Cytokine Dysregulation and Increased Oxidation Is Prevented by Dehydroepiandrosterone in Mice Infected with Murine Leukemia Retrovirus (44186)

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Abstract. **The effects of murine leukemia retrovirus infection on production of cytokines was investigated in mice fed different doses of dehydroepiandrosterone (DHEA). Young C57BL/6 female mice were injected with LP-BM5 murine retrovirus or were kept as uninfected controls. Two weeks later, each group was divided into subgroups: fed unsupplemented AIN 93 diet as the control, or diets supplemented with 0.02% DHEA (0.9 mg/mouse/day) or 0.06% DHEA (2.7 mg/mouse/day). The uninfected mice supplemented with 0.06% DHEA showed a significant ($P < 0.05$) increase in interleukin-2 (IL-2) and γ-interferon (IFN-γ) production, and hepatic vitamin E levels. Retroviral infection induced severe oxidative stress that was reduced by DHEAS supplementation in retrovirally infected mice. DHEA supplementation prevented the retrovirus-induced loss of cytokines (IL-2 and IFN-γ) secretion by mitogen stimulated spleen cells. DHEA also suppressed the production of cytokines interleukin-6 (IL-6) and tumor necrosis factor–α (TNF-α) by T helper 2 (Th2) cells which were otherwise stimulated by retrovirus infection. Thus, immune dysfunction and increased oxidation induced by murine retrovirus infection were largely prevented by DHEA.**

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Dehydroepiandrosterone (DHEA) and its sulfate (DHEA-S) play an important role in immunomodulation of animals (1) and humans (2). They are the most abundant adrenal secretory products in humans, yet their biological functions are not well defined. DHEA levels are decreased during aging and retroviral infection (1, 2). DHEA has important immunoregulatory roles, as its levels decline in human immunodeficiency (HIV)–infected people concomitantly with lowering of $CD4^+$-cell numbers (3). DHEA has significant immunomodulatory activities in old mice, with DHEA supplementation overcoming their cytokine dysregulation and immune dysfunction (1, 2). DHEA is a powerful antioxidant (4). Oxidation increases, causing vitamin E deficiencies in human and murine acquired immunodeficiency syndrome (MAIDS) (5). Oxidation results in damage to lymphocyte DNA, inhibiting immune functions. In addition, oxidation stimulates the release of nuclear factor κ B (NF-κB) which promotes synthesis of cytokines by T helper 2 (Th2) cells. These cytokines reduce the synthesis of Th1 cells' cytokines and lower cellular immune functions. DHEA supplementation of retrovirus-infected mice should prevent immune dysfunction, cytokine dysregulation, oxidative damage, and loss of antioxidant nutrients.

To test these hypotheses, young female mice were infected with LP-BM5 murine leukemia. Some were given DHEA in their diets to overcome the loss in antioxidants that occurs with this infection as it progresses and induces lymphoma and murine AIDS (6).

Methods and Materials

Animals and Retrovirus Infection. Female C57BL/6 mice, 8 weeks old, were obtained from Charles River Laboratories Inc. (Wilmington, DE). Animals were

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cared for as required by the University of Arizona Committee on Animal Research. The housing facility was maintained at 20°–22°C and 60%–80% relative humidity, with a 12:12-hr light:dark cycle. Water and diet were freely available to the mice 24 hr a day. After 2 weeks of being housed and fed the control diet (AIN 93A) (Beitlaham PA), half of the mice were randomly assigned to the infected groups and half remained uninfected. LP-BM5 retrovirus was administered intraperitoneally to mice in 0.1 ml with an esotropic titer (XC) of 4.5 $\log_{10}$ PFU/ml, which induces disease with a time course comparable to that previously published (5). Infection of female C57BL/6 mouse with LP-BM5 leads to the rapid induction of clinical symptoms with virtually no latent phase (7). Uninfected, normal mice were injected with complete culture medium used for LP-BM5 virus growth as a control. Administration of DHEA was begun 2 weeks after LP-BM5 infection and continued until the mice were sacrificed. Young mice (8 mice/group) were randomly assigned to the following treatments groups for 10 weeks: (A) uninfected and fed unsupplemented diet (AIN 93A); (B) uninfected and fed 0.02% DHEA–supplemented diet; (C) uninfected and fed 0.06% DHEA–supplemented diet; (D) retrovirus-infected, fed the unsupplemented diet (AIN 93A); (E) retrovirus-infected, fed the 0.02% DHEA–supplemented diet; and (F) retrovirus-infected, fed 0.06% DHEA–supplemented diet.

ELISA for Cytokines. γ-Interferon (IFN-γ), interleukin-2 (IL-2), IL-6, and tumor necrosis factor–α (TNF-α) were produced by mitogen-stimulated splenocytes *in vitro* as described previously (5). Briefly, spleens were gently teased with forceps in culture medium (RPMI-1640 containing 10% fetal calf serum (FCS), 2 m*M* glutamine, 100 units/ml penicillin and streptomycin, CM [complete medium]), producing a single cell suspension of spleen cells. Red blood cells were lysed by the addition of a lysis buffer (0.16 *M* ammonia chloride Tris buffer, pH 7.2) at 37°C for 3 min. Then the cells were washed twice with CM. Cell concentration was counted and adjusted to 1×10^7 cells/ml. Splenocyte viability was more than 95% as determined by trypan blue exclusion. One-tenth of a milliliter per well of splenocyte (1×10^7 cells/ml) were cultured in triplicate on 96-well flat-bottomed culture plates (Falcon 3072, Lincoln Park, NJ) with CM. Splenocytes were then stimulated with concanavalin A (Con A, at 10 μg/ml, with 0.1 ml/well; Sigma Chemical Co., St. Louis, MO) for induction of IL-2 and TNF-α with 24 hr of incubation, IFN-γ with 72 hr of incubation at 37°C, 5% CO_2 incubator. Splenocyte were also stimulated by lipopolysaccharide (LPS, at 10 μg/ml, with 0.1 ml/well (Gesco, Grand Island, NY) for 24 hr induction for IL-6 production. After incubation, the plates were centrifuged for 10 min at 800*g*. Supernatant fluids were collected and stored at −70°C until analysis. They were determined by sandwich ELISA as described previously (5). Rat anti-murine IFN-γ, IL-2, IL-6, hamster anti–TNF-α monoclonal antibody, standard recombinant TNF-α rabbit anti-murine TNF-α serum, purified antibodies, rat anti-murine IFN-γ, IL-2, TNF-α, and IL-6 biostimulated antibodies, and recombinant murine IFN-γ, IL-2, IL-6, TNF-α were obtained from Pharmingen (San Diego, CA).

Determination of Conjugated Dienes and Lipid Fluorescence. Approximately 0.2 g of tissue was homogenized in 5.0 ml of Folch solution (8) (2:1 v/v, chloroform:methanol). After protein separation, a 0.1-ml fraction was dried in a steady flow of nitrogen gas at 55°C, analyzed to determine conjugated dienes and lipid fluorescence as previously described (8). Conjugated dine fatty acids were determined by obtaining the absorbency of the solution at 237 nm (Shimadzo UV 160 UV recording spectrophotometer, Tokyo, Japan). Lipid fluorescence was measured in a Aminco Bowman fluorescence spectrophotometer (Bethesda, MD). Maximum fluorescence at 470 nm was measured. The activation wavelength was at 395 nm.

Determination of Phospholipid. The phospholipid contents of the livers and hearts were determined by the method of Raheja *et al.* (9). This method does not require the predigestion of the phospholipid. Dipalmitoyl phosphatidylcholine (Sigma) was used to produce standard curve.

Determination of Total Cholesterol. The total cholesterol of the liver and heart was determined by the method of Zack (10). Briefly, 0.3 ml of Folch extract was dried under air at 70°C. Three milliliters of Zak's reagent was added followed by 2 ml of sulfuric acid after mixing throughly. Total cholesterol was determined by obtaining the absorbency of the solution at 560 nm in a Shimadzo UV 160 UV recording spectrophotometer using cholesterol standards (Sigma).

Determination of Vitamin E and A. Vitamin E (α-tocopherol) and vitamin A (retinol) in liver and heart tissues were determined by the fluorometric method as described by Dugan *et al.* (11). Briefly, approximately 0.2 g of tissue was homogenized in 5 ml of folch extract. Then 0.3 ml of Folch's extract was dried under N_2, and 1.0 ml of ethanol was added, followed by the addition of 0.5 ml of 25% ascorbic acid. One milliliter of 10 *N* KOH was added and incubated for 15 min at 70°C. Five milliliters of *n*-hexane were added. To determine vitamin A level, 2.0 ml of the *n*-hexane layer was removed and measured at an emission of 430 nm and an excitation of 365 nm in fluorometer (Aminco Bowman fluorescence spectrophotometer) using an appropriate blank reagent. To the solution, 0.6 ml (10 *N*) of 60% sulfuric acid was added and vortexed for 30 sec, centrifuged and then the fluorescence intensity for vitamin E was determined in the hexane at an emission of 340 nm and excitation maximum of 295 nm. External standards of *d*-α-tocopherol and all-*trans* retinol (Eastman Chemical, Rochester, NY) were used for preparing standard curves. All levels of vitamins were represented by micrograms per gram of wet tissue.

Statistics. All parameters were compared using a one-way analysis of variance (ANOVA), followed by a *t* test (two-sample, assuming unequal variances) for compari-

sons between any two groups. $P < 0.05$ was considered significantly different between groups.

Results

Body Weight and Food Consumption. There were no significant changes in food or water consumption due to infection or DHEA supplementation (data not shown). Supplementation with 0.02% DHEA resulted in intake of 0.9 mg/mouse/day while the 0.06% DHEA diet yielded intakes of 2.7 mg/mouse/day. Spleen weight was elevated and splenocytes per gram of spleen were significantly ($P < 0.05$) lower in the infected mice as shown previously (12), indicating that infection had progressed towards murine AIDS. The cells per gram spleen and spleen weight were not affected by DHEA consumption (data not shown).

Th1-Cell Cytokine Production by Splenocytes. T helper 1 (Th1) cells produce cytokines that promote cellular immunity. It is suppressed in human (13) and murine AIDS (7). *In vitro* production of IL-2 and IFN-γ by Con A–stimulated splenocytes was significantly ($P < 0.05$) inhibited in cells from retrovirus-infected mice (Fig. 1). Cells from retrovirus-infected young mice consuming 2.7 mg DHEA/mouse/day for 10 weeks increased Th1 cytokine production significantly ($P < 0.05$) higher than those consuming 0.9 mg DHEA/mouse/day (Fig. 1). Retrovirus-infected young mice consuming 0.9 and 2.7 mg/mouse/day DHEA had 10% and 110%, respectively, greater IL-2 production. They also had 50% and 160% more IFN-γ production, respectively (Fig. 1). DHEA treatment significantly ($P < 0.05$) increased IL-2 and IFN-γ release by mitogen-stimulated splenocytes from uninfected mice compared with cells from uninfected mice that consumed the unsupplemented diet (Fig. 1).

Th2 Cell Cytokine Production by Splenocytes. Th2 cells' cytokines can suppress production of Th1 cytokines. Release of Th2 cytokines IL-6 and TNF-α was significantly ($P < 0.05$) increased in the retrovirus-infected (Fig. 2) compared with uninfected mice. Supplementation with DHEA (Fig. 2) significantly ($P < 0.05$) decreased secretion of IL-6 and TNF-α compared with secretion by cells from infected or uninfected mice fed the unsupplemented diet. Serum IL-6 levels were increased by retrovirus infection (Fig. 3). Treatment with DHEA significantly ($P < 0.05$) reduced serum IL-6 level and TNF-α production by mitogen-stimulated splenocytes (Fig. 2) of retrovirus-infected mice.

Hepatic Vitamin E and A. The hepatic vitamin E levels (Fig. 4) were significantly ($P < 0.05$) reduced by retrovirus infection as shown previously (5). DHEA consumption of 2.7 mg/mouse/day significantly ($P < 0.05$) retarded the loss of tissue vitamin A and E during infection. Retrovirus-infected mice treated with the higher DHEA supplement had 11% and 12% increased hepatic levels of vitamin E and A, respectively (Fig. 4). Uninfected mice fed 2.7 mg DHEA/day showed increased ($P < 0.05$) hepatic vitamin E and A compared with untreated mice (Fig. 4). Similar changes were observed in cardiac tissue (data not shown).

Hepatic Lipid Peroxidation. Retrovirus infection significantly ($P < 0.05$) increased hepatic free radical reactions products: lipid fluorescence and diene conjugates. DHEA supplementation significantly ($P < 0.05$) prevented the increases in both products (Fig. 5). There was a dose-effect relationship as both infected and uninfected mice treated with 2.7 mg DHEA/mouse/day of DHEA had significantly ($P < 0.05$) lower conjugated dienes and lipid fluorescence than mice fed 0.9 mg DHEA/mouse/day (Fig. 5).

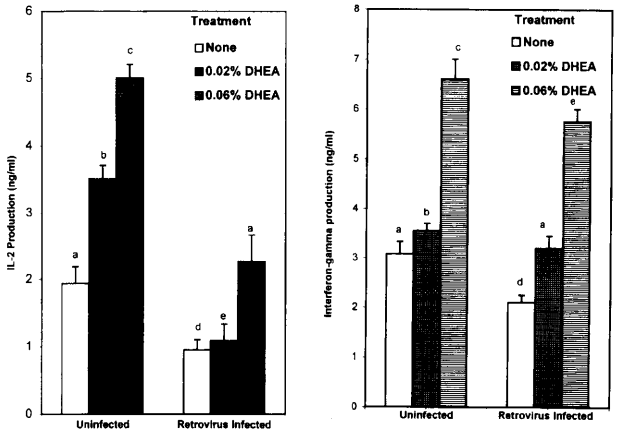

Figure 1. The effects of DHEA administration on Th1 cytokine production by splenocytes. The results are averages from triplicate assays of cells from each mouse in a group and are expressed as mean ± SE; $n = 8$. Immune responses were significantly different ($P < 0.05$) in IL-2 production (A) by splenocytes among certain groups: a < b < c < d < e. Also immune responses were different in IFN production (B) by spleen cells among certain groups: a < b < c, a > d, and c > e.

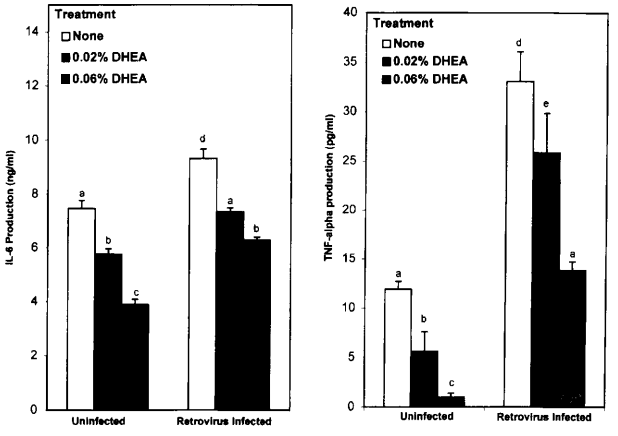

Figure 2. The effects of DHEA administration on Th2 cytokine responses by splenocytes. The results are averages from triplicate assays of cells from each mouse in a group and are expressed as mean ± SEM; $n = 8$. Immune responses were significantly different ($P < 0.05$) in IL-6 production (A) by splenocytes between certain groups: a < b < c and a < d. Also immune responses were different in TNF-α (B) by spleen cells among certain groups: d > e > a < b < c.

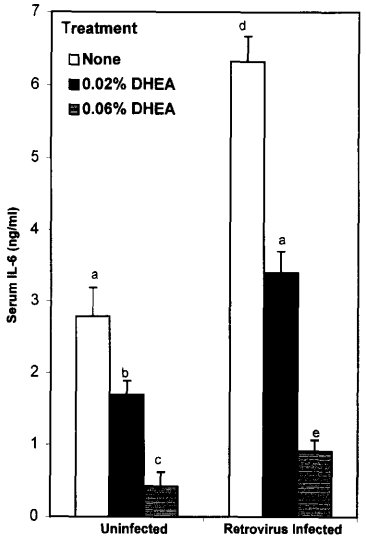

Figure 3. The effects of DHEA administration on serum IL-6. The results are averages from triplicate assays from each mouse in a group and are expressed as mean ± SE; $n = 8$. Immune responses were significantly different ($P < 0.05$) among certain groups: a > b > e > c and d > a.

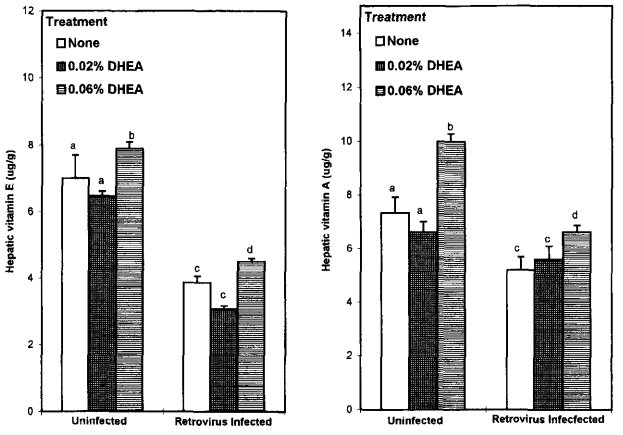

Figure 4. The effects of DHEA administration on hepatic vitamin E (A) and A (B) levels. The results are averages from duplicate assays done for each mouse in a group and are expressed as mean ± SE; $n = 8$. Vitamin levels were significantly different ($P < 0.05$) due to DHEA supplementation among certain groups: (A) a < c < b, b > d > c; (B) a > d > c, a < b.

Hepatic Total Cholesterol. At 10 weeks postinfection, the mice had increased total cholesterol levels ($P < 0.05$) in hepatic tissue (Fig. 6). Supplementation with 2.7 mg DHEA/mouse/day significantly ($P < 0.05$) prevented these increases by maintaining total cholesterol levels similar to those of uninfected mice. DHEA treatment of uninfected young mice did not cause a significant change in total hepatic cholesterol (Fig. 6).

Hepatic Phospholipid. Retrovirus infection significantly ($P < 0.05$) lowered phospholipids in hepatic tissues (Fig. 6). Uninfected mice treated with 2.7 more than those fed 0.9 mg DHEA/mouse/day significantly ($P < 0.05$) increased phospholipids levels in hepatic tissues (Fig. 6). However, supplementation with 2.7 and 0.9 mg DHEA/

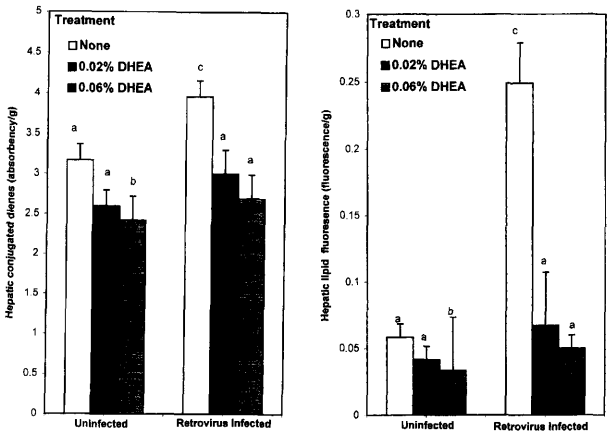

Figure 5. The effects of DHEA administration on hepatic lipid peroxidation (A and B). The results are averages of duplicate assays from each mouse in a group and are expressed as mean ± SE; $n = 8$. Lipid peroxidation responses were significantly ($P < 0.05$) higher in retrovirus-infected mice. Lipid peroxidation was different due to DHEA supplementation among certain groups: (A) a > b, a < c; (B) a > b, c > a.

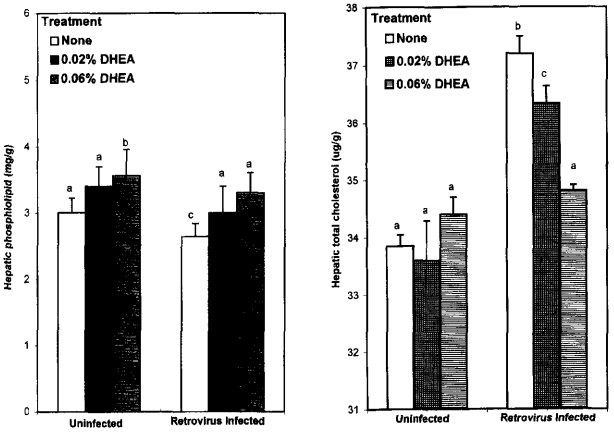

Figure 6. The effects of DHEA administration on hepatic phospholipid (A) and total cholesterol (B) levels. The results are averages from duplicate assays done for each mouse in a group and are expressed as mean ± SE; $n = 8$. Phospholipid (A) changes were significantly lower in retrovirus-infected mice, while total cholesterol changes were significantly increased ($P < 0.05$). Phospholipid changes were different due to DHEA supplementation among certain groups: (A) a < b, a > c ($P < 0.05$). Also total cholesterol changes were different due to DHEA supplementation among groups: (B) a < b < c < d.

mouse/day normalized phospholipids levels to become like those uninfected mice. Similar changes were noted in cardiac tissues (data not shown).

Discussion

The retrovirus infection increased cytokine dysregulation, lipid oxidation, and loss of vitamin E. DHEA supplementation prevented retrovirus-induced suppression of immune responses, stimulation of lipid peroxidation, loss of vitamin E, and normalized the lipid concentrations. Similar changes occurred in the uninfected young mice. DHEA supplementation significantly reduced the excessive IL-6 and TNF-α production by mitogen-stimulated splenocytes

from retrovirus infected young mice. DHEA enhanced IL-2 that should inhibit Th2 cells, reducing their cytokine secretion (14). DHEA supplementation also substantially increased cytokine secretion by Th1 cells from young infected mice whose increased IL-2 may have helped T cell mitogenesis.

Aberrant cytokine production promotes progression to murine (7, 15) and human (16) AIDS. When stimulation of Th2 cells by chronic retroviral antigen exposure prevented by T-cell receptor peptide immunization, immune dysfunction was also stopped (17). Similarly cytokine production changes due to retrovirus infection were prevented by DHEA in dose-dependent manner, suggesting that it regulates Th1 and Th2 cells. DHEA deficiency in HIV-infected patients occurs concomitantly with exacerbation of cytokine dysregulation and immune dysfunction (16). IL-6 secretion by cells from uninfected and retrovirus-infected mice supplemented with 2.7 mg DHEA/mouse/day was reduced by over 75% compared with similar unsupplemented controls. Dysregulation of IL-6 production is a major contributing factor to lymphoid malignancies (18) that occur more frequently during retrovirus infection (7). As supplemental DHEA overcame the excessive secretion of cytokines by Th2 cells in our mice leukemia virus–infected mice lymphoma development should be reduced (12). There was a normalization, lowering, of serum IL-6 and autoantibody production by DHEAS supplementation of aged mice (19). DHEA reduced the high IL-6 production by mitogen-stimulated cells from old infected mice (19). DHEA also lowered IL-6 serum in both infected and uninfected mice. IL-2 is an important growth factor for T cells. Its increased release during DHEA consumption is in accord with restoration of T-cell mitogenesis in both uninfected and retrovirus-infected mice. IFN-γ has antiviral activity, stimulating macrophage and cytotoxic T lymphocytes in HIV-infected people (20). Increased IFN-γ production by cells from retrovirus-infected young mice treated with DHEA should restore suppressed cell-mediated immunity (20, 21). The stimulation in uninfected mice shows that DHEA modifies Th1 cells' cytokine production. IFN-γ inhibits cytokine secretion by Th2 cells, which thus may explain the significant prevention of retrovirus-induced IL-6 and IL-10 production during (21) DHEA replacement. An antioxidant, glutathione, was reduced in murine (22) and human AIDS (23), facilitating increased lipid peroxidation. Oxidative stress increasing lipid peroxidation has been implicated in stimulating HIV replication (24) and immunodeficiency. Excessive free radical production induced by TNF (25) activated macrophages for increased NF-κB activity and HIV replication (26). Dietary supplementation by DHEA lowered hepatic oxidative stress in uninfected mice. It normalized oxidative damage in infected mice to levels of uninfected young mice. Moreover, the higher DHEA supplementation prevented vitamin E losses in uninfected as well as infected mice. Vitamin E prevents oxidation of cellular components by free radicals and singlet oxygen as the most effective antioxidant at high partial pressures (27). Vitamin E is an immune enhancer associated with cancer and tissue damage (5). Inhibition of vitamin E loss may delay disease progression by murine (7) and human retrovirus infection (6). Increased oxidation stimulates more NF-κB activity and thus retrovirus replication (24). Vitamin E supplementation in uninfected and retrovirus-infected mice reduced oxidative damage, while decreasing IL-6 production (28), much as occurred with our DHEA treatments that simultaneously prevented vitamin E loss. Supplementation by 2.7 mg DHEA/mouse/day significantly prevented oxidative damage and TNF-α and IL-6 increases. It maintained total cholesterol levels similar to those of uninfected mice. In our study, DHEA reduced the excessive TNF-α production during murine retrovirus infection. Lowered TNF-α production should reduce hepatic triglycerides, total cholesterol, and lipid peroxidation while maintaining vitamin E and phospholipid levels. TNF inhibits lipoprotein lipase synthesis (29), acetyl-CoA carboxylase (30), fatty acid synthase (31), and glycerol phosphate dehydrogenase (32). It also stimulates liver lipogenesis (33) and hepatic VLDL (34), causing severe hypertriglyceridemia. TNF has shown to stimulate muscle proteolysis and decrease protein synthesis during cancer cachexia (35). DHEA may function by binding to the specific DHEA receptor in T-cell clones that mediate increased IL-2 production (36). The activated B cells and macrophages from HIV patients produce high levels of IL-6 and TNF, as do with LPS-stimulated splenocytes and peritoneal macrophages for retrovirus-infected mice (7, 16).

DHEA supplementation of retrovirus-infected mice increased Th1 cells' cytokine secretion and lowered that by Th2 cells, significantly correcting immune deficiencies associated with murine leukemia and AIDS (37, 38). Prevention of retrovirus-induced oxidation, lipid changes, and loss of tissue antioxidants by DHEA may be an important regulator of Th2 cells and thus cellular immune functions.

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