Modulation of Cytokine Production by Dehydroepiandrosterone (DHEA) Plus Melatonin (MLT) Supplementation of Old Mice (44270)

PAULA INSERRA,* ZHEN ZHANG,* SUSSAN K. ARDESTANI,* MOHSEN ARAGHI-NIKNAM,* BAILIN LIANG,* SHUGUANG JIANG,* DON SHAW,† MARK MOLITOR,† KERRY ELLIOTT,† AND RONALD R. WATSON*,[1]

Arizona Prevention Center and Family and Community Medicine,† University of Arizona, Tucson, Arizona 87524*

Abstract. **Tissue levels of the antioxidants melatonin (MLT) and dehydroepiandrosterone (DHEA) decline with age, and this decline is correlated with immune dysfunction. The aim of the current study is to determine whether hormone supplementation with MLT and DHEA together would synergize to reverse immune senescence. Old (16.5 months) female C57BL/6 mice were treated with DHEA, MLT, or DHEA + MLT. As expected, splenocytes were significantly ($P < 0.05$) higher in old mice as compared to young mice. DHEA, MLT, and DHEA + MLT significantly ($P < 0.005$) increased B cell proliferation in young mice. However, only MLT and DHEA + MLT significantly ($P < 0.05$) increased B cell proliferation in old mice. DHEA, MLT, and DHEA + MLT help to regulate immune function in aged female C57BL/6 mice by significantly ($P < 0.05$) increasing Th1 cytokines, IL-2, and IFN-γ or significantly ($P < 0.05$) decreasing Th2 cytokines, IL-6, and IL-10, thus regulating cytokine production. DHEA and MLT effectively modulate suppressed Th1 cytokine and elevated Th2 cytokine production; however, their combined use produced only a limited additive effect.** [P.S.E.B.M. 1998, Vol 218]

Levels of the hormones, melatonin (MLT) (1) and dehydroepiandrosterone (DHEA) (2) decline with age and are associated with immune dysfunction (3, 4). The thymus is the center for growth and differentiation of T cells and thymic involution is a major cause of immune dysfunction in the elderly. Thymic involution is accompanied by alterations in the levels of thymic growth and inhibitory factors, and these factors are regulated by hormones. Therefore, age-related changes in hormone levels alter the thymic microenvironment and subsequently the development of naive T cells. Understanding the mechanisms of action these compounds have in the aging process must be ascertained in order to demonstrate a cause and effect relationship.

Melatonin (MLT), the main hormone secreted by the pineal gland, has many well-established roles (5, 6). MLT appears to be an effective scavenger of hydroxyl free radicals (7), as well as being two times more effective at scavenging peroxyl radicals than vitamin E (6). Although MLT receptors are present on a variety of cells, MLT being lipid soluble can readily pass membranes without the aid of carrier proteins. This property implies that MLT could have a ubiquitous antioxidant role in the body. Once inside the cell, MLT binds calmodulin (8) and scavenges hydroxyl radicals. Additionally, MLT might bind nuclear receptors (9) and ultimately regulate gene expression. MLT may also regenerate the antioxidant enzyme, glutathione peroxidase by supplying $NADPH_2$ (10). $NADPH_2$ is necessary for generating the reduced form of glutathione.

Increasing survival has been the focus of many studies; however, only one experiment demonstrated improved survival rates. Rats fed diets deficient in calories and protein lived significantly longer than controls (11). Dietary restriction conserves normal melatonin rhythms (12). During fasting, tryptophan is mobilized and can be converted to sero-

[1] To whom requests for reprints should be addressed at Arizona Prevention Center, PO Box 245052, University of Arizona, Tucson, AZ 85724. E-mail:rwatson@u.arizona.edu

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tonin, which is then converted to MLT. MLT levels rise during nighttime fasts and possibly during states of starvation. MLT has also been shown to enhance IL-2 production and T-helper-cell activity. Increased IL-2 and T helper cells leads to increased antibody production (13).

The adrenal hormone DHEA and insulin-like growth factor (IGF-1) also decline with age (2). DHEA replacement in older humans resulted in significantly increased IGF-1, which may facilitate an anabolic state in the elderly. Anabolism and preservation of lean body mass (LBM) can decrease susceptibility to, and improve recovery from, infectious diseases. Reduced *de novo* DHEA synthesis results in an altered ratio of DHEA:cortisol. Normally, corticotrophin releasing hormone (CRH) from the hypothalamus acts on the pituitary gland, resulting in the release of adrenocorticotropic hormone (ACTH). ACTH stimulates the adrenal cortex increasing both DHEA and cortisol production. During aging, DHEA synthesis is impaired and cannot negatively feedback on cortisol, stimulation of the adrenal cortex results in aberrant cortisol synthesis. This subsequently leads to immunosuppression, decreased lean body mass, increased body fat, and glucose intolerance.

DHEA in old mice has been shown to increase natural-killer-(NK)-cell cytotoxicity, decrease IL-6, and alter T-lymphocyte subsets (14). Additionally, T cells from young mice typically produce more IL-2, IL-3, and GM-CSF and less IL-4, IL-5, and IFN-γ than those from older mice (14). We (15) and others (14) have shown that this can be normalized by oral or iv administration of DHEA or DHEA-S, respectively. The aim of the current study is to determine the individual, as well as synergistic, immunological effects of DHEA and MLT replacement in old mice, as compared to its effect on young mice.

Material and Methods

Animals and Diets. Female C57BL/6 mice, 1.5 and 16 months old, were obtained from the Charles River Laboratories Inc. (Wilmington, DE). In this mouse strain, mice become sexually mature within 2–3 months of age. By the age of 3 months they exhibit a near maximal immune response that peaks at the age of 5–6 months. Therefore, mice 2 months of age, at the start of the treatments, were considered young. Additionally, immune response declines gradually after 8–9 months of age in this strain, and we have observed that 90% of mice die prior to 28 months resulting in a median lifespan of 24–25 months. Consequently, we used mice 16.5 months old in the old mice group, since they would already be experiencing immuno-senescence. The mice were housed in transparent plastic cages with stainless steel wire lids (three to four mice per cage) at the University of Arizona animal facility. Animals were 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 hr light:dark cycle. Water and diet were freely available. After 2 weeks of housing and being fed the control diet (AIN 93A), the mice were randomly assigned to the following treatments: Groups A–D were young mice (eight mice/group) fed (A) unsupplemented (control) AIN 93A diet and 0.05% ethanol in the drinking water, (B) 0.02% DHEA supplemented diet for the first 3 weeks (6.2 μg/mouse/day) and then 0.06% DHEA diet for the next 9 weeks (18.66 μg/mouse/day) with 0.05% ethanol in the drinking water, (C) unsupplemented diet with 10 μg/ml melatonin (MLT) dissolved in 0.05% ethanol drinking water (49.8 μg/mice/day) for 12 weeks, and (D) 0.02% DHEA supplemented diet for the first 3 weeks and then 0.06% DHEA thereafter with 10 μg/ml MLT in 0.05% ethanol drinking water (for 12 weeks). Four groups of old mice (four mice/group) were provided with the same supplemented diets and treated water as described for young mice. DHEA was donated by Edenland Inc. (Baybush, Kildore, Ireland). The 0.02% DHEA diet and 0.06% DHEA diet were prepared by Diets Inc. (Bethlehem, PA) using the same AIN 93A diet, pelted and color coded. MLT was purchased from Sigma (St. Louis, MO) and dissolved in 95% ethanol. It was then diluted in distilled water. The final concentration of MLT in the drinking tap water was 10 μg/ml with 0.05% ethanol. The treatment period was 12 weeks for all groups.

Standard Cytokines and their Antibodies. Rat antimurine IFN-γ, IL-2, IL-4, IL-6, IL-10 purified antibodies, rat antimurine IFN-γ, IL-2, IL-4, IL-6, IL-10 biotinylated antibodies, and recombinant murine IFN-γ, IL-2, IL-4, IL-6, IL-10 were obtained from Farmington (San Diego, CA).

ELISA for Cytokines. IFN-γ, IL-2, IL-4, IL-6, and IL-10 were produced by splenocytes as described previously (16). Briefly, spleens were collected after sacrifice under ether anesthesia. Mononuclear cells were obtained by gently teasing with forceps in culture medium (RPMI 1640 containing 10% FCS, 2 m*M* glutamine, 100 units/ml penicillin and streptomycin, CM), 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. Splenocytes, 0.1 ml/well (1×10^7 cell/ml), were cultured in triplicate on 96-well flat-bottom culture plates (Falcon 3072, Lincoln Park, NJ) with CM. Splenocytes were then stimulated with concanavalin A (Con A, 10 μg/ml, 0.1 ml/well, Sigma) for induction of IL-2, IL-4, and IL-10 with 24 hr incubation, IFN-γ with 72 hr incubation at 37°C in a 5% CO_2 incubator. Splenocytes were also stimulated by lipopolysaccharide (LPS, 10 μg/ml, Gebco, Grand Island, NY) for 24 hr induction for IL-6 and TNF-α 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 (17) as we have described previously (16).

Mitogenesis of Splenocytes. Splenic T- and B-cell proliferation was determined by [^{3}H]thymidine incorporation as described previously (18). Briefly, splenocytes in 0.1 ml of CM (1×10^7 cell/ml) were cultured in 96-well flat-bottom cultured plates (Falcon) with Con A and LPS (10 μg/ml). They were incubated at 37°C in a 5% CO_2 incubator for 44 hr for Con A and LPS-induced T- and B-cell proliferation respectively, and then pulsed with [^{3}H]thymidine (0.5 μCi/well, New England Nuclear, Boston, MA). After 6 hr, they were harvested by a cell sample harvester (Cambridge Technology, Cambridge, MA). Radioactivity was determined by a liquid scintillation counter (Tri-Carb, 2200 CA, Packard, Lagunahills, CA). Data were presented as counts per minute (CPM).

Natural Killer (NK) Cell Cytotoxicity. NK-cell function was measured by a fluorescent concentration release assay modified from the method of Wierda *et al.* (19). Briefly, this method measures the calcein AM (Molecular Probes, Eugene, OR) remaining in the target cells using the Pandex Fluorescence Concentration and Analyzer (FCA) (IDEX, Portland, ME). YAC-1 target cells were washed twice with PBS and labeled with the calcein AM derivative. Effector to target (E:T) ratios were adjusted to 100:1, 50:1, and 25:1, and plated in U-bottom microtiter plates (Falcon 3077, Lincoln Park, NJ) containing 4×10^4 target cells/100 μl. The plate was centrifuged (90 *g*) for 3 min to facilitate cell-to-cell interaction. The cells were then incubated at 37°C in a humidified atmosphere of 5% CO_2 for 3 hr. After incubation, 20 μl of 1% inert fluoricon polystyrene assay particles were added to each well of the plate (Pandex Harvesting Plate, IDEX, Portland, ME), and 70-μl aliquots from each well of the irradiation plate were transferred to a Pantex plate. Epifluorescence of each well in the harvest plate was automatically read at 485/533 nm excitation/emission wavelengths for calcein AM using the Pantex FCA. Specific cytotoxicity (%) was calculated as follows:

$$\frac{\text{Spontaneous Release} - \text{Experimental Release}}{\text{Spontaneous Release} - \text{Maximum Release}} \times 100 = \text{Specific Cytotoxicity (\%)}$$

Lymphocyte Subpopulation Measurement. Thymus were collected after sacrifice under ether anesthesia. Mononuclear cells were obtained by gently teasing with tweezers in CM. Cell suspensions were washed with CM. Red blood cells were lysed by lysing buffer. The remaining cells were washed twice with cold CM. The number of viable cells was determined by using trypan blue exclusion. Cell concentration was then adjusted to $1–2 \times 10^5$/0.1 ml/tube for subsequent lymphocyte surface marker determinations as described by Lopez *et al.* (20). The following directly conjugated rat anti-mouse monoclonal antibodies were used: phycoerythrin (PE)-CD8, cy-chrom-CD3, fluorescein isothiocyanate (FITC)-CD4 and FITC-CD5 (PharMingen, San Diego, CA). Tissues from each mouse were counted and assessed separately, with four mice/group. Samples were analyzed using a FacStar flow cytometer (Becton Dickinson, San Jose, CA) with the consort 40 program.

Statistics. The statistical tests for comparison among groups were finished in NCSS program (Kaysville, UT) using Friedman's Block/Treatment test, followed by Duncan's Multiple Range Test between any two groups. $P < 0.05$ was considered significant difference between two groups.

Results

Weights. No change in weight was noted for either old or young mice throughout the study period.

Spleen and Thymic Cell Numbers. Spleen weights were significantly higher ($P < 0.005$) in untreated old mice 106 ± 13 mg than in untreated young mice 66 ± 14 mg. Treatment with DHEA + MLT did not alter spleen weights in either old or young mice (data not shown). Old mice had a significantly higher number of splenocytes ($P < 0.05$) than young mice (Fig. 1). Young mice treated with DHEA + MLT had a significantly ($P < 0.05$) lower number of splenocytes than their respective controls (Fig. 1). The percentage of CD_3^+/CD_8^+ cells from thymic glands was not found to be significantly different and was not affected by treatments (data not shown). The percentage of CD_5^+ cells was higher in old mice (19.3 ± 2.85) than young mice (13.8 ± 3.5); however, this did not reach significance ($P = 0.06$) nor was it affected by hormone supplementation.

Spleen Cell Function. B-cell proliferation, in response to *in vitro* mitogen stimulation with LPS, did not differ between untreated old and young mice (Fig. 2A). B-cell proliferation was higher in young mice supplemented with DHEA ($P < 0.0005$), MLT ($P < 0.0005$), and DHEA + MLT ($P < 0.05$) as compared to control young mice (Fig.

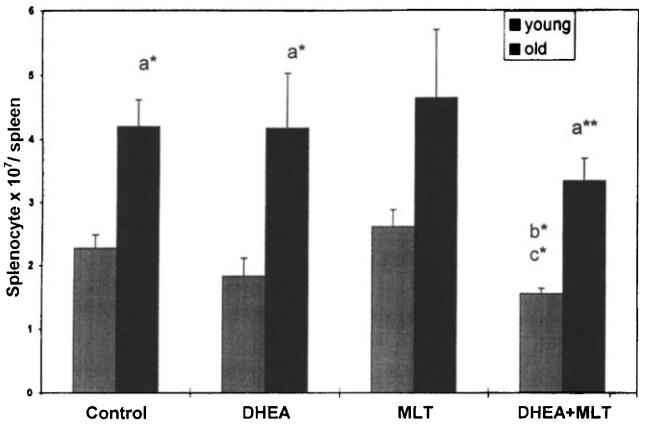

Figure 1. Effect of DHEA, MLT, and DHEA + MLT on splenocyte numbers in old and young mice. The values are mean ± SE. The data represent eight young mice per group and four old mice per group. (a) *P* value compares old mice with young mice receiving the same treatment. (b) *P* value compares DHEA + MLT treated young mice with untreated young mice. (c) *P* value compares DHEA + MLT treated young mice with young mice treated with MLT alone. $^*P < 0.05$, $^{**}P < 0.005$.

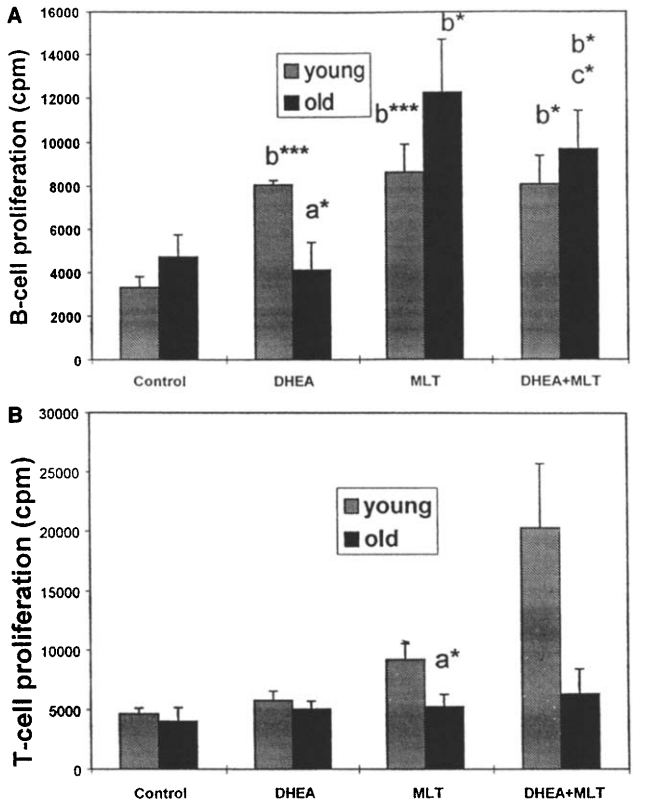

Figure 2. Effect of DHEA, MLT, and DHEA + MLT on (A) B cell proliferation by LPS-stimulated and (B) T-cell proliferation by ConA-stimulated splenocytes from old and young mice. Each sample was done in triplicate and averaged. The values are mean ± SE. The data represent eight young mice per group and four old mice per group. (a) *P* value compares old mice with young mice receiving the same treatment. (b) *P* value compares treated mice with their respective controls. (c) *P* value compares DHEA + MLT treated old mice with old mice treated with DHEA alone. $^{*}P < 0.05$, $^{**}P < 0.005$.

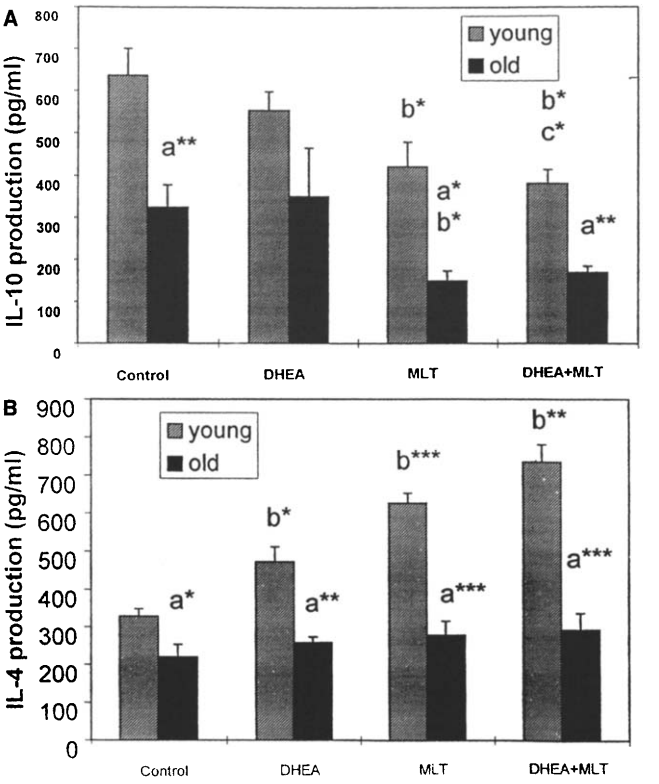

Figure 3. Effect of DHEA, MLT, and DHEA + MLT on (A) IL-10 and (B) IL-4 production by ConA-stimulated splenocytes from old and young mice. Each sample was done in triplicate and averaged. The values are mean ± SE. The data represent eight young mice per group and four old mice per group. (a) *P* value compares old mice with young mice receiving the same treatment. (b) *P* value compares treated mice with their respective controls. (c) *P* value compares DHEA + MLT treated old mice with old mice treated with DHEA alone. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

2A). Old mice treated with MLT and DHEA + MLT had significantly ($P < 0.05$) higher B-cell proliferation as compared to old controls (Fig. 2A). T-cell proliferation, in response to *in vitro* mitogen stimulation with ConA, did not differ between untreated old and young mice (Fig. 2B). However, MLT supplementation significantly ($P < 0.05$) increased T-cell proliferation in young mice as compared to MLT-treated old mice (Fig. 2B). Natural-killer-cell cytotoxicity did not differ between young and old mice and was not found to be altered by treatments (data not shown).

Cytokine Production by Splenocytes. Th2 cells predominantly produce the cytokines IL-4, IL-6, and IL-10, which function by regulating B cells and suppressing Th1 cells. Mitogen (ConA)-stimulated splenocytes from untreated, MLT, and DHEA + MLT-treated old mice produced significantly lower amounts of IL-10 than cells from untreated, MLT, and DHEA + MLT-treated young mice (Fig. 3A). Additionally, IL-10 production was significantly ($P < 0.05$) decreased in old mice treated with MLT as compared to untreated old mice and did not quite reach significance in the DHEA + MLT group ($P = 0.06$) (Fig. 3A). Mitogen (ConA)-stimulated splenocytes significantly increased in young mice treated with DHEA ($P < 0.05$), MLT ($P < 0.0005$), and DHEA + MLT ($P < 0.005$) as compared to untreated young mice. However, no differences in IL-4 production were observed in old mice (Fig. 3B). Mitogen (LPS)-stimulated splenocytes in all treatment groups of both old ($P < 0.005$) and young ($P < 0.0005$) mice produced decreased amounts of IL-6 as compared to their old and young respective controls (Fig. 4).

Th1 cells predominantly produce the cytokines interferon-γ (IFN-γ) and IL-2. These cytokines are capable of activating T cells and therefore can regulate cell mediated immunity. Mitogen (ConA)-stimulated splenocytes from untreated, DHEA and MLT treated old mice produced significantly ($P < 0.005$) lower amounts of IFN-γ than young mice treated similarly (Fig. 5A). DHEA, MLT, and DHEA + MLT significantly increased IFN-γ production in young mice ($P < 0.005$, $P < 0.0005$, $P < 0.05$) as compared to untreated young mice as well as increased IFN-γ production in old mice ($P < 0.0005$, $P < 0.005$, $P < 0.05$) as compared to untreated old mice (Fig. 3A). Mitogen (ConA)-stimulated splenocytes from untreated and DHEA-treated old mice produced significantly lower ($P < 0.005$) amounts of IL-2 as compared to untreated young mice (Fig. 5B). IL-2 production also significantly ($P < 0.05$) increased in young and old

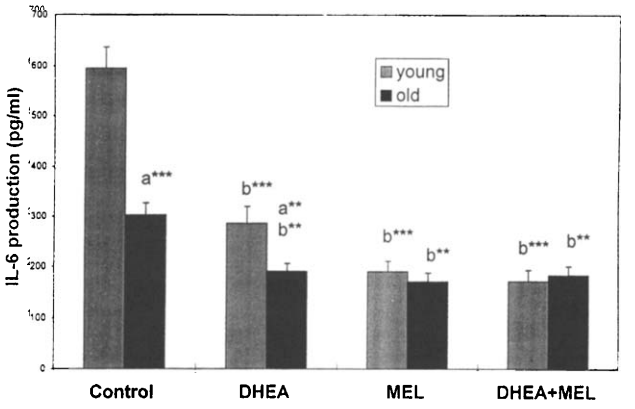

Figure 4. Effect of DHEA, MLT, and DHEA + MLT on IL-6 production by ConA-stimulated splenocytes from old and young mice. Each sample was done in triplicate and averaged. The values are mean ± SE. The data represent eight young mice per group and four old mice per group. (a) *P* value compares old mice with young mice receiving the same treatment. (b) *P* value compares treated mice with their respective controls. $^{*}P < 0.005$, $^{***}P < 0.0005$.

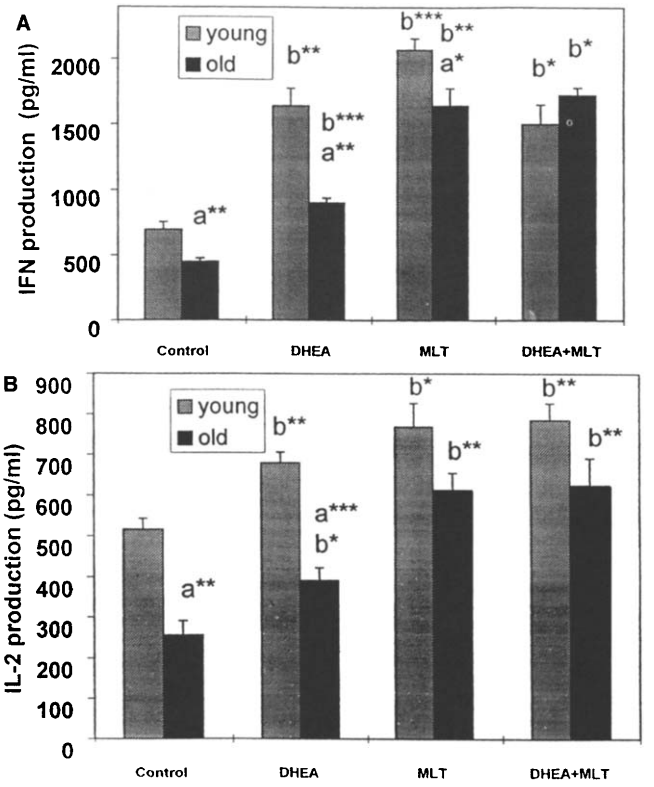

Figure 5. Effect of DHEA, MLT, and DHEA + MLT (A) IFN-γ and (B) IL-2 production by ConA-stimulated splenocytes from old and young mice. Each sample was done in triplicate and averaged. The values are mean ± SE. The data represent eight young mice per group and four old mice per group. (a) *P* value compares old mice with young mice receiving the same treatment. (b) *P* value compares treated mice with their respective controls. $^{*}P < 0.05$, $^{**}P < 0.005$.

mice treated with DHEA, MLT, and DHEA + MLT as compared to their respective controls (Fig. 3B).

Discussion

In the current study DHEA or MLT alone, or in combination, was able to stimulate Th1 cell cytokines and suppress Th2 cell cytokines in young mice, thereby improving cellular immune function. This is the first report studying the simultaneous supplementation of both of these immunoregulatory hormones whose production declines with age. Their synergistic effects may be more evident in primates that synthesize, and thus, may require much larger doses than mice. Additionally, since DHEA or MLT supplementation alone restored immune function, a substantial increase was not observed when the two hormones were administered together. DHEA and MLT were also able to normalize aberrant cytokine production in aged female C57BL/6 mice. Young and old mice supplemented with DHEA and/or MLT had increased production of IL-2 and IFN-γ. Treatments in old mice restored these cytokine levels to that of young untreated mice. Decreased IL-2 production occurs with aging (21–28) and decreased IFN-γ production by PHA and ConA-stimulated lymphocytes also occurs with aging (29–31). Aging is frequently associated with decreased levels of DHEA (32, 33) and MLT (34) with increased oxidative damage during the development of immunosenescence. Aging includes increased production of autoantibodies and decreased cellular immunity due to an increase in Th1 (35) and a decrease in Th2 cells. Th1 cells generally produce a different subset of cytokines (IL-2, IFN-γ) than Th2 (IL-4, IL-6, IL-10) cells do. Older individuals generally have increased Th1 cytokines and decreased Th2 cytokines. Th2 cytokines stimulate B-cell proliferation and humoral immunity and ultimately antibody production. Th1 cytokines stimulate T cells and cellular immunity. This increase in humoral immunity results in the production of autoantibodies and is the major cause of arthritis and other autoimmune diseases associated with aging. Additionally, a lack of cellular immunity results in the ability of cancers and viruses to proliferate. Previously, we have shown that Th2 cytokines decrease in old mice when DHEA + MLT are replaced (15). Although we have now shown that both Th1- and Th2-type cytokines are suppressed in old mice, only Th1 cytokines can increase to levels of young control mice. Additionally, the Th2 cytokines IL-6 and IL-10 can be further decreased with treatments. DHEA and MLT may therefore be useful treatments for conditions where cellular immunity is suppressed. Further studies, with additional age groups, as well as different mouse strains and animal species are still needed before these results can be generalized.

Production of IL-6 is usually substantial in aged subjects, so that its presence can be readily detected in the plasma of aged animals and people (36–39) although it has also been found to be decreased (40). IL-6 is involved in T-cell activation, growth, and differentiation. It also serves as an inducer of both B-cell proliferation and maturation (41) and for the development of mucosal immunity (42). Unregulated IL-6 production can have adverse effects, such as immune function suppression. DHEA, MLT, and DHEA + MLT significantly decreased IL-6 production in young and old mice. These results demonstrate that supple-

mentation with DHEA and/or MLT can regulate IL-6 production.

Many of the age-associated changes in T cells, macrophages, and B cells are linked to excess endogenous IL-10. IL-10 can directly inhibit IL-2 gene expression by activated T cells (43), reduce expression of class II major histocompatibility complex molecules (44), and depress B7 costimulatory molecule expression on activated macrophages (45). $CD5^+$ B cells, rather than Th2 cells, are the major producers of IL-10 following cellular activation (46), and the number of $CD5^+$ B cells increases with advance aging (47). Our study is in agreement with the increase in IL-10 production by activated splenocytes in old mice. However, supplementation with these hormones did not change the number of $CD5^+$ cells in old mice but nevertheless lowered IL-10 production. Perhaps, the decrease in IL-10 was due to suppressed Th2 function.

DHEA, MLT, and DHEA + MLT increased B-cell mitogenesis in old and young mice. However, this may not represent all of the *in vivo* effects of these hormones. For instance, spontaneous mitogenesis was also measured and was not found to change in either young or old mice. DHEA + MLT also increased T-cell mitogenesis in cells from young mice.

Modifying the ratio of DHEA:cortisol, as well as decreasing free radicals, are possible mechanisms by which DHEA and MLT restore immune function. Hormone replacement with DHEA in the aged may restore the optimal DHEA:coritsol ratio, thereby reducing the immunosuppressive effects of relatively high cortisol found in aged animals. As our data demonstrate, DHEA may accomplish this by regulating cytokines. MLT, on the other hand, decreases the free-radical load. Reduced free radicals should suppress their reaction with DNA in naive T cells and the aberrant activation of B cells. This is also supported by our data, as maintaining and/or regulating T and B cells would ultimately lead to a change in the cytokine profile. Additionally, MLT's antioxidant properties may prevent the production of cytokines directly, as free radicals can activate signal transduction pathways leading to cytokine synthesis. Furthermore, since our results demonstrate an additive effect between DHEA and MLT, it is likely that they have different mechanisms of action. Our data demonstrate that DHEA and MLT regulate immune function in C57BL/6 mice by suppressing Th2 and increasing Th1 cytokines. This shift in cytokines results in a regulation of immune function typically seen in the young, thereby normalizing humoral and cellular immunity. The importance of further hormone replacement studies in the elderly, as well as throughout the aging process, is therefore merited.

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