Stimulation of Th2 Response by High Doses of Dehydroepiandrosterone in KLH-Primed Splenocytes

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Although dehydroepiandrosterone (DHEA) has long been considered as a precursor for steroid hormones, it has also been shown to have regulatory effects in immune homeostasis. We have examined the effect of high DHEA doses on T cell proliferation, differentiation, and cytokine secretion patterns following stimulation with mitogens and soluble antigens. DHEA profoundly inhibited T cell receptor-mediated T cell proliferation in the upstream of IL-2R signaling. Addition of DHEA to KLH-primed splenocytes stimulated Th2 response, indicated by an increase of IL-4 or a decrease of IFN-γ production in the cultures. Further studies showed that DHEA enhanced IL-4, but inhibited IL-12-mediated T cell proliferation and IL-12 production in antigen-presenting cells (APCs). Our data demonstrated that supraphysiologic levels of DHEA favored Th2 immune responses *in vitro* by inhibition of IL-12 production from APCs and/or stimulation of Th2 proliferation during the interactions of T cells with APCs. [Exp Biol Med Vol. 226(11):1051–1060, 2001]

Key words: DHEA; T lymphocytes; Th1; Th2

Dehydroepiandrosterone (DHEA) is a C_{19} adrenal steroid secreted by the adrenal glands, gonadal tissue, and cells in the central nervous system (CNS) (1, 2). DHEA is biosynthetic precursor of virtually all steroid hormones, including testosterone and 17β-estradiol (3–5). In the plasma, DHEA is predominantly present as DHEA-S (stored form). DHEA-S is converted to the active form DHEA by intracellular sulphatases that are expressed in a number of cell types, including monocytes and macrophages (6).

Although DHEA/DHEA-S is one of the most abundant steroid hormones in the circulation, its physiological role remains unclear. In young adults, the levels of DHEA-S and DHEA in plasma reach to 5–6 μg/ml (DHEAS) and 2–4 ng/ml (DHEA), considerably higher than any other steroids, and they then decrease with old age (7). Therefore, DHEA/DHEA-S has been thought to play a role in the aging process (8–12). In murine model systems of aging, DHEA supplements appear to reverse the immunological defects seen as a consequence of aging (13–16). In particular, DHEA supplements increase the ability of old mice to resist experimental viral and bacterial disease (14, 17–21). Low plasma DHEA is seen in oncologic disorders, diabetes mellitus, and chronic inflammatory diseases (22–25). These observations suggest that endogenous adrenal DHEA may play an important role in the normal immune homeostasis, though the mechanism by which this is achieved remains unknown.

Our current study was designed to formally examine the regulation of T cell functions by a supraphysiologic range of DHEA (5–20 μM). The DHEA concentrations were chosen based on our initial studies showing inhibition of mitogen-stimulated T cell proliferation, and have been reported to regulate cytokine (IL-6 and IL-2) production in human spleen mononuclear cells (26). Since T cells express specific receptors for DHEA and form the lynch pin of the adaptive immune response (27), we predicted that if DHEA regulated immune effector functions, it would very likely be at the level of T cell function. Therefore, we examined the effect of high DHEA doses on the proliferative response and the cytokine profile of primed and unprimed T cells in antigen-specific or mitogen-mediated immune responses.

Materials and Methods

Animals. Female SJL/J mice (4–6 weeks old) were purchased from the National Institutes of Health (Bethesda, MD) and female Balb/c (4–6 weeks) were obtained from

This work was supported by the William Weaver Fund.

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Received May 2, 2001.
Accepted August 9, 2001.

1535-3702/01/22611-1051$15.00

The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in the animal care facility at Vanderbilt University.

Reagents, Cytokine, and Antibodies. Con A (concanavalin A) was purchased from Pharmacia Biotech (Uppsala, Sweden), and DHEA and SEB (Staphylococcal enterotoxin B) were obtained from Sigma (St. Louis, MO). The DHEA stock solution (10 mM) was prepared by being dissolved in dimethyl sulfoxide (DMSO, Sigma), and was diluted to the experimental concentrations (5–20 μM) in the culture medium. rIL-2 was a gift from Dr. R. Budd (University of Vermont, Burlington, VT), rmIL-12 was provided by Genetics Institute (Cambridge, MA), and rmIL-4 was from PharMingen (San Diego, CA). Anti-IFN-γ and anti-CD3 antibodies were purified by QAE gel and Sephadex G-25M column (Pharmacia Biotech) from B cell hybridomas. The anti-mouse IFN-γ hybridoma clone (R4-6A2) was obtained from the American Type Culture Collection (Rockville, MD), and anti-mouse CD3 hybridoma was a kind gift from Dr. Mark Boothby (Department of Microbiology and Immunology, Vanderbilt University).

Antigen-Primed Splenocytes. Female SJL/J mice were immunized subcutaneously on the dorsum with 400 μg of KLH, emulsified in complete Freund's adjuvant (CFA; Sigma) on Days 0 and 7 at three separate sites. The KLH-primed splenocytes were harvested on Day 14 after removal of dead cells and erythrocytes by Histopaque-1077 gradient centrifugation (Sigma) at 320*g* for 10 min.

Preparation of Activated T Cells and Macrophages. Splenic lymphocytes were harvested from naïve mice and were cultured in the presence of Con A (2×10^6 cells/ml with 4 μg/ml of Con A) in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco-BRL, Gaithersburg, MD) at 37°C in 5% CO_2. After 3 days, cells were harvested and maintained in RPMI containing low serum (0.5% FBS) for 18 hr in order to synchronize T cells to G1 phase of the cell cycle. The resting T cells were harvested and dead cells were removed by centrifugation through Histopaque-1077 (Sigma) at 320*g* for 10 min. The viable cells were collected and washed with PBS. These isolated cells contained >98% T cells based on flow cytometric assay for the presence of CD3 antigen. Splenic macrophages were isolated by adherence of splenocytes to a plastic petri dish as described previously (28) and were grown at 37°C in atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20% LADMAC supernatant containing CSF-1 (colony stimulating factor). The macrophages were harvested when the cultures had reached confluence. The LADMAC supernatant was prepared after the confluent LADMAC cells had grown in the Dulbecco's modified Eagle's medium for 5–7 days.

Proliferation Assay. Splenic lymphocytes (2×10^5) were added to each well of a 96-well flat-bottom plate and were cultured in RPMI 1640 medium in triplicate with antigen/mitogen in the absence or presence of DHEA. During the last 18 hr of culture, [^{3}H]thymidine (0.5 μCi/well; ICN, Costa Mesa, CA) was added, and the amount of radioisotope incorporation was determined by counting on the liquid scintillation counter (Wallac, Turku, Finland).

Flow Cytometry for Apoptosis. Splenocytes (1×10^6 cells/well), collected from naïve SJL/J mice were stimulated in anti-CD3- (2 μg/ml) coated 6-well plate in the presence of various concentrations of DHEA. After incubation overnight at 37°C, apoptotic cells were stained with Annexin V-PE (PharMingen) according to the manufacturer's protocol and were counted by FACS analysis.

ELISA for Cytokines. Cytokines (IL-4 and IFN-γ) were measured by a sandwich ELISA (enzyme-linked immunosorbent assay) as described previously (29). The matched antibody pairs and cytokine standards were purchased from Endogen (Woburn, MA). Briefly, ELISA plates (Corning, Corning, NY) were coated with capture antibody in 0.06 M bicarbonate buffer (pH 9.6) overnight at 4°C, and were blocked with 3% bovine serum albumin in PBS for 1 hr after being washed with PBS-0.05% Tween 20 (PBST). The samples and cytokine standards were added in triplicate and were incubated overnight at 4°C. After washing again with PBST, biotinylated-detecting antibody was added and incubated for 3 hr at 25°C or room temperature, and was then incubated with avidin-alkaline phosphatase (1:10,000, Sigma) for 1 hr after washes with PBST. The absorbance at 405 nm for each sample was read after addition of substrate *p*-nitrophenyl phosphate (Sigma) for 30 min. The amount of cytokine in the supernatant was calculated by interpolation from a standard curve. IL-12p70 in the culture supernatants was quantitated using ELISA kit (R&D Systems, Minneapolis, MN) following the manufacturer's protocol.

Induction of T Cell Differentiation. Naïve splenic T cells from Balb/c mice were enriched by "panning" as described by Lewis and Kamin (30) and were differentiated into Th1 or Th2 by an established procedure described previously (31). Briefly, anti-CD3 antibody (4 μg/ml in PBS) was immobilized to a 12-well plate by incubation for 1 hr at 37°C, and was then washed twice with PBS to remove unbound antibody. Naïve T cells (5×10^5 cells/ml, 4 ml/well) were stimulated in this anti-CD3 antibody-coated plate in the presence of DHEA in the culture medium supplemented with either 10 ng/ml IL-4 and 5 μg/ml anti-IFN-γ antibody (R4-6A2; Th2 differentiation) or 10 ng/ml IL-12 (Th1 differentiation). After 7 days of incubation, the differentiated T cells were washed three times with culture medium to remove exogenous cytokines, and then an equal number of these cells (5×10^5 cells/ml) from each sample was restimulated with plate-bound anti-CD3 antibody for 24 or 48 hr. The level of differentiation (Th1 or Th2) in each culture was determined by amount of the marker cytokine (Th2: IL-4; Th1: IFN-γ) production measured by ELISA.

Results

DHEA Inhibits T Cell Receptor (TcR)-Mediated T Cell Proliferation. T cell proliferation is activated by a complicated network of signals initiated from TcR, CD28, and then IL-2R. The primary stimulus is from the TcR/CD3/CD4 complex interacting with its cognate antigen-MHC (major histocompatibility complex) molecule expressed on antigen-presenting cells (APCs). Integration of TcR/CD3- and CD28-signaling pathways results in the transcription of IL-2 and IL-2R genes and entry of resting cells to G1 phase of the cell cycle (32–34). Our first study was to define the effect of DHEA on T cell proliferation induced by interactions with antigen and APCs.

As shown in Figure 1, addition of DHEA to *in vitro* cultures decreased the proliferative response of lymphocytes to KLH. The effect of DHEA on T cell proliferation was dependent upon the concentrations of both antigen and DHEA. In the cultures with 100 μg/ml of KLH, all doses of DHEA inhibited lymphocyte proliferation in response to KLH. Twenty micromoles of DHEA completely suppressed the proliferation, and 5 μM of DHEA reduced proliferative response from 72,951 ± 2,173 cpm (in the absence of DHEA) to 311 ± 234 cpm (98% inhibition). In the cultures

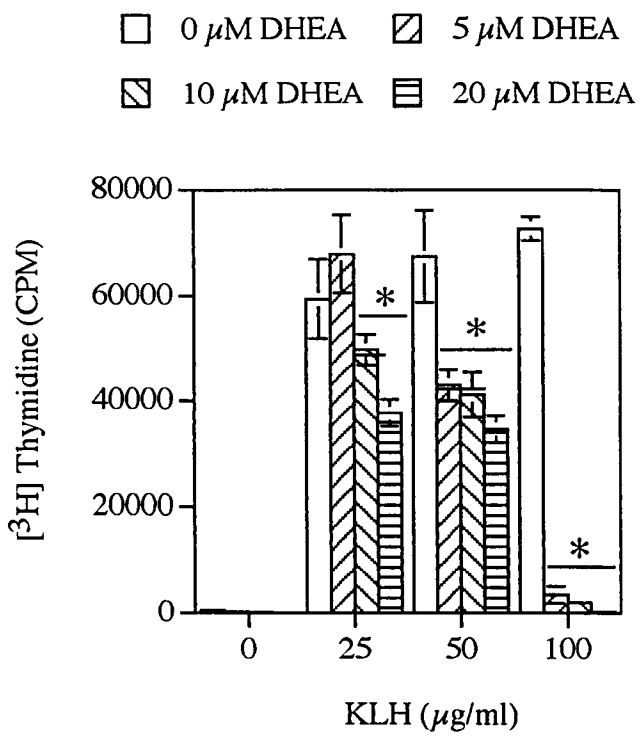

Figure 1. DHEA inhibits antigen-stimulated T cell proliferation. Splenocytes (2×10^5 cells/well) from KLH-immunized SJL/J mice were cultured with antigen KLH in triplicate in 96-well plates in the presence of DHEA for 96 hr, and the antigen-specific T cell proliferation was counted after incubation with 0.5 μCi [^{3}H]thymidine for the last 18 hr. The data represent the mean value and SD (error bar) of [^{3}H]thymidine uptake (cpm) of triplicate determinations at each point from a representative of three separate experiments. *Significantly reduced from the controls (0 μM DHEA), $P < 0.001$ ($n = 9$ for each group).

with less antigen (25 μg/ml), the inhibition of proliferation by the same dose of DHEA was less as well. Thirty-six percent inhibition of proliferative response was observed in the presence of 20 μM DHEA, which was from 59,432 ± 7,616 cpm in untreated cultures to 37,749 ± 2,499 cpm in 20 μM DHEA-treated cultures, and no significant reduction of proliferation was noted in the presence of 5 μM DHEA. These observations imply that DHEA inhibits T cell proliferation against antigen and its effect is dependent upon the strength and dose of the signal mediated by TcR/CD3 complex.

To find the inhibitory site(s) of DHEA on T cell proliferation induced by antigen and APCs, we examined the effect of DHEA on naïve T cell activation induced by cross linking the TcR/CD3 complex with anti-CD3 antibodies or superantigen (SEB), which specifically binds Vβ domain of TcR (35). As shown in Figure 2A, DHEA inhibited anti-CD3 antibody-stimulated T cell proliferation most strongly. Addition of as low as 5 μM DHEA reduced 99.6% of T cell proliferation in the cultures stimulated with 2 μg/ml antibody, showing incorporation of radiolabel decreased from 76,483 ± 7,571 cpm in the absence of DHEA to 828 ± 349 cpm in the presence of 5 μM of DHEA, and complete inhibition was noted in the cultures with 20 μM DHEA. Similar results were also seen when DHEA was added to the cultures stimulated with less anti-CD3 antibody (0.2 or 0.02 μg/ml). In the experiments with SEB (Fig. 2B), DHEA inhibited SEB-stimulated T cell proliferation in the similar pattern as seen in Fig. 2A. SEB (1 μg/ml)-induced T cell proliferation was reduced from 102,756 ± 15,233 cpm in untreated lymphocyte cultures to 34,835 ± 3,087 cpm (68% suppression) in 5 μM DHEA-treated cultures, and further to the baseline level in the cultures with 20 μM DHEA. Similar effect of DHEA was also observed in the cultures stimulated with 0.1 or 0.01 μg/ml SEB (Fig. 2B). These experiments indicate that DHEA efficiently inhibits proliferation of naïve T cells induced by the primary stimulus of cross-linking TcR/CD3/CD4 complex.

We next examined whether the potent antiproliferative effects of DHEA were due to induction of apoptosis. Splenocytes were stimulated by plate-bound anti-CD3 antibodies in the presence of 5 or 20 μM DHEA for 24 hr. As shown in Figure 3, the number of apoptotic cells (M2) in the cultures with DHEA (5 or 20 μM) did not increase as compared with those without DHEA, suggesting that inhibition of T cell proliferation by the concentrations of DHEA used in cultures is not through induction of cell death.

To elucidate whether DHEA affected IL-2R signaling, Con A-activated T cells, in which expression of IL-2R was upregulated (36), were stimulated by IL-2 in the presence or absence of DHEA. As indicated in Figure 4, in the cultures with less IL-2 (1 u/ml), the inhibition of T cell proliferation was clearly observed, 20 μM DHEA reduced proliferation from 12,122 ± 1,228 cpm in untreated cultures to 5,639 ± 492 cpm, which was close to the baseline level. However, in

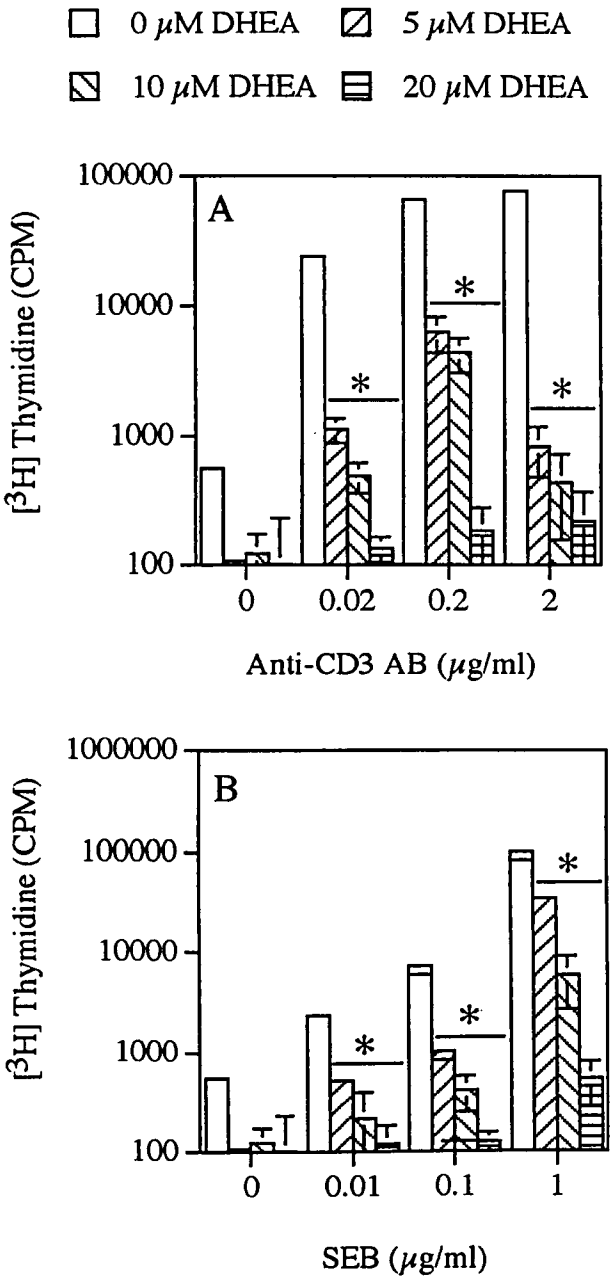

Figure 2. DHEA inhibits T cell proliferative response induced by ligands of TcR/CD3 complex. Naïve splenocytes (2×10^5 cells/well) from SJL/J mice were cultured in 96-well plates with DHEA in the presence of anti-CD3 antibody (A) or SEB (B) overnight, and T cell proliferation was counted after incubation with 0.5 μCi [^{3}H]thymidine for 18 hr. The data represent the mean value and SD (error bar) of [^{3}H]thymidine uptake (cpm) of triplicate determinations at each point from a representative of three separate experiments. *Significantly reduced from the controls (0 μM DHEA), $P < 0.001$ ($n = 9$ for each group).

the cultures stimulated with high amount of IL-2 (10–100 u/ml), DHEA did not inhibit IL-2R-mediated T cell proliferation. For example, in the cultures stimulated with 100 u/ml IL-2, the [^{3}H] incorporation in the cultures treated with 20 μM DHEA was 171,960 ± 15,122 cpm, which was not significantly different from 165,540 ± 13,253 cpm counted in the untreated cultures. These data suggest that DHEA has

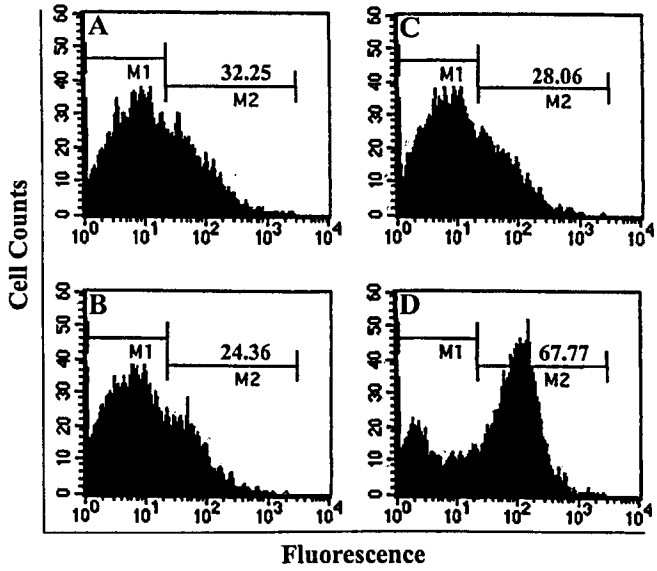

Figure 3. No induction of apoptosis by DHEA in the cultures of splenic T cells. Naïve T cells from SJL/J mice were activated by plate-bound anti-CD3 antibodies in the presence of DHEA for 24 hr: (A) 0 μM; (B) 5 μM; and (C) 20 μM. The same number of T cells in PBS (D) was used as a positive control for apoptotic cells. M2 represents percentage of apoptotic cells in total from a representative of four separate experiments. No statistical difference was found between DHEA-treated and -untreated cultures.

no effect on IL-2R-mediated T cell proliferation, particularly in the cultures stimulated with 10–100 U/ml IL-2. Conversely, the addition of the exogenous IL-2 might rescue inactivation of T cells by high doses of DHEA. Taken together, our data suggest that inhibition of TcR-mediated T cell proliferation by DHEA occurs in the upstream of IL-2R signaling.

DHEA Favors Development of Th2 Cells Induced by Interactions between T Cells and APCs. When CD4$^+$ T helper (Th) cells encounter antigens presented by APCs, they become activated and differentiate into two functionally distinct subsets: Th1 and Th2. Th1 cells secrete IFN-γ and lymphotoxin, whereas Th2 cells produce IL-4, IL-5 and IL-10 (37). The direction of differentiation depends the strength of signal through TcR and costimulations, including cytokines and hormones (34, 38). Our next step was to investigate the roles of DHEA on Th differentiation and development.

KLH-primed splenocytes were stimulated with 50 μg/ml of KLH in the presence or absence of DHEA, and the culture supernatants were collected after 24, 48, 72, and 96 hr. Analysis of Th2- or Th1-secreted cytokines (IL-4 or IFN-γ) by ELISA indicated that the presence of DHEA stimulated Th2 response in these cultures in which T cell proliferation was shown to be decreased (Fig. 1). As shown in Figure 5, increase in IL-4 production was seen in the cultures of 72 and 96 hr with 10–20 μM DHEA. In the presence of 20 μM DHEA, IL-4 production increased from 0.934 ± 0.033 ng/ml (in the absence of DHEA) to 1.526 ± 0.044 ng/ml (with 20 μM DHEA) (63.4% increase) at 72 h, and from 0.511±0.039 ng/ml (in the absence of DHEA) to

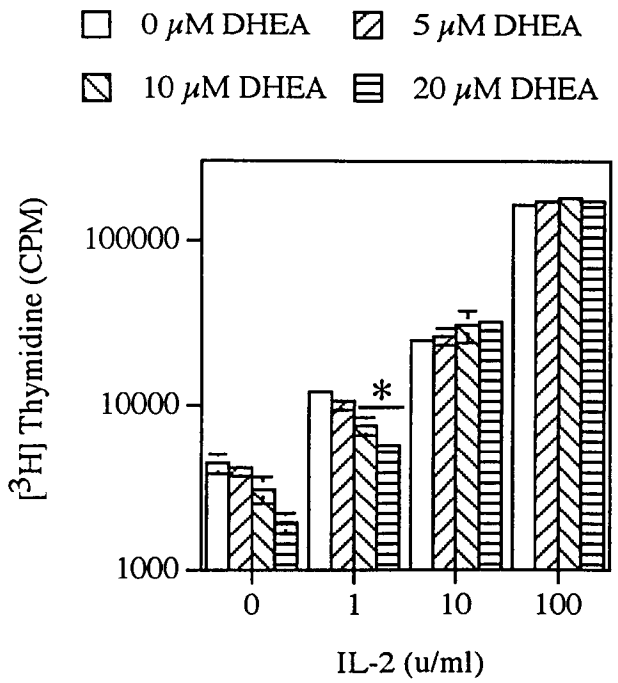

Figure 4. Effect of DHEA on IL-2 stimulated T cell proliferation. Con A-activated T cells (2×10^5 cells/well in a 96-well plate) were stimulated with IL-2 in the presence of DHEA for 24 hr, and IL-2-induced T cell proliferation was counted after incubation with 0.5 μCi [^{3}H]thymidine for 18 hr. The data represent the mean value and SD (error bar) of [^{3}H]thymidine uptake (cpm) of triplicate determinations at each point from a representative of three separate experiments. *Significantly reduced from the controls (0 μM without DHEA), $P < 0.001$ ($n = 9$ for each group).

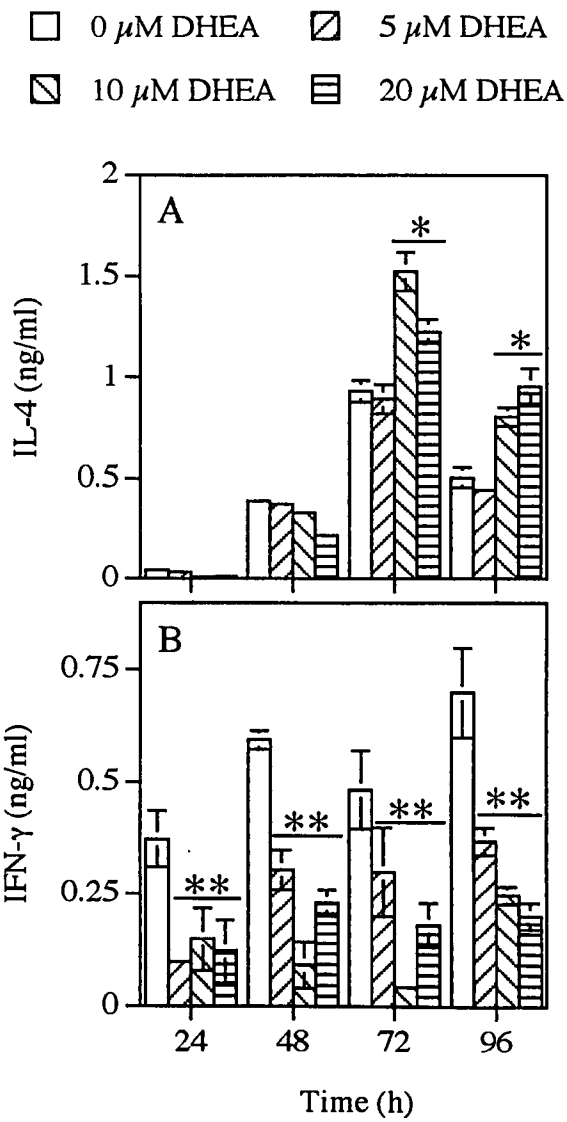

Figure 5. Effect of DHEA on cytokine profile of KLH-primed splenocytes. The KLH-primed splenocytes (2.5×10^6 cells/ml) as described in Figure 1 were stimulated with 50 μg/ml soluble KLH in the presence of DHEA and were harvested after 24, 48, 72, or 96 hr. (A) IL-4 and (B) IFN-γ in the supernatants were measured by ELISA. The data represent the mean value and SD (error bar) of triplicate determinations at each point from a representative of three separate experiments. *Significantly increased from the controls (0 μM DHEA), $P < 0.01$ ($n = 9$ for each group). **Significantly decreased from the controls (0 μM DHEA), $P < 0.001$ ($n = 9$ for each group).

0.956 ± 0.09 ng/ml (with 20 μM DHEA; 87% increase) at 96 hr (Fig. 5A). However, the production of IFN-γ decreased at all concentrations of DHEA and at all time points examined (Fig. 5B). These experiments suggest that there is a preferential activation of Th2 (IL-4-producing) cells in the presence of DHEA (10–20 μM).

To understand the mechanisms of DHEA action on Th differentiation, we first examined the effect of DHEA on Th cell differentiation driven by exogenous cytokines IL-12 or IL-4. Naïve T cells, purified from splenocytes of Balb/c mice, were stimulated by plate-bound anti-CD3 antibodies in the presence of either IL-4 with anti-IFN-γ antibodies (Th2 response) or rIL-12 (Th1 response) along with DHEA for 7 days as described in "Materials and Methods." Viable T cells were harvested and recultured in equal number of cells for 24 or 48 hr in plates precoated with anti-CD3 antibodies. The culture supernatants were harvested and the amount of IFN-γ and IL-4 secreted in the cultures was measured. As shown in Figure 6A, DHEA did not change IL-4 production under conditions that favored a Th2 response. However, in the presence of DHEA, there was an increase of IFN-γ-producing Th1 cells in a dose-dependent manner (Fig. 6B). IFN-γ production in the presence of 20 μM DHEA increased from 16.83 ± 3.2 to 444.5 ± 23.7 9 pg/ml (26-fold) at 24 hr, and from 52.7 ± 2.7 to 630 ± 30 pg/ml (12-fold) at 48 hr. These observations suggest that under the stimulation of exogenous IL-12 there is a preferential skewing of naïve T cells to a Th1 phenotype in the presence of DHEA, and that Th2 differentiation is not affected by DHEA in these *in vitro* systems.

The different readout of Th1 development between T cells-APCs (Fig. 5) and T cells-IL-12 (Fig. 6) systems in the presence of DHEA led us to study effect of DHEA on IL-12 production. IL-12 is exclusively produced by APCs (e.g., macrophages) in the cultures of splenocytes, and development of Th1 cells is closely linked to the production of IL-12 (39, 40). We examined the effect of DHEA on IL-12 secretion of LPS-activated macrophages. Splenic macro-

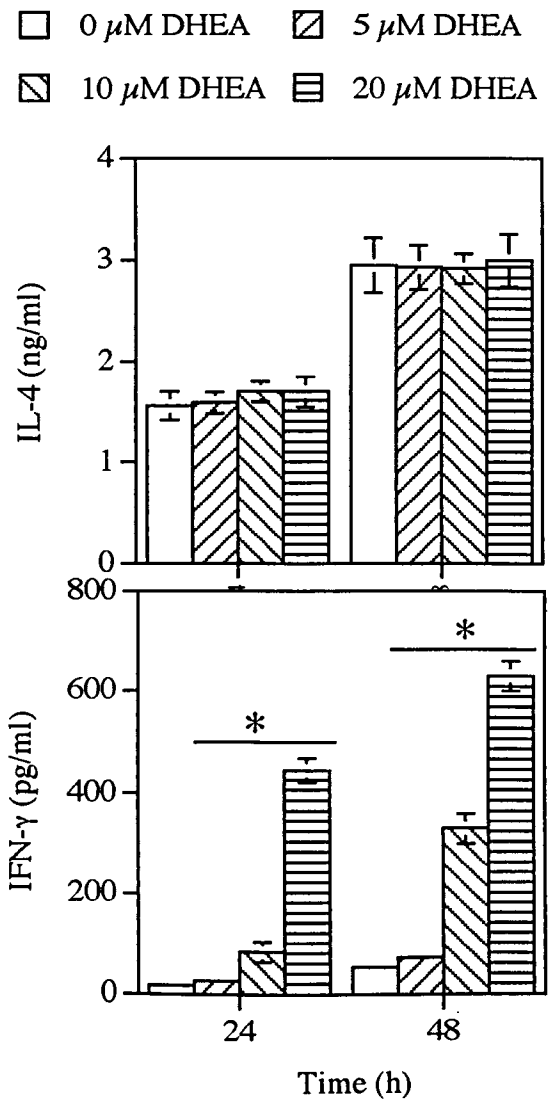

Figure 6. Effects of DHEA on differentiation of T cell subsets Th1 or Th2. Naïve T cells (5×10^5 cells/ml) were differentiated into Th1 or Th2 in the presence of DHEA in an established *in vitro* system as described in "Materials and Methods." (A) Th2 differentiation indicated by IL-4 production; (B) Th1 differentiation indicated by IFN-γ production. The data represent the mean value and SD (error bar) of triplicate determinants at each point from a representative of three separate experiments. *Significantly increased from the controls (0 μM DHEA), $P < 0.001$ ($n = 9$ for each group).

phages, isolated from splenocytes of SJL/J mice, were treated with DHEA for 30 min before addition of 0.5 μg/ml LPS. Addition of as low as 5 μM DHEA decreased IL-12 production from 59 ± 2.68 to 16.84 ± 0.47 pg/ml (71.5% inhibition; Fig. 7), suggesting that in the antigen-presenting cultures, addition of DHEA reduces IL-12 secretion from APCs.

The development of Th1/Th2 immune response not only relates to differentiation of Th1/Th2 phenotype from a naïve precursor, but also relies on growth of activated Th1/Th2 subsets stimulated by Th1/Th2 cytokines in an autocrine matter. We examined the effect of DHEA on IL-12/IL-4-mediated proliferation of activated T cells, which were

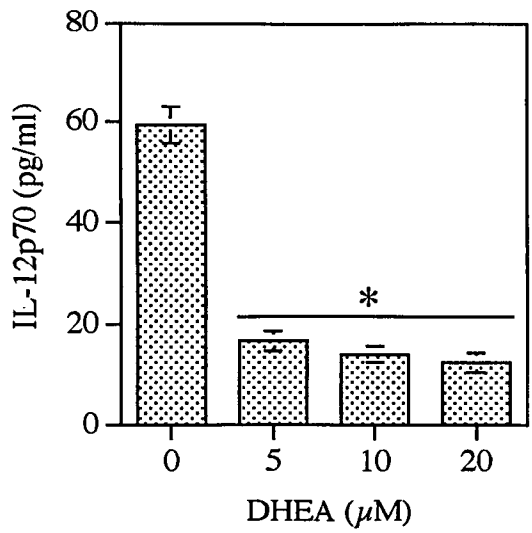

Figure 7. Inhibition of IL-12 production from activated macrophage. Splenic macrophages (1×10^6 cells/well in a 24-well plate) were stimulated by 0.5 μg/ml LPS in the presence of DHEA for 48 hr. IL-12p70 in the culture supernatants were measured by ELISA. The data represent mean value and SD (error bar) of triplicate determinants at each point from a representative of three separate experiments. *Significantly reduced from the controls (0 μM DHEA), $P < 0.001$ ($n = 9$ for each group).

obtained from splenocytes of SJL/J mice as described in "Materials and Methods." After gradient centrifugation to remove dead cells, activated T cells were stimulated with recombinant IL-4 or IL-12 in the absence or presence of DHEA. As seen in Figure 8A, the presence of DHEA enhanced IL-4-stimulated T cell proliferation: the proliferation increased from 32,459 ± 2553 cpm (in the absence of DHEA) to 89,148 ± 3898 cpm when DHEA (10 or 20 μM) was added along with IL-4 (10 ng/ml; 2.8-fold). Significant increases were also seen in the cultures treated with less DHEA or stimulated with less IL-4 (5 ng/ml; Fig. 8A). In contrast, DHEA suppressed IL-12 induced T cell proliferation in a dose-dependent manner (Fig. 8B). Following stimulation with 10 ng/ml of IL-12, T cell proliferation decreased from 35,333 ± 2,031 cpm (in the absence of DHEA) to 6,225 ± 1,430 cpm following the addition of 20 μM of DHEA (85% suppression). Similar results were also seen in the cultures stimulated with 0.1 or 1 ng/ml IL-12 (Fig. 8B). These data indicate that the presence of DHEA enhances IL-4-stimulated Th2 but decreases IL-12-induced Th1 cell proliferation.

Our preliminary studies demonstrate that in addition to enhancement of IL-4-stimulated T cell (Th2) proliferation (Fig. 8A), the supraphysiologic levels of DHEA favor Th2 response by suppressing Th1 development via inhibition of IL-12 production from APCs (Fig. 7) and IL-12-mediated expansion of activated T cells (Th1; Fig. 8B). Therefore, the stimulation of Th2 response in antigen-presenting cultures by high DHEA doses is probably mediated by multiple actions described above, among these it primarily targets APCs to reduce IL-12 production and enhances IL-4 stimulated proliferation of Th2 cells.

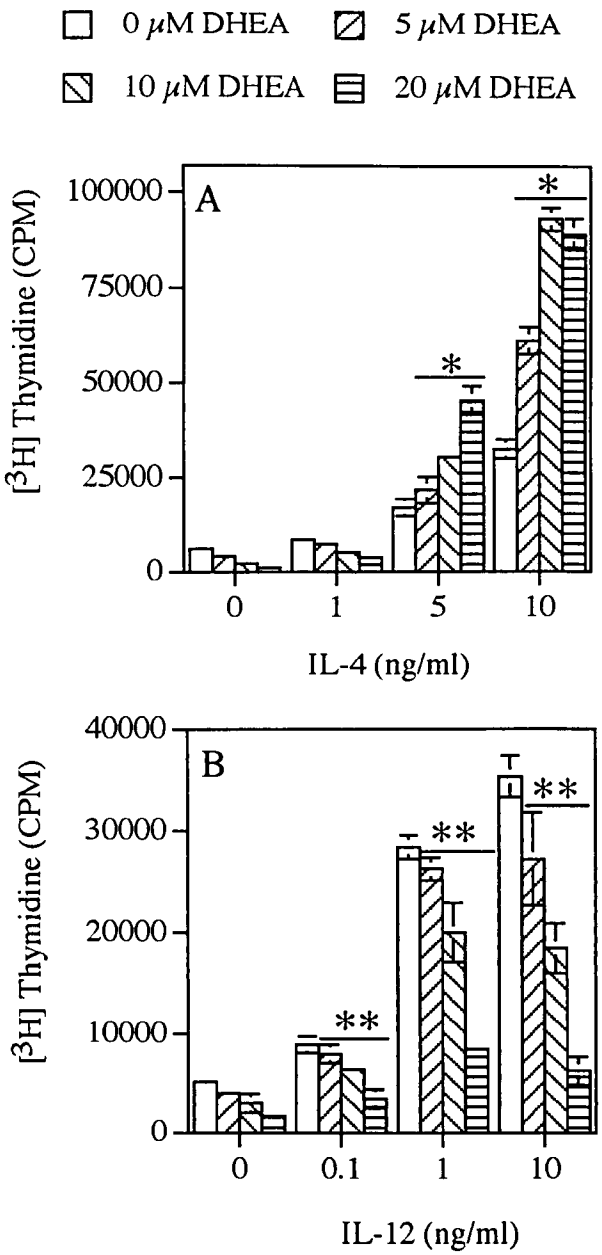

Figure 8. Effects of DHEA on cytokine-driven T cell growth. Naïve T cells were activated with Con A and underwent a period of resting to ensure that all T cells were synchronized to G1 phase. The activated T cells (2×10^5 cells/well in a 96-well plate) were stimulated with (A) IL-4 or (B) IL-12 in the presence of DHEA for 24 hr. The data (cpm) represent the mean value and SD (error bar) of triplicates at each point from a representative of three separate experiments. *Significantly increased from the controls (0 μM DHEA), $P < 0.001$ ($n = 9$ for each group). **Significantly decreased from the controls (0 μM DHEA), $P < 0.001$ ($n = 9$ for each group).

Discussion

Our studies show the profound effect of DHEA on the regulation of T cell proliferation and differentiation *in vitro*. We have shown four major effects of high doses of DHEA *in vitro*: DHEA inhibited proliferation of naïve and antigen-primed T cells; its anti-proliferative effect on T cells occurred prior to IL-2R signaling and was not due to induction of apoptosis; the addition of DHEA to the antigen-stimulated splenocytes enhanced Th2 responses; and DHEA stimulated IL-4R-mediated (Th2 cells) but inhibited IL-12R- (Th1 cells) mediated T cell proliferation, and reduced IL-12 production from APCs.

DHEA/DHEA-S is one of the major circulating adrenal cortical hormones in humans and many other warm-blooded animals (41). DHEA levels in plasma reach to the peak in early adulthood (2–4 ng/ml), then decrease with old age (7). In the sera of adult mice, DHEA levels are considerably less than in humans, and maximal amounts do not exceed 1 ng/ml (42). In the present investigation, the DHEA doses (5–20 μM) we tested are undoubtedly high when compared with the normal serum levels of this hormone, but the local and intracellular DHEA levels in the steroid target tissue are uncertain. The average serum DHEA-S level in healthy "younger" adults is about 7.5 μM, and increases to 20–70 μM following administration of pharmacological dosages of DHEA without any sign of systemic toxicity (43). The recirculating DHEA-S is converted to DHEA by ubiquitous steroid sulfatase. Therefore, DHEA levels in plasma may not reflect the actual concentrations of DHEA in the target tissue, which could be much higher than that observed in the circulation.

The antiproliferative activity of DHEA has been reported in many different mammalian cell lines (44–47), but the effect of DHEA on T cell function has been controversial. Our results are in agreement with that of Loria *et al.* (48), who showed that DHEA suppressed the proliferation of Con A- or LPS-activated mixed splenocytes cultures in a dose-dependent manner. Other have shown that preincubation of T cells in the presence of DHEA caused an enhancement of the proliferative response (49). Similar conflicting results on cytokine production in T cells in the presence of DHEA have been noted (49, 50). One of the reasons for the discrepancy could be due to the differences in the assays used to determine the effect of DHEA. Our studies have shown that the inhibition of T cell proliferation by high doses of DHEA *in vitro* could not be the result of directly inducing cells to undergo apoptosis (Fig. 3), which also was shown in the cultures of peripheral lymphocytes (51). With a structure similar to DHEA, glucocorticoid has been demonstrated to inhibit T cell proliferation, but through induction of apoptosis (52, 53), suggesting that the inhibitory effect of DHEA at the concentrations we used was not due to the nonspecific binding to glucocorticoid receptor in T cells. DHEA inhibited T cell proliferation following ligation of TcR (Fig. 2), but did not affect IL-2-mediated proliferation (Fig. 4), suggesting that the inhibition of T cell proliferation by DHEA occurs at a point proximal to IL-2R signaling, including downregulation of CD28 and/or IL-2R expression.

Following antigenic challenge, Th0 cells differentiate into two mutually exclusive lineages, Th1 and Th2. Factors that modify lineage commitment depend to a large extent on the cytokine milieu, strength of the costimulatory signals,

antigen concentration, and the presence or absence of other factors including hormones (37, 54, 55). The most potent regulators of T cell differentiation are cytokines, and of these, IL-4 is a central to induce T cells to a Th2 phenotype (56). In contrast, IL-12 is a key cytokine in the regulation of Th1 development. Mice lacking IL-12 or its receptor show a markedly reduced Th1 response (57). In the antigen-presenting cultures, addition of DHEA inhibited IFN-γ production in all cultures, but it increased IL-4 production in antigen-dependent matter (Fig. 5), indicating that under these particular conditions *in vitro*, the presence of DHEA stimulated Th2 immune responses. However, DHEA has been shown an opposite effect *in vivo*, in which downregulation of Th2 (or upregulation Th1) immune response by administration of DHEA has been found in old or retrovirus infected mice (16, 17, 20). This discrepancy could be in part due to that DHEA is rapidly cleared from the blood and converted in peripheral tissue to other steroids (5), which effect on T cells may be different from that of DHEA, whereas *in vitro* DHEA is protected from this kind of biomodification.

In the antigen-presenting cultures, DHEA could act on both T cells and APCs. In the pure macrophage cultures, Wu *et al.* (58) have shown that DHEA induces macrophages to produce TGF-β, which can inhibit IL-12 production by an autocrine matter (59). DHEA also directly inhibited LPS-induced IL-1 and TNF-α (60) and IL-12 production (Fig. 7). These cytokines are critical effectors in eliciting and priming IFN-γ production and Th1 development (39, 61). Therefore, in the microenvironment of *in vitro* cultures induction of other regulatory cytokines such as TGFβ and/or inhibition of IL-12 from APCs by DHEA may contribute to its amplification of a Th2 phenotype. In the aspect of its action in T cells, DHEA enhanced IL-12-induced Th1, but did not change IL-4-induced Th2 differentiation from naïve T cells following ligation of TcR (Fig. 6). In contrast, DHEA inhibited IL-12R but enhanced IL-4R signalings leading to cell growth (Fig. 8). In terms of IL-4R signalings, it has been clearly demonstrated that there are two independent pathways associated with IL-4R, one is Jak/Stat pathway for gene activation (phenotype development), and another is IRS/Ras leading to proliferation (62). Our data suggest that DHEA may block the inhibitory factor(s) of IRS/Ras pathway to enhance IL-4-stimulated cell proliferation, but not affect regulation of Jak/Stat (Jak1/Jak3-Stat6/Stat6) pathway from IL-4R for Th2 phenotype development. Just like IL-4R signalings, IL-12R triggers two separate intracellular pathways: Jak/Stat pathway (Jak2/Tyk2-Stat3/Stat4) (63, 64) and MKK-p38 MAPK pathway (65); both are required for IL-12-induced IFN-γ expression. Again, it is possible that DHEA acts on MKK-p36 MAPK rather than Jak/Stat of IL-12R signalings. Figure 6 indicates that addition of DHEA stimulated IL-12-induced Th1 phenotype development, suggesting that DHEA may benefit expression of IFN-γ gene by deactivation of inhibitory factor(s) on MKK-p38 MAPK pathway.

The steroid hormone receptor superfamily has been characterized, and the intracellular receptors undergo structural alternation upon binding to the hormone, conferring upon it the ability to bind DNA and regulate gene transcription (66). Unfortunately, the receptor for DHEA has not been identified yet, but specific DHEA binding activity has been detected not only in T cells (27, 67), but also in mouse B16 melanoma cell line and rabbit vascular smooth muscle cells (47, 68). The data we present here indicate that DHEA has multiple functions on T cells and macrophages, which are mediated through binding to a group of intracellular receptors, or a specific receptor mediating many different pathways at the same time.

In conclusion, our current investigation suggests that DHEA is a potent regulator of T cell immune response, and elevated levels of DHEA favor the development of a Th2 immune response. DHEA is currently classified as a food additive by the FDA and hence is not closely regulated. Although there have been no untoward effects of DHEA reported so far, unsupervised use of high doses of DHEA is likely to give to rise to effects that could be both useful and perhaps detrimental.

We wish to thank Ms. Sandy Watkins for administration assistance and Mrs. Monica Schwarz for editorial help.

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