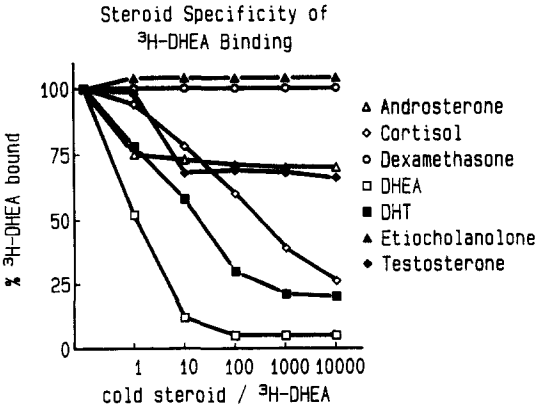

Fig. 3. Steroid specificity of [^{3}H]DHEA binding. Hd11.2 cells in triplicate were incubated with 8 nM [^{3}H]DHEA alone or in the presence of various unlabeled steroids as listed employing concentrations from 1- to 10,000-fold excess of the labeled DHEA. Each tube (0.5 ml) contained 3.46×10^6 cells. The non-specific binding was approx. 30% of the total.

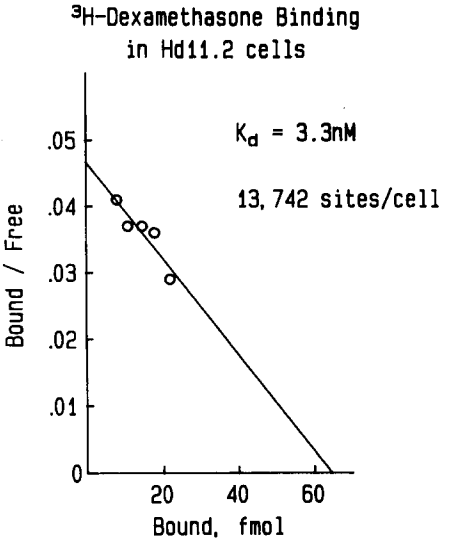

Fig. 4. Scatchard analysis of specific [^{3}H]dexamethasone binding in Hd11.2 cells. Binding studies were performed in triplicate with and without a 500-fold excess of unlabeled dexamethasone as described in Materials and Methods.

to compete for specific [^{3}H]DHT binding sites present within these same cells (data not shown). The distinct specificities of the glucocorticoid and androgen receptor in this cell line suggest that they are independent of one another. Additional support for the presence of a DHEA binding complex, distinct from the glucocorticoid or androgen receptors, came from functional studies. Exposure of the T-cell hybridoma 30.17 to DHEA, DHT, or glucocorticoids prior to activation, resulted in steroid hormone-specific changes in their ability to produce IL-2 and IL-4. The results are presented in Fig. 6 and clearly demonstrate steroid effects that are unique to each species of

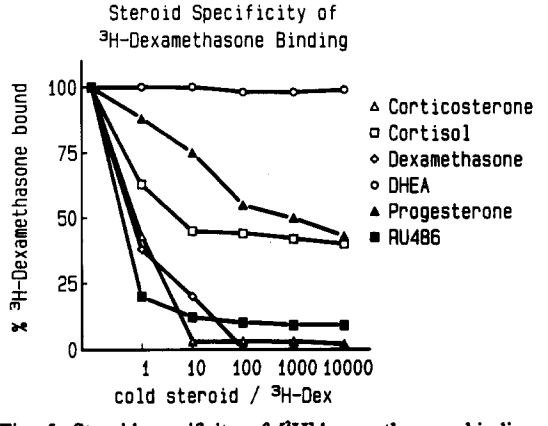

Fig. 5. Steroid specificity of [^{3}H]dexamethasone binding. Hd11.2 cells in triplicate were incubated with 10 nM [^{3}H]dexamethasone alone or in the presence of various unlabeled steroids as listed above at concentrations from 1- to 10,000-fold excess of the labeled dexamethasone.

hormone. These results are totally consistent with our previous findings [3, 10–12].

[^{3}H]DHEA binding and IL-2 responses to DHEA preincubation in normal CD4$^+$ and CD8$^+$ T cells

[^{3}H]DHEA-specific binding was measured in normal murine peripheral lymph node T cells to question whether these cells also contained high affinity DHEA binding complexes. A short preincubation with DHEA was found to increase IL-2 production by unfractionated CD4$^+$ and CD8$^+$ cells (Fig. 7) following activation by antiCD3ϵ. As shown in Figs 8 and 9, the existence of high affinity [^{3}H]DHEA binding sites were observed in both normal CD4$^+$ (K_d: 1.4 nM with 362 sites per cell) and CD8$^+$ cells (K_d: 2.7 nM with 189 sites per cell). This indicates that both major subsets of normal T cells possess low numbers of DHEA binding complexes and will also respond to DHEA influences by increasing their production of IL-2 when activated. Normal murine thymocytes failed to demonstrate any specific binding to [^{3}H]DHEA (data not shown).

Sucrose density gradient studies

Many steroid hormone receptor proteins exhibit shifts in sedimentation constants when incubated with high and low ionic strength buffer systems [8, 9]. The sucrose density sedimentation characteristics of the [^{3}H]DHEA cytosol binding complex obtained from the cytosolic fraction of the Hd30.17 hybridoma cell line were evaluated in both high (400 mM KCl, pH 7.6, 10 mM Tris–Cl buffer) and low (30 mM KCl) ionic strength buffer systems. The [^{3}H]DHEA-specific binding complexes were found to sediment at 7–8s in low ionic strength and 3–4s in high ionic strength sucrose density gradients (Fig. 10).

DNA-cellulose binding

Cytosolic [^{3}H]DHEA binding complexes from the Hd30.17 hybridoma cell lines were preincubated at a low temperature (4°C for 4 h), or at 25°C for 1 h with or without sodium molybdate as shown in Fig. 11. Warming to 25°C is known to activate steroid hormone receptors and facilitate binding to DNA-cellulose, whereas the presence of sodium molybdate, even with warming, inhibits steroid receptors from binding to DNA-cellulose. Binding of the complex to DNA-cellulose was enhanced by elevating the temperature to 25°C and was inhibited by the

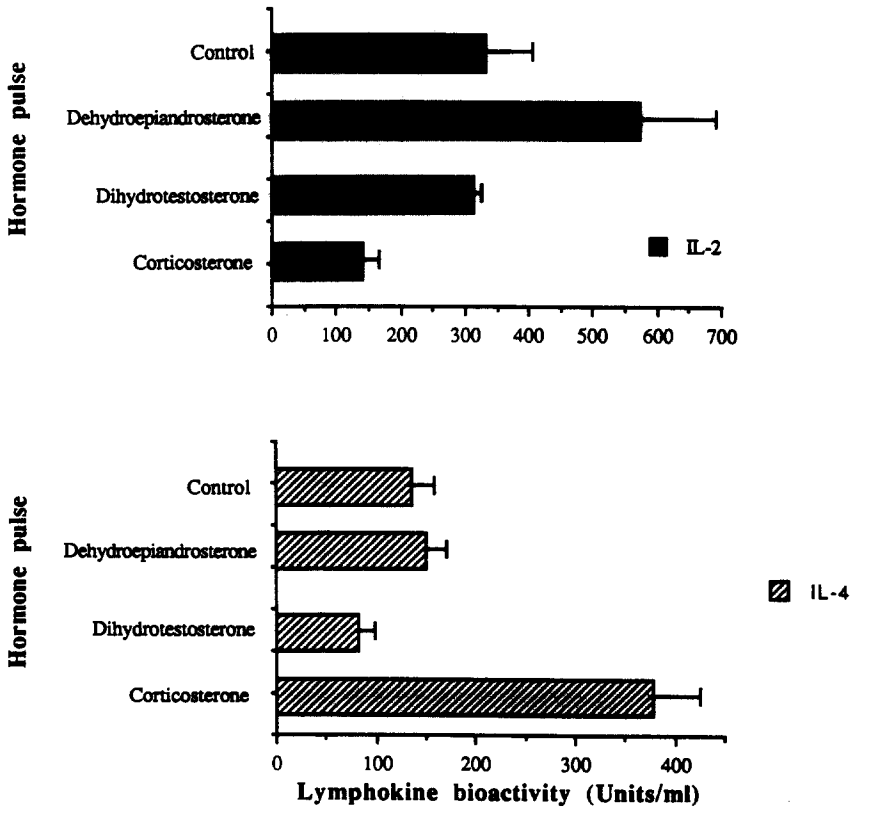

Fig. 6. The effect of DHEA on the capacity of T-cell hybridoma cells to produce IL-2 and IL-4 *in vitro* is distinct from the effects of corticosterone and DHT. A single clone of T-cell hybridoma (30.17) was separated into distinct fractions that were pulsed with either 10^{-7} M DHEA, 10^{-7} M DHT, 10^{-8} M corticosterone, or serum-free media alone, for 45 min at 37°C. After extensive washing, 5×10^5 T cells from each group were cultured with accessory cells and antiCD3ϵ to stimulate IL-2 and IL-4 production. The activity of the lymphokines was assessed using a quantitative bioassay as described in Materials and Methods.

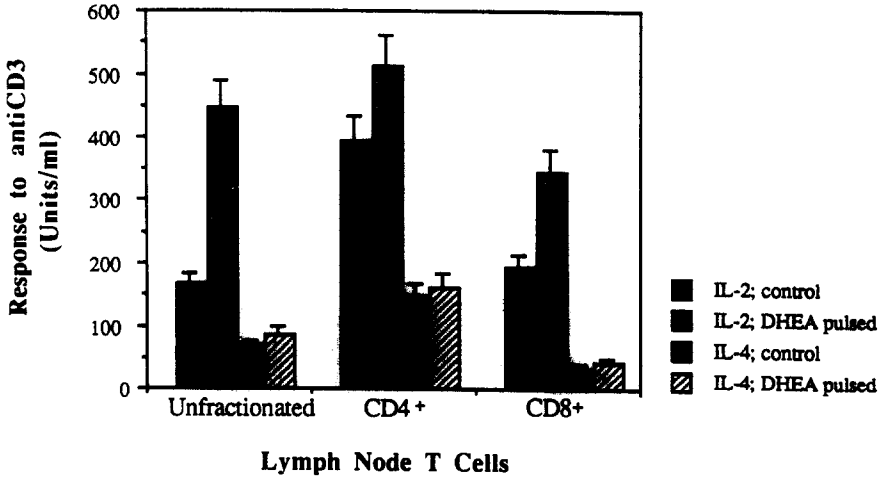

Fig. 7. DHEA stimulation of IL-2 production in CD4$^+$ and CD8$^+$ lymph node T cells. Lymph node T cells from mature adult C3H (18 weeks of age) were prepared for culture with antiCD3. In addition, lymph node cells from C3H mice were enriched for CD4$^+$ and CD8$^+$ cells by negative selection and then cultured with antiCD3 as described in Materials and Methods. DHEA (10^{-7} M) was used in a 30 min pulse where indicated as described in Materials and Methods. IL-2 and IL-4 bioactivities in unstimulated spleen cell cultures were $<20\ \mu$/ml. The cytokine production of IL-2 and IL-4 in response to antiCD3 by unfractionated T cells is shown on the left, CD4$^+$ cells in the middle, and CD8$^+$ cells on the right.

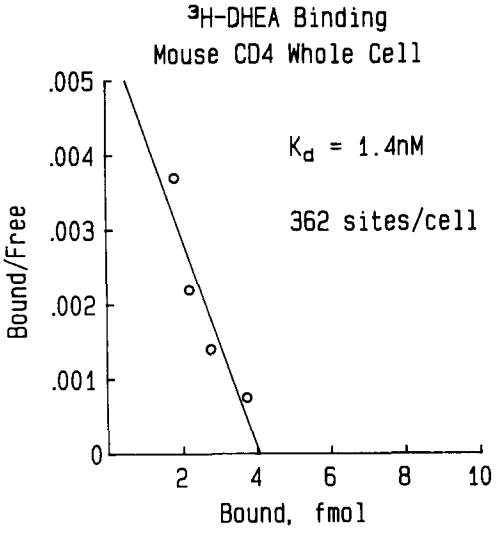

Fig. 8. Scatchard analysis of mouse CD4+ peripheral lymphocytes. CD4+ lymphocytes from normal mice were incubated in triplicate with 2–20 nM [3H]DHEA alone or in the presence of unlabeled DHEA at a 500-fold excess. Each tube (0.5 ml) contained 0.6×10^{-6} cells, and the non-specific binding was 50–66% of the total binding.

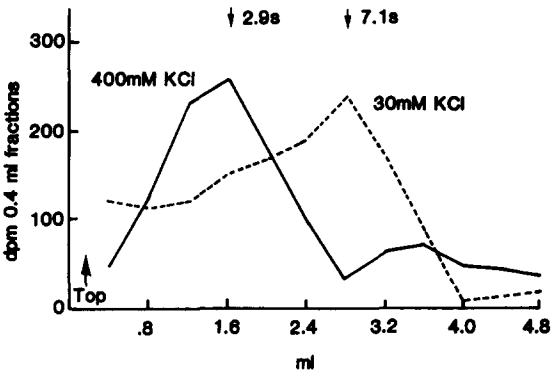

Fig. 10. Sucrose density gradient analysis of [3H]DHEA binding complex in cytosol from Hd30.17 hybridoma cells. Cytosol was saturated with 10 nM [3H]DHEA at 4°C for 4 h and then treated with charcoal dextran. 0.3 ml of supernatant sample was applied to either a high salt (400 mM KCl, solid line) or low (30 mM KCl, dashed line) gradients. Samples (0.4 ml) were centrifuged at 4°C for 18 h in a Spinco rotor at 350,000 g. An Isco density gradient fractionator apparatus was used to collect 4-drop samples beginning from the top. 14C-labeled bovine carbonic anhydrase (CA, 2.9*s*) and human gamma globulin (7.1*s*) were applied as reference markers. The non-specific binding was 70% of the total.

addition of sodium molybdate at 10 mM. These results indicate that the DHEA binding complex from murine T cells possess DNA-cellulose binding characteristics similar to other steroid hormone receptor complexes.

Effect of temperature and enzymes on the DHEA binding complex

Steroid hormone receptors are proteins that can be inactivated by heat or by incubation with proteolytic enzymes [8, 9]. The effects of heat and various hydrolytic enzymes on the specific binding of the Hbd30.17 cytosol [3H]DHEA binding complex were studied, and the results are summarized in Table 2. Heating the cytosol for 60 min to 60°C eliminated [3H]DHEA-specific binding to the DHEA binding complex. Specific binding was also affected by a preincubation with the proteolytic enzymes, chymotrypsin and trypsin, which reduced specific binding to <13% of the control. In contrast, treatment of the cytosolic preparation with phospholipase A, deoxyribonuclease, or

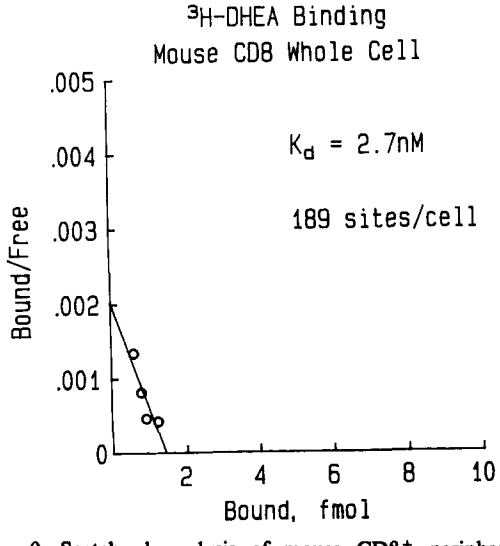

Fig. 9. Scatchard analysis of mouse CD8+ peripheral lymphocytes. CD8+ lymphocytes from normal mice were incubated in triplicate with 2–20 nM [3H]DHEA alone or in the presence of unlabeled DHEA at a 500-fold excess. There were 4.6×10^6 cells per tube (0.5 ml), and the non-specific binding was 14–35%.

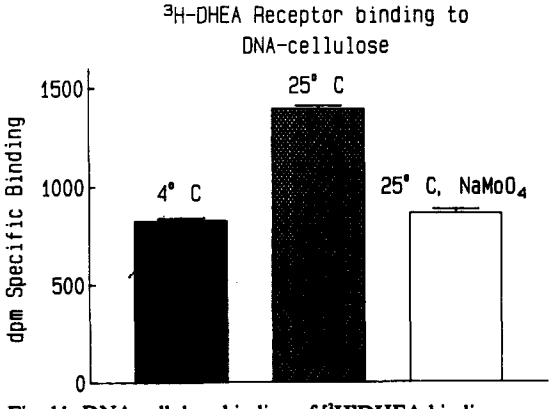

Fig. 11. DNA-cellulose binding of [3H]DHEA binding complex. Cytosol from hybridoma cells were incubated for 4 h at 4°C in the presence of 10 nM [3H]DHEA before application to the column as shown on the left. Samples were further incubated at 25°C for 45 min (as shown in the middle column) or with 10 mM sodium molybdate (as shown in the column on the right).

Table 2. Effects of hydrolytic enzymes on hybridoma cytosol [^{3}H]DHEA binding

Enzyme	[^{3}H]DHEA specifically bound	%
Untreated control	500[a]	100
Trypsin	60	12
Chymotrypsin	11	7
Phospholipase A	525	105
Deoxyribonuclease	550	110
Ribonuclease	535	107
Heat 60 min at 60°C	11	7

Cytosol from the 30.17 hybridoma cells was incubated for 2.5 h at 4°C with 1 mg/ml of enzyme or buffer (0.03 M KCl, 0.01 M Tris–HCl, pH 7.6 at 4°C). Samples in duplicate were studied. The specific binding was determined as listed in Materials and Methods.

[a]dpm/mg protein.

ribonuclease did not affect specific [^{3}H]DHEA binding to the DHEA binding complex.

DISCUSSION

The best characterized biologic function of DHEA is to serve as a precursor for conversion to the sex steroids. We have recently reported, however, that DHEA also possesses the capacity to enhance the ability of murine T cells to produce the lymphokine IL-2 following activation [3], and have provided evidence to suggest that this steroid hormone may represent a natural regulator of T-cell function *in vivo* [24, 25]. The results of the current study on a DHEA binding macromolecular complex in DHEA-responsive murine T-cell hybridomas, as well as normal murine T cells, indicate there is a DHEA binding macromolecule (or protein) that has steroid hormone receptor-like characteristics. This receptor was observed to exist both in intact cells, cytosolic fractions, and isolated nuclei of DHEA-responsive T cells. The DHEA binding complex appears to be highly specific for DHEA.

There is ample evidence for the existence of a complex interplay between the endocrine and immune systems. It has been recently documented [3, 23–25] that glucocorticoids, DHEA, and DHT are capable of altering the quantity of lymphokines (IL-2, IL-4, and others) produced by activated murine T cells. Glucocorticoids are well known inhibitors of γIFN and IL-2 production by activated murine T cells, suggesting their influences may suppress the development of cellular immune responses [23]. The suppressive effects of glucocorticoids on IL-2 production can be reversed by RU486 or DHEA treatment of T cells before activation [3], suggesting that DHEA might function as an endogenous antiglucocorticoid. Direct DHEA exposure of responsive T cells is known to enhance their capacity to produce IL-2 following activation ([3] and Table 1). These results add to evidence indicating that T-cell production of lymphokines can be regulated in defined ways by a variety of steroid hormones, including glucocorticoids, DHT, and DHEA. Agents that are able to alter cytokine production by T cells would be expected to exert influences on both cellular and humoral types of immune responses.

DHEA and DHEAS are secreted into the circulation by the adrenal cortex in humans [26] and by the gonads in rodents [27]. Their pattern of secretion in humans is well characterized. The levels of DHEA and DHEAS rise in late childhood in both sexes and reach their peak concentrations between the ages of 20–30 years. The endogenous synthesis of DHEA then declines, reaching low to very low levels in the elderly [28].

The binding characteristics of the DHEA binding complex suggest that DHEAS requires conversion to DHEA before it can bind to the putative DHEA receptor [24]. T cells may not contain the sulfatase required to render DHEAS biologically active for the T cell. Macrophages have been found to contain a sulfatase capable of converting DHEAS to DHEA and can thereby influence the function of T cells ([24] and unpublished results).

Similarities exist between the characteristics of the DHEA binding macromolecule observed in the murine T-cell hybridoma cells and the rat liver cytosolic DHEA binding complex reported by Kalimi and Regelson [9]. The apparent dissociation constants were similar for both. Furthermore, sucrose density sedimentation characteristics (3–4*s* in high ionic strength buffers and 7–8*s* in low ionic buffers) were also similar. Specific binding was inhibited in both by heating and protein digestion but not by treatment of cytosolic fractions with other enzymes, suggesting that the DHEA binding molecules are proteins. The cytosol complexes had higher binding to DNA-cellulose on thermal activation at 25°C than after incubating in the cold or with sodium molybdate.

Several differences are, however, apparent between our findings and those previously reported on DHEA binding complexes [9]. These authors observed that β-etiocholanolone, androsterone, progesterone, 17β-estradiol, and testosterone were effective in inhibiting DHEA binding of the rat liver cytosol complexes. Protein binding of DHEA in T-cell hybridomas was

not inhibited by these steroid hormones. We have also observed high affinity binding to mouse liver cytosol (Meikle *et al.*, unpublished data), but have not yet characterized it fully.

DHT was able to displace DHEA from the DHEA binding complex, although less effective than DHEA itself. Normal DHEA levels in rodents are about 5% of the level observed in humans [27, 28]. Male rodents have plasma testosterone levels that are also about 20-fold higher than plasma DHEA, however, circulating DHT and DHEA levels are nearly equivalent [27]. *In vivo* DHT levels would not be expected to exceed those of DHEA or to functionally modulate the DHEA binding complex. Neither DHT, glucocorticoids, nor any other steroid tested were able to duplicate the effects of DHEA on IL-2 production by T cells (see Fig. 6) [3, 23–25]. It therefore seems unlikely that the major metabolites of DHEA are activating the putative DHEA receptor to produce the biologic response observed.

Whether biologic responses are mediated by a specific DHEA receptor in tissues other than T cells is presently unknown. The reported effects of DHEA on bone [29], the cardiovascular system [30, 31], lipids [32], obesity [5,32], cancer resistance [4], and susceptibility to diabetes [6] suggests that additional biologic responses by DHEA, in addition to those on the immune system, may exist. Further studies are required to investigate this hypothesis and to characterize the molecular structure of the putative DHEA receptor.

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