EFFECTS OF DEHYDROEPIANDROSTERONE AND MELATONIN SUPPLEMENTATION AND T CELL RECEPTOR PEPTIDE TREATMENT ON IMMUNE RESPONSE AND LIPID PEROXIDATION IN RETROVIRUS-INFECTED AND AGED MICE

by

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Zhen Zhang

entitled Effects of Dehydroepiandrosterone and Melatonin Supplementation as well as T Cell Receptor Peptide Treatment on Immune Response and Lipid Peroxidation in Retrovirus-infected and Aged Mice

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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DEDICATION

This dissertation is dedicated to all of my family members: My husband, Mr. Gang Chen; my parents, Mr. Qitian Zhang, Ms. Pingxin Lu; and my brother, Mr. Ping Zhang, whose love and encouragement enabled me to finish my Ph.D. program. Throughout my life, they have always been very supportive and understanding.

TABLE OF CONTENTS

Page

TABLE OF CONTENTS — *continued*

Page

ABSTRACT

Aging and acquired immune deficiency syndrome (AIDS) are associated with significant immune dysfunction and increased oxidative stress, resulting in increased susceptibility to opportunistic infections, cancers, autoimmune diseases, and death. Aging and retrovirus infection lower host defenses by modulating cytokine production to alter T and B cell functions. Increased oxidative stress may result in damage to lymphocyte DNA, further compromising an already suppressed immune system in the aged and retrovirally infected. The overall objective of this study is to determine the potential therapeutic roles of immunomodulators including the hormone dehydroepiandrosterone (DHEA) and melatonin (MLT), and also the T cell receptor (TCR) peptides, in ameliorating the immunological and nutritional abnormality caused by aging and retrovirus infection.

Female C57BL/6 mice infected with murine LP-BM5 leukemia retrovirus (MuLv) develop murine AIDS. Similar to human AIDS, murine AIDS is characterized by immune deficiency with dysregulated cytokine production. This retrovirus-infection inhibits release of Th1 cytokines (IL-2, INF-γ), stimulates secretion of Th2 cytokines (IL-4, IL-6, IL-10) as well as TNF-α, and also increases hepatic lipid peroxidation and induces tissue vitamin E deficiency. Decreased Th1 cytokine production and elevated Th2 cytokine secretion induced by retrovirus infection was largely prevented by dietary supplementation with DHEA and MLT as well as with TCR peptide injection. During retrovirus infection these treatments reduced hepatic lipid peroxidation and prevented vitamin E loss. The use of DHEA plus MLT was more effective in preventing retrovirus-induced immune

dysfunction than either DHEA or MLT alone. These results suggest that retrovirus-induced immune dysfunction and increased lipid peroxidation are largely prevented by treatment with DHEA, MLT and TCR peptide. Similarly, it was observed that DHEA, MLT and TCR peptide treatments largely prevented immunosenescence by stimulating Th1 cytokine production and inhibiting Th2 cytokine secretion in aged mice. Although there are several hypotheses proposed to interpret the immunoregulatory effect of DHEA, MLT, or TCR peptides in retrovirus-infected and/or aged mice, the detailed molecular mechanisms are still largely unknown and need to be explored by further studies.

In addition, the effects of vitamin E supplementation on the development of lung injury and hepatic lipid peroxidation in immunodeficient (Nude) mice exposed to side-stream cigarette smoke (SSCS) was studied. Dietary vitamin E supplementation increased vitamin E levels in lung and liver and attenuated SSCS-mediated pulmonary injury and lipid peroxidation, which may be due primarily to increased antioxidant activity in the supplemented mice.

CHAPTER I

INTRODUCTION

PROBLEM STATEMENT

Aging presents a progressive decline in physiological homeostasis resulting in increased vulnerability to a myriad of infections and cancers which are caused, in large part, by immunosenescence. A progressive decline in overall immune competence and regulation is a unifying factor that contributes to the susceptibility of aged people to disease. Age-related changes in immunity primarily involve alterations in T cell function, including dysregulation of cytokine production and decreased proliferation response of T cells to mitogens. In addition, a number of alterations in B cell function are also observed in aging. There is an increased likelihood of autoantibody production and other lymphoproliferative disorders. Immunosenescence can be defined as impaired cytokine production, evidenced by decreased Th1 cytokines (IL-2, INF-λ) with increased Th2 cytokines (IL-4, IL-6, IL-10). Th1 cytokines are primarily involved in activating cellular immune defenses. Th2 cytokines inhibit Th1 cytokine production, and also have major roles in stimulating B cells to mature and produce immunoglobulins. Dysregulated cytokine production can facilitate immunosuppression, as well as growth of leukaemia and autoantibodies, which are common in older individuals.

Acquired immune deficiency syndrome (AIDS) is caused by the human immunodeficiency virus (HIV) and is characterized by immune deficiencies that render the body highly susceptible to opportunistic infections and tumors. Murine AIDS is induced by infection of mice with the LP-BM5 murine leukemia retrovirus mixture (MuLv). It shares many similarities to the pathogenesis of human AIDS, even though HIV and MuLv represent different types of retrovirus. Murine AIDS is characterized by splenogegaly,

lymphadenopathy, deficient B cell response to T-independent antigens *in vitro*, reduced T cell function, dysregulated cytokine production, and tissue vitamin E deficiency. In HIV-infected patients and MuLv-infected mice, Th1 cytokine production declines, while Th2 cytokines and TNF-α increase. Th2 cytokines suppress Th1 cells, causing anergy of cell mediated immunity, thus allowing for the continued reproduction of retrovirus and the stimulation of oxidative radical secretions by macrophages.

Aging and AIDS involve excessive free radical and reactive oxygen species production, thus decreasing cellular antioxidant levels. These reactive oxygen-containing molecules are produced at high levels when immune defenses are breached by increased exposure to bacterial mitogens and endotoxins, and may facilitate disease progression during aging and retrovirus infection.

Pharmacological therapy with agents like zidovudine (AZT), offered in clinical AIDS treatment, have frequently resulted in deleterious side effects. They have shown an inability to improve immune dysfunction initiated by the retroviral infections, and have also proven ineffective against AZT-resist HIV strains. Thus, new co-therapies are needed for the clinical treatment of AIDS. Immunomodulators with low toxicity such as hormones (dehydroepiandrosterone (DHEA) and melatonin (MLT)) and T cell receptor (TCR) peptide may help to normalize immune dysfunction, as well as increased lipid peroxidation and malnutrition, secondary to retrovirus infection. Since immunosenescence is characterized by immunodysfunction similar to that induced by retrovirus infection, these immunomodulators may also play an important role in slowing the aging process.

The hypothesis of this study is that retrovirus infection and aging induce immune

dysfunction, excessive lipid peroxidation, and tissue vitamin E loss. In humans, the decline of DHEA and MLT levels with aging may in part contribute to immunosenescence. Since DHEA and MLT are antioxidant immunomodulators, it has been proposed that supplementation with these hormones may provide beneficial effects on immune function during retrovirus infection and aging, by preventing dysregulated cytokine production and increased lipid peroxidation. To address the therapeutic effects of these hormones, murine models of retrovirus infection and aging were used in this study. These murine models mimic the immune dysfunction and metabolic status changes seen in similarly afflicted humans. The primary objective of this work is to investigate the effects of DHEA and MLT supplementation on the prevention of immune dysfunction and excessive lipid oxidation caused by murine retrovirus infection and/or aging. In addition, the immunomodulatory effect of TCR peptide has also been determined in aged or retrovirally infected mice.

The results of this study demonstrated that DHEA, MLT and TCR peptide treatments significantly prevented the reduced Th1 cytokine secretion while suppressing the elevated Th2 cytokine production during murine retrovirus infection and/or aging. These treatments also inhibited excessive lipid peroxidation and tissue vitamin E loss induced by retrovirus infection.

Most circulating DHEA exists in an immunologically inactive sulfated form (DHEAS) which is not capable of directly diffusing across the cell membranes of most cell types. Appropriate biochemical mechanisms exist that allow tissue-localized desulfation of extracellular DHEAS followed by diffusion of DHEA across the plasma membrane and its

intracellular resulfation in those cell types. DHEAS effects on T cells are compartmentalized in mice to lymphoid organs containing cells having the greatest DHEA sulfatase activity, supporting the concept that DHEA has an important immunomodulatory activity.

DHEA is an important regulator of murine and human T cell responses as well as cytokine production. DHEA has been shown to directly stimulate T cell proliferation and IL-2 production by enhanced transcription of IL-2 gene and stabilization of IL-2 mRNA. The primary immunologic target of DHEA is CD4+ T helper cell population. Stimulation of CD4+ T cells by DHEA leaded to increased IL-2 production with enhanced cytotoxic activity and reduced IL-4 levels. It has been reported that DHEA increased IL-2 production by binding to a specific DHEA binding complex in the cytosol and/or nuclei. DHEA could also increase IL-2 and IFN-γ production but inhibit IL-4 secretion by counteracting the immunosuppressing effects of glucocorticoids (GCS). Glucocorticoid exerts its immunosuppressive effects directly through inhibition of many T cell cytokines, which is mediated by impairing the functions of some nuclear transcriptional factors. Glucocorticoid binds to specific cytosolic receptor heat shock protein 90 (HSP90). This complex translocates into the nucleus and binds to the glucocorticoid response element (GRE) in the 5' regulatory region of some cytokine genes. Occupation of the GRE inhibits nuclear factor of activated T-cells (NFAT) and AP-1 binding. In addition, glucocorticoid treatment of T-cells prevents nuclear factor-κB (NFκB) translocation into the nucleus probably by inducing an increased synthesis of the inhibitor of NFκB (IκB). Thus, by

inhibiting translocation of these transcriptional factors, glucocorticoid blocks gene expression of IL-1, IL-2, IL-3, IL-6, TNF-α, and INF-γ. The antiglucocorticoid properties of DHEA could be explained by the observed down-regulation of glucocorticoid receptors by DHEA (Figure 1). The inhibiting effect of DHEA on IL-6 production in aged mice may be mediated by estrogens which are implicated in the regulation of IL-6 secretion, or by inhibition of corticotrophin releasing factor which stimulates monocytic output of IL-6. In addition, immunomodulatory effects of DHEA might be related to its capacity to function as a natural modulator of peroxisome activities. DHEA, by enhancing peroxisome activity, could normalize fatty acid metabolism and increase antioxidant potential in the cell, therefore to modify lymphoid cell activities.

In addition, DHEA modestly downregulated HIV-1 expression in infected human cells. DHEA inhibited TNFα-induced NFκB activation by inhibiting NADPH-dependent oxidative intermediates, thus preventing HIV-gene expression.

MLT also plays an important immunoregulatory role via both direct and indirect action on immune system. Specific binding sites for MLT have been described on human and rodent thymocytes, splenocytes, bone marrow cells, as well as blood lymphocytes and granulocytes. Human circulating CD4+ T lymphocytes contains specific and high-affinity binding sites for MLT. MLT receptors are coupled to guanine nucleotide binding proteins which modulate guanine nucleotides in human lymphocytes. MLT has been shown to enhance IL-2 and IFN-γ production by modulating the activity of Th1 cells via nuclear receptor-mediated transcriptional control. MLT acts on Th2 cells in mouse inducing the

production of IL-4. Th2 cells are sensitive to IFN-γ, which stimulates MLT production and selectively inhibits Th2 cytokine production. It is also suggested that MLT's immunomodulatory effect is mediated through the hypothalamic-pituitary-adrenal axis, hypothalamic thytropin-releasing hormone, as well as the opioid system. In addition, MLT may restore age-associated immune dysfunction by rejuvenating the zinc pool.

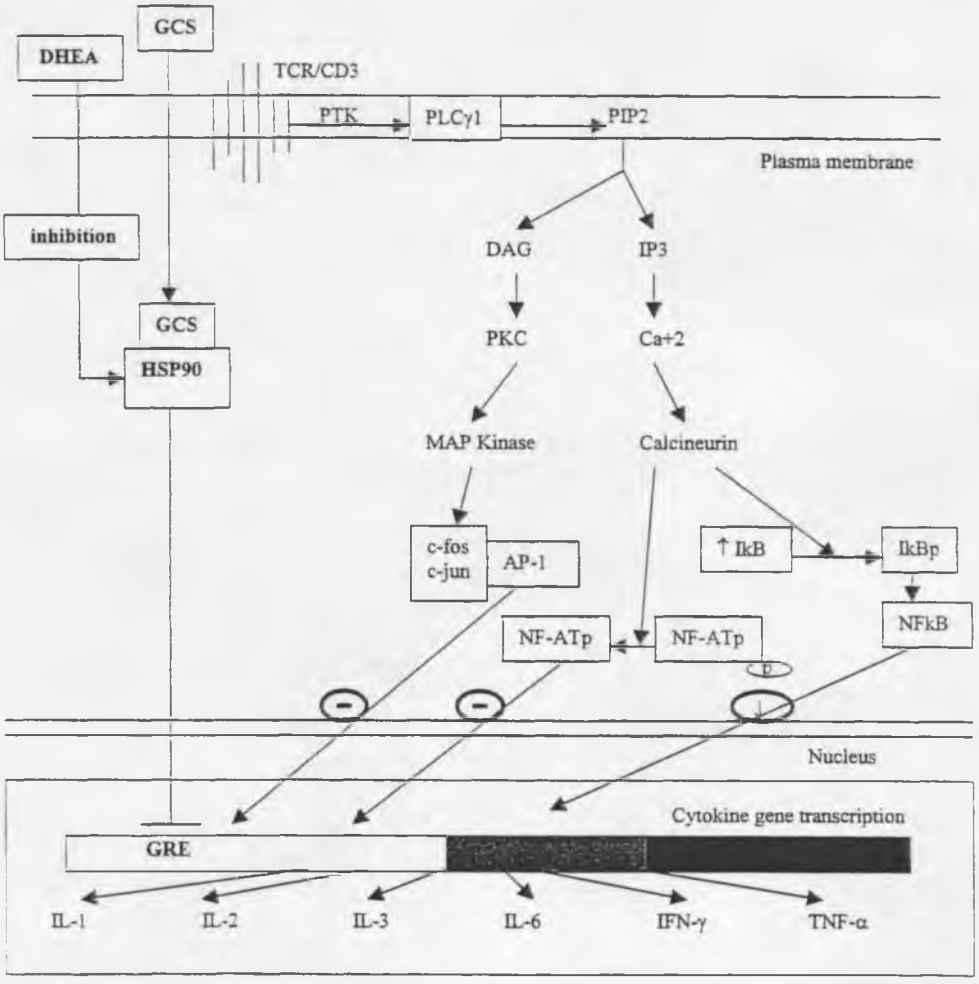
MLT appears to be an effective scavenger of hydroxyl free radicals, as well as being more effective at scavenging peroxyl radicals than vitamin E. Although MLT receptors are present on a variety of cells, MLT being lipid soluble can readily pass membrane without the aid of carrier proteins. This property implies that MLT could have a ubiquitous antioxidant role in the body. Once inside the cell MLT binds calmodulin and scavenges hydroxyl radicals. Additionally, MLT might bind nuclear receptors and ultimately regulate gene expression. MLT regenerate the antioxidant enzyme glutathione peroxidase by supplying $NADPH_2$. Reducing free radicals by MLT 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 cytokine profile. MLT could also inhibit HIV replication by reducing the binding activity of NFκB, suggesting that it may have the potential to be an effective antioxidant in the treatment of AIDS.

Both human and murine AIDS are related to selective activation of some CD4+ T cell via retroviral superantigens. An aberrant stimulation of several subsets of Th2 cells may lead to polyclonal activation of T and B cells at an early stage and subsequent aberrant cytokine production, which eventually leads to profound immune deficiency with

suppressed cell-mediated immunity. Because of the crucial importance of TCR peptides in the initiation of immune responses, autoantibodies to TCR peptides should play an important role in the development of immunological disease. Although the detailed molecular mechanism of TCR peptide treatment remains unknown, two potential mechanisms have been proposed for prevention of immune dysfunction during murine retrovirus infection (Figure 2 and 3). Firstly, TCR peptides corresponding to the CDR1 and Fr2 segments of the Vβ chain may interact with MHC molecules, altering the antigen presentation process. Secondly, autoantibodies against TCR Vβ induced by TCR Vβ peptide treatment may slow the selective expansion of T cell clones by obstructed binding of the antigen to TCR Vβ chain. It has been reported that infected mice produced high levels of autoantibodies against two human TCR peptides to suppress T cells bearing homologous murine TCR Vβ peptides. This study tested whether treatment with synthetic peptides based on different human TCR Vβ CDR1 sequences could prevent immunodysfunction during murine retrovirus infection or aging. It was hoped that the use of a human TCR peptide, which is essentially homologous to the murine one, would facilitate its development for effective and safe use in humans.

In summary, our results demonstrated that DHEA, MLT and TCR peptide treatments could prevent immunodysfunction and excessive lipid peroxidation induced by retrovirus infection and aging. However, the precise cellular and molecular mechanisms responsible for the immunoenhancing properties of DHEA, MLT and TCR peptides are still not well-defined. The findings of this study would be useful in gauging the need for

further studies aimed at completely understanding the mechanisms of the immunomodulatory effect of DHEA, MLT or TCR peptide on aged and/or AIDS patients, as well as the possible effectiveness of using these immunomodulators in patients with immunodeficient status.



– inhibit binding of AP-1and NFAT to cytokine genes by GCS-HSP90
↑ increase IkB synthesis by GCS
↓ inhibit NFkB translocation to nucleus by reducing its release from IkB

Figure 1. Possible mechanism of DHEA treatment for preventing glucocorticoids (GCS)-induced inhibition of T cell cytokine gene expression

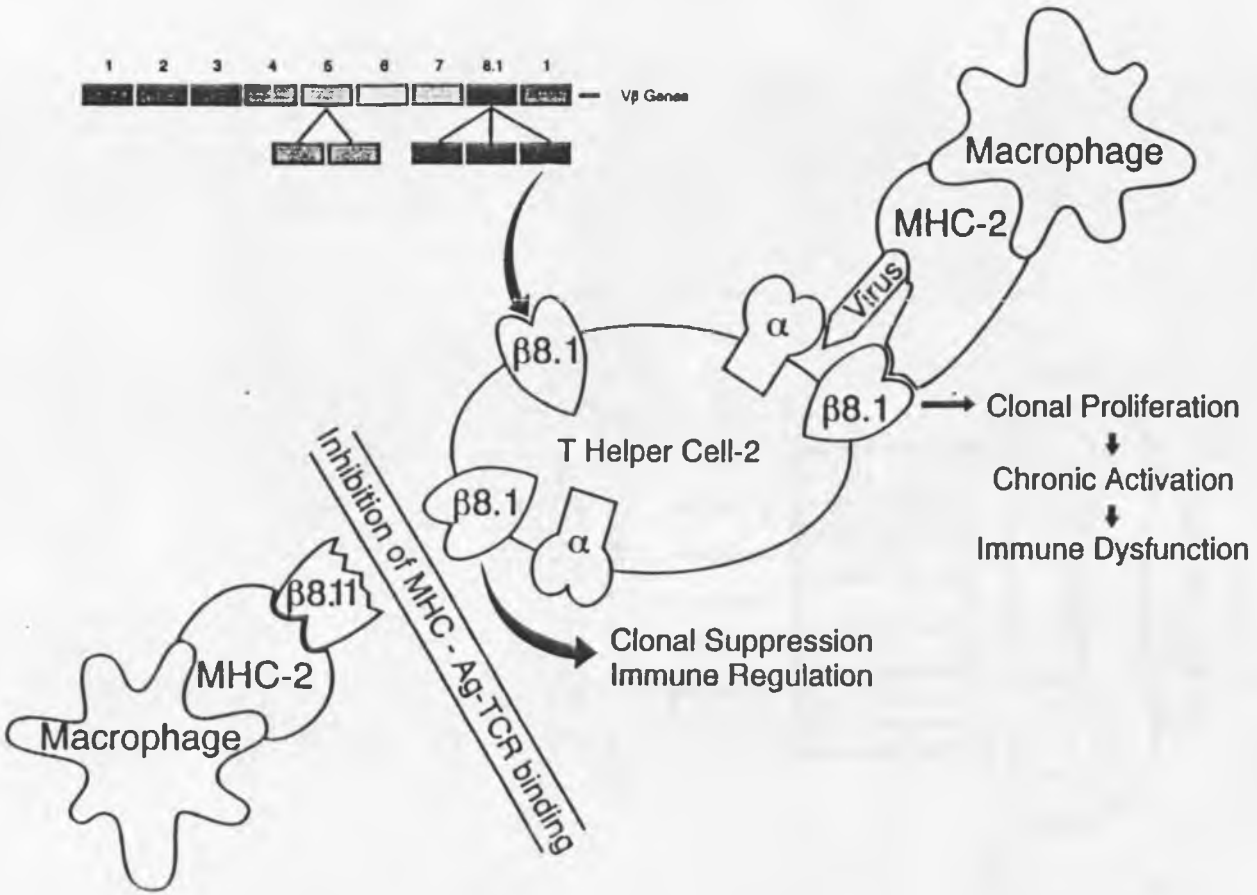


Figure 2. Possible mechanism of TCR Vβ8.1 peptide treatment for preventing immune dysfunction in murine AIDS

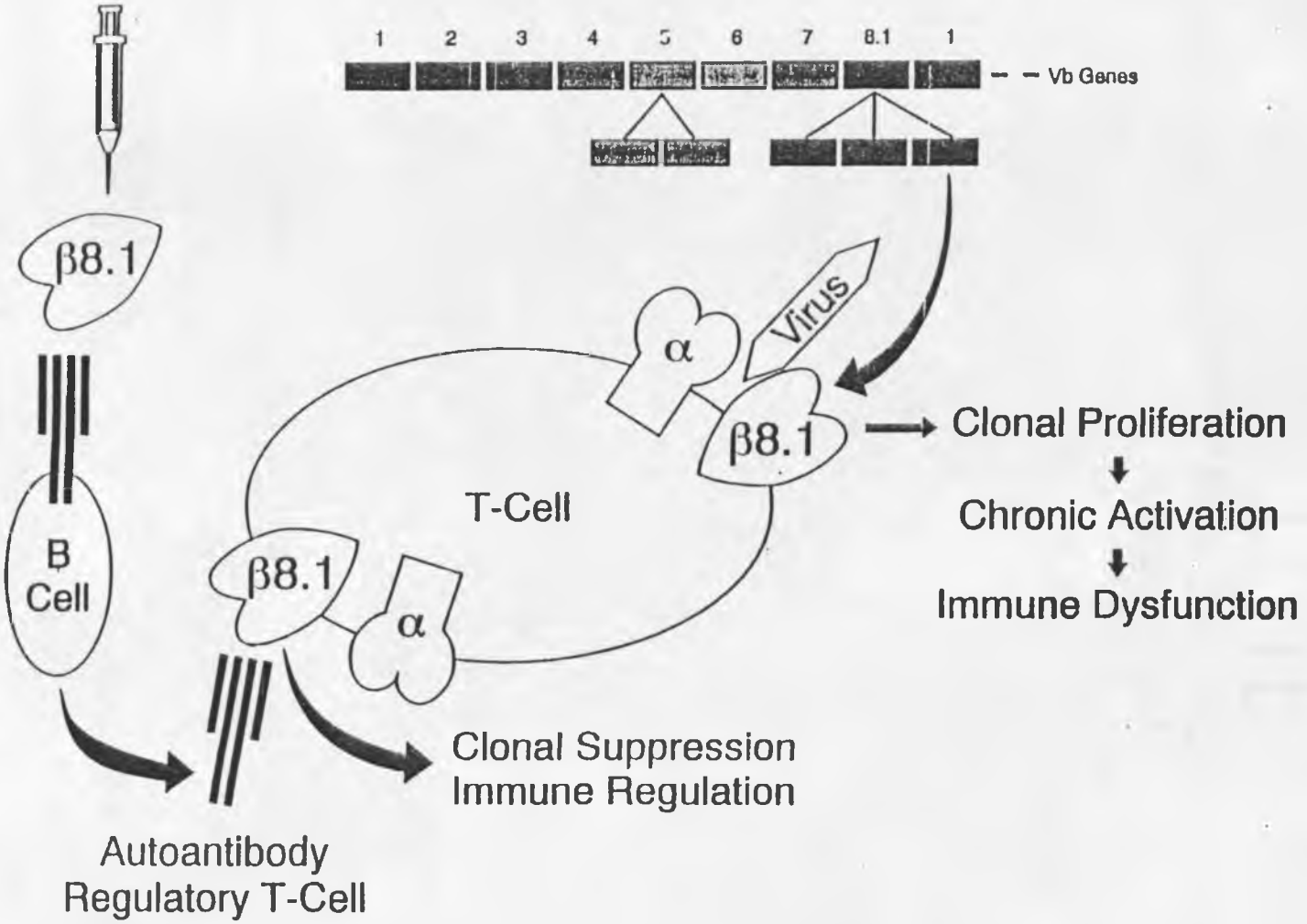


Figure 3. Possible mechanism of TCR Vβ8.1 peptide treatment for preventing immune dysfunction in murine AIDS

LITERATURE REVIEW

I am the first author of the three review papers which are attached in the appendices of this dissertation. Among them, the first one (DHEA in immune modulation and aging) has been accepted in the book: DHEA: Health Promotion & Aging. The second one (Melatonin, immune modulation and aging) has been published in Autoimmunity 26; 43-53, 1997. The third one (Antioxidants and AIDS) is a chapter of the book: Nutrients and Food in AIDS.

FORMAT EXPLANATION

1. Relationship of research papers

Six original research papers I am involved in are used in my dissertation. Paper 1 investigated the effects of dietary DHEA supplementation on cytokine dysregulation and increased lipid oxidation in young female C57BL/6 mice infected with murine leukemia retrovirus. While Paper 2 studied the influences of dietary supplementation with DHEA and MLT on immune function and lipid peroxidation in retrovirus-infected young mice, Paper 3 studied the same issues with respect to cytokine production in aged mice.

The immunomodulatory effect of TCR peptide treatment in retrovirally infected young or aged mice was studied on Papers 4 and 5. Paper 4 tested whether TCR Vβ peptide injection could prevent retrovirus-induced immune dysfunction, excessive lipid peroxidation, and tissue vitamin E deficiency in young mice. The modulatory action of TCR Vβ peptide on immunosenescence was considered in Paper 5.

In addition, Paper 6 demonstrated the preventive effects of vitamin E supplementation on smoking-induced lung damage and tissue lipid peroxidation in immunodeficient (Nude) mice.

2. Contribution to each research paper

I am the first author of Paper 2 (accepted in Immunology) and Paper 6 (accepted in Nutrition Research). In Paper 1, I was in charge of designing the experiment, feeding and infecting mice, performing all the assays, analyzing data, and writing the paper. In

Paper 6, I was responsible for tissue vitamin E measurement by HPLC, data analysis, and manuscript preparation.

I was the second author of the other four papers which have been published in different journals. In these papers, I measured lymphocyte proliferation, cytokine production by splenocytes, as well as tissue vitamin E levels. In addition, I assisted my colleagues in sacrificing mice, taking serum, isolating and culturing splenocytes, as well as measuring lipid profiles and lipid peroxidation products.

CHAPTER II

PRESENT STUDY

SUMMARY OF IMPORTANT FINDINGS

The methods, results, and conclusions of this study are presented in the research papers appended to this dissertation. The following is a summary of the most important findings in these papers.

Overall summary: Young (4-8 weeks old) or aged (15-16 months old) female C57BL/6 mice infected with murine leukaemia retrovirus developed murine AIDS. The aim of this study was to investigate the effects of DHEA, MLT, and TCR peptide treatments on immune function and lipid peroxidation in retrovirus-infected and/or aged mice. Each research paper listed in the appendices illustrated different aspects of this study.

Paper 1. The effects of different doses of DHEA supplementation on cytokine dysregulation and increased oxidation were studied in young mice during retrovirus infection. Feeding mice with diets supplemented with 0.02% DHEA (0.9 mg/mouse/day) or 0.06% DHEA (2.7 mg/mouse/day) for 10 weeks largely prevented retrovirus-induced suppression of Th1 cytokine (IL-2, IFN-γ) secretion, and the stimulation of Th2 cytokine (IL-6) and TNF-α production. It also prevented increased lipid peroxidation, and loss of hepatic vitamin E. Similar changes in immune function and tissue vitamin E levels also occurred in uninfected young mice treated with DHEA. The modulatory effects of DHEA on immune function and lipid peroxidation were dose-related.

Paper 2. The effects of DHEA and MLT supplementation on immune function and lipid peroxidation were investigated in retrovirally infected young mice. Dietary

treatment with DHEA or MLT alone, as well as together, for 12 weeks largely prevented the retrovirus-induced reduction of B and T cell proliferation, suppressed production of Th1 cytokine (IL-2, IFN-γ), and elevated secretion of Th2 cytokine (IL-4, IL-6, IL-10) and TNF-α. DHEA and MLT treatment also inhibited excessive lipid peroxidation and loss of hepatic vitamin E. The use of DHEA plus MLT was more effective in preventing retrovirus-induced immune dysfunction than either DHAE or MLT alone. Similarly, hormone supplementation also increased immune function and tissue vitamin E levels in uninfected mice.

Paper 3. The modulatory effect on cytokine production by DHEA plus MLT supplementation was determined in aged mice as compared against young mice. Old mice were fed with DHEA, MLT, or DHEA+MLT for 12 weeks. As expected, the number of spleen cells was significantly higher in old mice than that in young mice. MLT and DHEA+MLT significantly increased B cell proliferation in old mice. DHEA, MLT, and DHEA+MLT helped to modify cytokine production in old mice by significantly increasing Th1 cytokines (IL-2, IFN-γ) or decreasing Th2 cytokines (IL-6, IL-10). Although DHEA or MLT alone effectively modulated suppressed Th1 cytokine and elevated Th2 cytokine production, their combined use produced only a limited additive effect during aging.

Paper 4. This paper tested whether TCR peptide treatment would prevent murine retrovirus-induced immune dysfunction, excessive lipid peroxidation, and malnutrition. Young mice were infected with LP-BM5 retrovirus. Two weeks after infection, TCR

peptides Vβ5.2, Vβ8.1, Vβ5.2+ 8.1, Vβ8.1(N), Vβ8.1(C) were injected to these mice at a dose of 200 μg/mouse. Vβ5.2 and Vβ8.1 treatments largely maintained lymphocyte proliferation and Th1 cytokine (IL-2, INF-λ) release, and prevented excessive Th2 cytokine (IL-6, IL-10) and TNF-α secretion in retrovirus-infected mice. These treatments also normalized hepatic and cardiac lipid profiles, reduced tissue lipid peroxidation, and thereby significantly maintained tissue vitamin E during retroviral infection. However, V β8.1 segment treatment did not prevent the immune dysfunction and oxidative damage caused by the retrovirus infection. These data suggest that intact TCR peptide injection during murine retrovirus infection largely prevents immune dysfunction by blocking the excessive stimulation of Th2 cell caused by retroviral superantigens. In addition, TCR peptide treatment also ameliorated malnutrition status by preventing excessive lipid peroxidation as well as tissue vitamin E loss.

Paper 5. It has been reported that retrovirally infected young mice may produce a high level of autoantibodies against certain TCR peptides to diminish retrovirus-induced immune abnormalities. Since autoantibodies to these peptides are also increased in uninfected old mice, TCR peptide treatment may reduce immunosenescence. This paper determined the effectiveness of TCR peptides in preventing aging-related immune dysfunction. Young and aged mice were injected with synthetic human TCR Vβ5.2 or V β8.1 peptides. Administration of these autoantigenic peptides to old mice prevented age-related reduction in splenocyte proliferation and IL-2 secretion while retarding the excessive production of IL-4, IL-6 and TNF-α induced by aging. Thus, immune

dysfunction and abnormal cytokine production, induced by the aging process, may be largely prevented by injection of selected TCR Vβ CDR1 peptides.

Paper 6. Sidestream cigarette smoke (SSCS) is a major component of environmental tobacco smoke. The purpose of this study was to investigate the preventive effects of vitamin E supplementation on the lung injury and tissue lipid peroxidation of immunodeficient (Nude) mice exposed to SSCS. A small amount of SSCS exposure increased pulmonary resistance and lipid peroxidation in the lung and liver of these mice. Dietary vitamin E supplementation increased vitamin E levels in lung and liver. In addition, supplementation attenuated SSCS-mediated pulmonary injury and lipid peroxidation. The enhanced resistance against SSCS-induced lung injury and lipid peroxidation may relate to the increased antioxidant capacity in vitamin E-supplemented mice.

APPENDIX A:

LITERATURE REVIEW

DHEA in immune modulation and aging

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Abstract

Dehydroepiandrosterone (DHEA) is a major adrenal cortical steroid in humans. Aging in human is associated with a decline in DHEA production. Low DHEA levels occur concomitantly with the development of a number of the problems of aging: immunosenescence, increased infectious diseases, increased incidence of several cancers, atherosclerosis, diabetes mellitus, and osteoporosis. Numerous animal data have showed that DHEA administration has protective effects against immunosenescence and other aging-related problems. In addition, these beneficial effects have also been demonstrated in some studies of DHEA replacement in elderly people. This paper reviewed the role and possible mechanisms of DHEA in aging-associated immune function.

Keywords : Dehydroepiandrosterone (DHEA), Dehydroepiandrosterone sulfate (DHEAS), Immunoregulation, Aging

Dehydroepiandrosterone (DHEA) is made in large quantities as the sulfated form (DHEAS) by the adrenal cortex only in primates and a few nonprimates species. In humans, DHEA is the most abundant circulating adrenal steroid. The decline in DHEA production is parallel to the development of immunosenescence and other aging-related diseases. Although some animal and human studies have indicated beneficial effects of DHEA supplementation on immune function, others did not. The precise biological functions of DHEA are still not known. In order to understand the role of DHEA in aging process, DHEA's effect on immunosenescence and its relationship to other aging-related physiological dysfunction are reviewed here.

Biochemical endocrinology of DHEA

DHEA is quantitatively one of the major adrenal cortical hormones synthesized from cholesterol in humans. Before being secreted into the plasm by the adrenals, most synthesized DHEA is efficiently sulfated to DHEA-3β-sulfate (DHEAS). DHEAS is the dominant species of steroid in the human plasma. More than 99% of circulating DHEA is in the form of DHEAS, which functions as an inactive reservoir. DHEAS has a half-life of 7-10 hours with a metabolic clearance rate (MCR) of 5-20 L/day. In contrast, DHEA has a relatively short half-life of 15-30 minutes due in large part to its higher MCR of approximately 2,000 L/day and its extensive conversion to the sulfate form. Most DHEA is produced in the adrenal cortex which produces 15 mg DHEAS per day, while the testes

and ovaries account for very little DHEAS entering the circulation (Migeon et al 1957). Free DHEA and DHEAS are metabolically interconvertible by sulfotransferase for conjugation and sulfatase for hydrolysis in many tissues.

Physiologically, DHEA concentration in the blood oscillates coincidentally with cortisol, consistently with the response of adrenal DHAE secretion to adrenocorticotrophic hormone (ACTH) during circadian and stress-related activation, but there is no feed-back control of DHEA secretion at the hypothalamo-pituitary (Baulieu 1996). DHEAS synthesis is also stimulated by ACTH, but because it's low MCR, its concentration remains at approximately the same 24 hours a day under normal circumstances. In the blood, neither DHEA nor DHEAS bind significantly to sex steroid binding protein, and the binding of DHEAS to albumin seems nonspecific. DHEAS is water-soluble and is easily transported by attaching to albumin.

DHEA and DHEAS are themselves less biologically active than other androgen (Herbert 1995). Secreted DHEA and DHEAS are metabolized (Figure 1 and Figure 2) by a number of enzymes widely distributed in the body. Besides the DHEA/DHEAS interconversion, there is the formation of active androgens, such as testosterone (T) and 5α-dihydrotestosterone (DHT) and ρ4-androstenedione (ρ4-A). T and ρ4-A can themselves be aromatized to produce estrogens. In addition, DHEA may be transformed to the weakly estrogenic ρ5-antrostene-3β, 17β-diol, and other derivatives of yet poorly defined function. The metabolism of DHEA into potentially active sex steroids may occur

in many cells containing androgen or estrogen receptors (Baulieu 1996); this is the case for adipose tissue, bone, muscle, breast, prostate, skin, brain etc, particularly the liver, where it is quantitatively important and from which part of these active DHEA metabolites may be released into the circulation and thus reach target tissues (Figure 2).

When DHEA is administered, orally or parenterally, metabolites are formed in different proportions (Baulieu 1996). After oral administration, DHEA is largely absorbed and converted to DHEAS in the hepato-splanchnic system, and the blood concentration of the latter increases rapidly (Longcope 1995). Orally administrated DHEAS could be largely hydrolyzed in the acidic medium of the stomach.

DHEA and aging

DHEA production is directly age-related. There is an increased production of DHEA at puberty, and the maximum blood concentration is reached during the third decade of life. Then it declines gradually, leaving a residual value of 15-20% of the maximum during the 8-9th decade of life (Hopper et al 1975). The blood levels are 2-4μg/L (~10 nmol/L) for DHEA and 2-6 mg/L (~10 μmol/L) for DHEAS in young adults (25-35 years old), approximately 10-20% more in men than in women. In healthy individuals aged 60-90 years old, the DHEAS concentration is 0.3-2 mg/L within the 80-85% confidence limits.

Since basal levels of DHEA and DHEAS in the human decrease with age, the adrenal gland and its hormones have been assumed to be involved in the aging process. This decrease is not the result of a metabolic change of DHEA or DHEAS, but of a decreased adrenal secretory rate. Challenge tests with ACTH indicate that in elderly subjects the DHEA or DHEAS response is decreased, unlike that of cortisol secretion, which is maintained constant (Baulieu 1996). This is because there is apparently, but unexplained, defect in the desmolase activity of the cytochrome P450c17 in the adrenal reticularis zona. The increase of the cortisol/DHEA(S) ration in the blood is possibly responsible for the immuno-deficiency and other metabolic changes which develop during aging.

DHEA and immunomodulation in the aged

Aging and immune function

Aging is associated with a decline in immune function that leads to an increased incidence of infection, cancer, and autoimmune disease (Ershler 1993). Age-related changes in immunity primarily involve alterations in T cell function, including a decreased proliferation response of T cells to mitogens (Staino-Coico et al 1994, Miller 1994), and dysregulation of cytokine production. In addition, a number of alterations in B cell function are also observed in aging, which may result primarily from changed regulation, e.g. by T cells (Currie 1992). There is an increased likelihood of autoantibody

production and the emergence of monoclonal antibodies and other lymphoproliferative disorders.

Immunosenescence can be significantly viewed as dysregulation in cytokine production, which is implicated in alteration the function of the immune system and many other organ systems. T helper type 1 (Th1) cells produce IL-2 and IFN-γ, whereas Th2 cells produce IL-4, IL-5, IL-6 and IL-10. Th1 cytokines are primarily involved in activating cellular immune defenses. IL-2 is vital for the proliferation of activated T cells, while IFN-γ is needed to activate macrophages involved in pathogen destruction. In animal models, suppression of Th1 cytokine production explains the loss of disease resistance that occurs with aging and in diseases such as AIDS (Figure 3) (Watson et al 1996). In elderly individuals IL-2 production and expression of IL-2 receptor (IL-2R) (Currie 1992) are also diminished, whereas IFN-γ secretion may be increased as in some animal studies (Daynes et al 1992). Both unstimulated and mitogen-stimulated cells from aged mice produce large amounts of IL-6 and IL-10 (Watson et al 1996). The dysregulated IL-6 production is so great that the IL-6 presence can readily be detected in the plasma of aged animals (Daynes et al 1993). Basal IL-6 production is also increased in normal aging people, whereas stimulated IL-6 secretion is either not affected (Daynes et al 1993) or reduced (Effros et al 1991).

IL-6 is a pleiotropic cytokine that plays an important role in the generation of the acute

phase and inflammatory responses. In the immune system, it is involved in T-cell activation, growth, and differentiation as well as in B-cell proliferation and maturation (Hirano 1994). The regulation of IL-6 gene expression is complex, and usually little or no IL-6 is secreted spontaneously. With age, however, this tight regulation seems to be relaxed, and measurable levels of IL-6 appear in the plasma without stimulus. Aberrant outcomes, such as a persistent acute phase response, continual nonspecific B-cell proliferation, or enhanced osteoclast activity, represent some of the pathologic conditions related with aging (Hirano 1994).

IL-10 appears to be constitutively expressed in old age and may therefore be continuously exerting its influences on all IL-10 responsive cell types (Spencer et al 1996). Many age-associated changes in B cell, T cell, macrophage functions that define the immunosenescent phenotype (Thoman et al 1989, Miller 1991) may be closely linked to dysregulated control over endogenous IL-10 production. IL-10 is produced by activated various lymphoid cells, particularly CD5+ B cell, numbers of which are elevated with advancing age (Booker et al 1992, Brohee et al 1991) and also implicated in the production of autoantibodies in the elderly (Stall et al 1992).

These Th2 cytokines can inhibit Th1 cytokine production and have major roles in stimulating B cells to mature and produce immunoglobulins. As shown in Figure 3, their actions facilitate immunosuppression and growth of leukaemia and autoantibodies, which are common in older individuals. Altered B cell function with aging is also evidenced by

a decreased ability to generate antibodies to antigens and decreased effectiveness of vaccines to confer immunity. In contrast to T cells and B cells, the number and activity of NK cells have been reported to either increase (Lighthard et al 1986, Krishnaraj et al 1987) or remain the same (Nagel et al 1981) in the aging population. The role of the immune system in degenerative processes, and susceptibility to malignant processes needs further investigation.

DHEA and immunosenescence

In aged mice and in retrovirally infected mice developing leukemia and murine AIDS, DHEA or DHEAS supplementation reduced excessive Th2 cytokine production and augmented Th1 cytokine secretion (Araghiniknam et al 1997a). *In vitro* studies have shown that DHEA exerted a stimulatory effect on IL-2 secretion, inhibited NK cell differentiation (Risdon et al 1991), and prevented the age-related IL-6 production (Daynes et al 1993). The relevance of these findings to human biology and diseases is perplexing, because adrenal production of DHEA or DHEAS in these experimental animals is either minute or does not exist; humans and nonhuman primates are the only species with the capacity to synthesize and secrete these adrenal androgens in quantities surpassing all other known steroids (Cutler 1978, van Weerden 1992, Roth et al 1993).

Human studies have demonstrated the beneficial effects of DHEA on immune function. However, epidemiological data examining DHEA effects are still limited. DHEA has

been shown to increase IL-2 production and cytotoxic effector function in human T cells (Suzuki et al 1991) and inhibit Epstein-Barr virus-induced morphologic transformation and stimulation of DNA synthesis in human lymphocytes *in vitro* (Henderson et al 1981). Administration of DHEA (200mg/day orally) for 3-6 months to patients with systemic lupus erythematosus reduced their corticosteroid requirements (van Vollenhoven et al 1994). In postmenopausal women DHEA treatment with a 50 mg daily oral dose for 3 weeks (Casson et al 1993) increased NK cell number and its cytotoxicity. However, T cell mitogenic and IL-6 responses were inhibited. In another study, replacement of oral DHEA (50 mg/day) for 5 months in elderly men significantly activated immune function. DHEA administration increased responsiveness to T- and B-mitogens, and increased number of cells expressing IL-2R. *In vitro* mitogen-stimulated release of IL-2 and IL-6 was enhanced without basal secretion being affected. Also there was a significant increase in the number of both monocyte and NK cell and the cytotoxicity of NK cell. Activation of immune system appeared to require a latency of several weeks and occurred within 2-20 weeks of DHEA treatment. It was proposed that the immunoregulatory activity of DHEA might be mediated by increasing the bioavailable growth hormone-insulin-like growth factor I (GHIGF-I) levels and/or the ratio of DHEA to glucocorticoids which are immunosuppressive (Khorram et al 1997). GHIGF-I system is recognized to promote cellular growth as well as metabolism at multiple levels (Corpas et al 1993, Rudman et al 1990), and to modulate the immune system in health and disease (Miller 1991, Gaillard 1994, Reichlin 1993, Ader et al 1995). The significance of these findings in terms of the ability to mount an immune response to a foreign antigen has not yet been determined.

The gender differences in immune response to DHEA recapitulate the importance of sex steroid milieu in modulating the action of DHEA to target cells.

Mechanism of immunoregulation by DHEA

Most DHEA in the circulation exists primarily in an immunologically inactive sulfated form (DHEAS) which is not capable of directly diffusing across the cell membranes of most cell types. Appropriate biochemical mechanisms exist that allow tissue-localized desulfation of extracellular DHEAS followed by diffusion of DHEA across the plasma membrane and its intracellular resulfation in those cell types (Falany et al 1989). The mechanism responsible for the immune-enhancing properties of DHEA is currently unknown. It is likely that DHEA response is mediated through binding to a specific receptor complex present in the cytosol and/or nuclei of lymphocytes (Kalimi et al 1990, Meikle et al 1992). DHEA may also modulate other steroid hormone receptors such as that of estrogens, androgens, or glucocorticoids. Alternatively, the effects of DHEA may be mediated by the elevation of GHIGF-I levels and/or by the reverse of the glucocorticosteroid-induced inhibition of immune function. The antiglucocorticoid properties of DHEA can be explained by the observed down-regulation of glucocorticoid receptors by DHEA (Kalimi et al 1990). In addition, the immunomodulatory effects of DHEA might be related to its antioxidant activity (Araghiniknam et al 1997b) and its capacity to function as a natural modulator of peroxisome activities (Spencer et al 1995).

DHEA is a mediator of murine (Daynes et al 1990a) and human (Suzuki et al 1991) T cell responses. DHEA has been shown to directly stimulate T cell proliferation and IL-2 production by enhanced transcription of IL-2 gene and stabilization of IL-2 mRNA. DHEAS effects on T cells are compartmentalized in mice to lymphoid organs containing cells having the greatest DHEA sulfatase activity (Daynes et al 1990b), supporting the concept that DHEA has an important immunomodulatory activity. The primary immunologic target of DHEA is CD4+ T helper cell population. Stimulation of CD4+ T cells by DHEA leaded to increased IL-2 production with enhanced cytotoxic activity and reduced IL-4 levels (Suzuki et al 1991). DHEA could also increase IL-2 and IFN-γ production but inhibit IL-4 secretion by counteracting the immunosuppressing effects of glucocorticoids (Daynes et al 1990c, Suzuki et al 1991, Daynes et al 1990b). The maintenance of IFN-γ production by DHEA may contribute to some of its *in vivo* antiviral activity (Daynes et al 1990b). It was reported that s.c. injection of DHEA antagonizes dexamethasone suppression of B and T cell blastogenesis as well as dexamethasone-mediated thymus and spleen atrophy (Blauer et al 1991). In aged mice, the reduced regulation of IL-6 production could be effectively prevented and/or reversed by DHEAS supplementation (Daynes et al 1993). The inhibiting effect of DHEA on IL-6 production may be mediated by estrogens which were implicated in the regulation of IL-6 secretion (Girosole et al 1992), or by inhibition of corticotrophin releasing factor which stimulated monocytic output of IL-6 (Lev et al 1992).

DHEAS is a natural regulator of peroxisome activity (Spencer et al 1995), which may

affect immune function by modulating oxidant production. Peroxisomes are involved in numerous intracellular processes including antioxidant activities and β-oxidative metabolism of very long chain fatty acid. Aged rats have reduced numbers of peroxisomes and reduced peroxisomal β-oxidation activity (Beier et al 1993), which may be responsible for in the increase in long chain fatty acids of plasma membrane. The elevation in phospholipid content of linoleic acid (18:2) results in accumulation in membrane arachidonic acid (20:4) which is readily converted into prostaglandins and leukotrienes (Goppelt-Struebe et al 1986). Prostaglandins are modulators of lymphocyte proliferative responses and lymphokine production, whereas leukotrienes are mediators of the inflammatory response (Goodwin et al 1977). Modifications of cellular phospholipid fatty acid composition can also result in alterations in membrane fluidity and capacity of phospholipid metabolites to serve in signal transduction processes. T cells obtained from aged rodents possesses a depression in proliferation and a reduced capacity to produce and respond to IL-2 following mitogenic activation, which is probably due to a decreased membrane fluidity and a defect in the generation of specific second messengers (Soyland et al 1993, Calder et al 1991, Proust et al 1987). DHEA, via its capacity to enhance peroxisome activity in aged tissues, would lead to normalization of fatty acid metabolism and an increase in antioxidant potential, therefore to modify lymphoid cell activities. Thus, reverse of immunosenescence by DHEA supplementation may be linked to the ability of this steroid to act as a modulator of peroxisomal activities.

DHEA and aging problems that may be associated with immunosenescence

DHEA replacement in aged mice significantly normalized immunosenescence, suggesting that this hormone plays a key role in aging and immune regulation. For most cancers in the elderly, especially at early stages of growth when immune defenses are important, low DHEA levels would be expected to permit accelerated tumor cell growth. Since maintenance of DHEA levels is required for normal cellular immunity, there should be greater cancer immune defense and lower cancer incidence in people with normal DHEA levels. DHEA blocked spontaneous and carcinogen-induced tumors in rodents (Schwartz et al 1986), and animal studies have suggested that anti-carcinogenic effect of DHEA is potentially via inhibition of cell growth and glucose-6-phosphate dehydrogenase (G6PDH) activity (Gordon et al 1987). Low serum DHEA levels are associated with breast cancer in premenopausal women (Zumoff et al 1981, Helzlsouer et al 1992). It has been proposed that DHEA may directly, or via conversion to androstanediol, which has antiestrogenic properties, inhibit breast cancer.

Aging process is associated with increased rates of cardiovascular diseases, diabetes and osteoporosis, which perhaps promote by Th2 cytokine-mediated inflammation. DHEA modulates energy metabolism, low levels should affect lipogenesis and gluconeogenesis, increasing the risk of heart disease and diabetes mellitus. DHEA inhibits G6PDH in the pentose-6-phosphate cycle, which reduces NADPH activity in tissues. NADPH is involved in cell division and syntheses of fatty acids, cholesterol,

phospholipids and steroids (Gordon et al 1987, Lopez et al 1967). Thus, DHEA may indeed prevent atherosclerosis and hypercholesterolaemia by inhibiting NADH activity. Low serum DHEAS levels are connected with increased cardiovascular morbidity in men (Barrett-Connor et al 1986). It has been reported that high levels of plasma DHEA inhibited atherosclerosis in rabbits (Gordon et al 1988).

DHEA increases insulin responsiveness by increasing tissue sensitivity to it (Casson PR et al 1995). There is an inverse correlation between serum insulin and DHEAS levels. Serum DHEAS is reduced in man with insulin resistance and hyperinsulinaemia (Nestler 1995, Nestler et al 1994), and insulin may lower DHEA and DHEAS levels in men and in women (Nestler et al 1989). Low DHEAS levels may indicate undetected, early insulin sensitivity problems. Although the beneficial effects of DHEA and DHEAS on diabetes mellitus during the aging process have been extensively studied, the mechanisms of their actions remain poorly understood.

Low levels of DHEA are also associated with bone loss in older women (Taelman et al 1989). DHEA may be converted by bone osteocytes into estrogen, affecting bone resorption (Nawata et al 1995). In addition, a metabolite of DHEA and DHEAS, 4-androstene-3β, 17β-diol, has an affinity for estrogen receptors and may act as an antiresorptive agent, as dose estrogen (Vermeulen et al 1986). Thus, DHEA may act to help increase calcium absorption in osteoporosis and play a role in conservation of bone mass in the absence of ovarian estrogens.

Summary

DHEA is a multi-functional steroid with immune and metabolic effects. Since aging is associated with a decline in DHEA secretion and DHEA may have potential beneficial effects as reviewed in this paper, its replacement in elderly people may prevent or even reverse some age-associated diseases (immunosenescence, cancer, heart disease, etc.). Although mounting evidence suggests that DHEA may have a broad range of clinical uses, the long-term effects of this steroid are unknown. Since the evidence supporting DHEA as an anti-aging therapy is still sparse, the use of DHEA supplementation remains controversial until it has been thoroughly studied for possible deleterious side effects.

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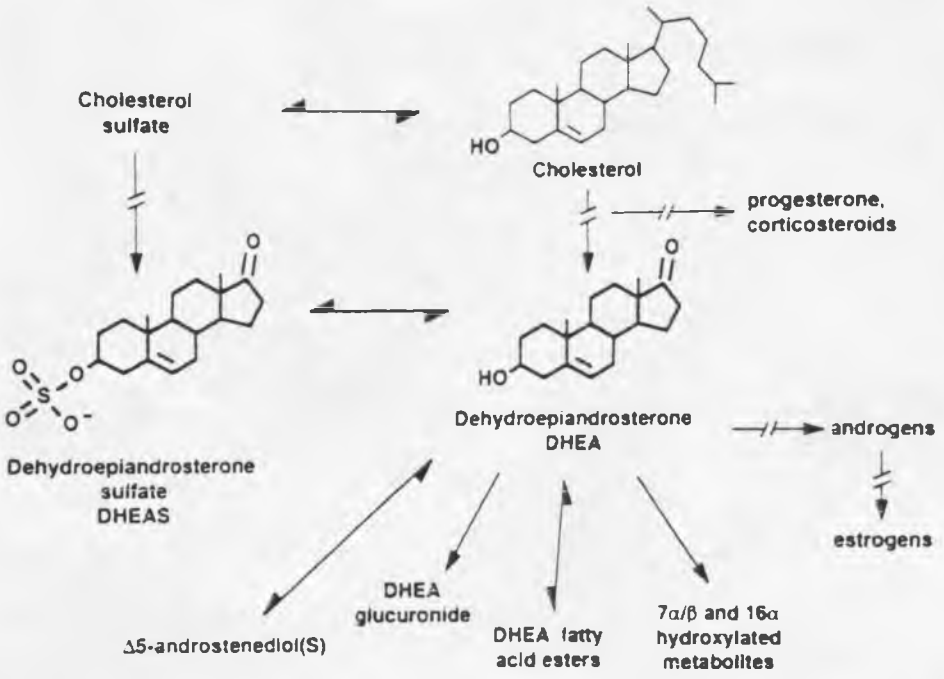
Figure legend

Figure 1. DHEA(S) METABOLISM

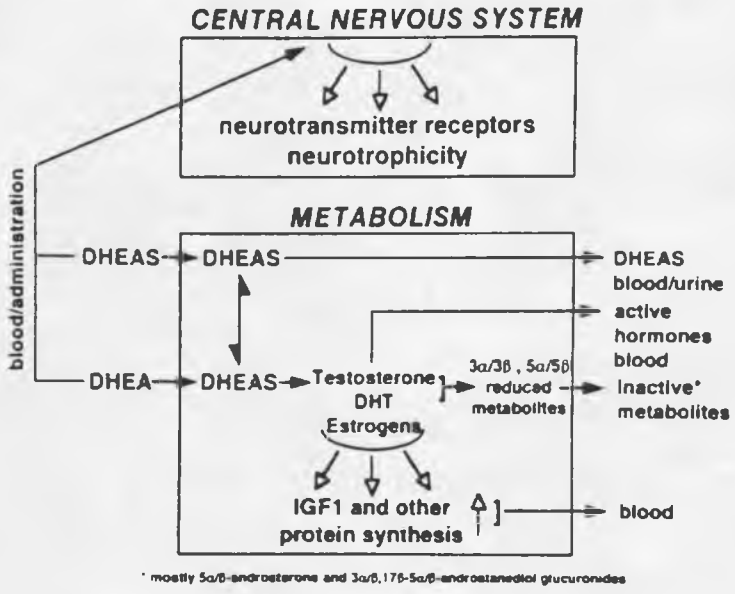
Figure 2. DHEA(S): POSSIBLE MECHANISMS OF ACTION

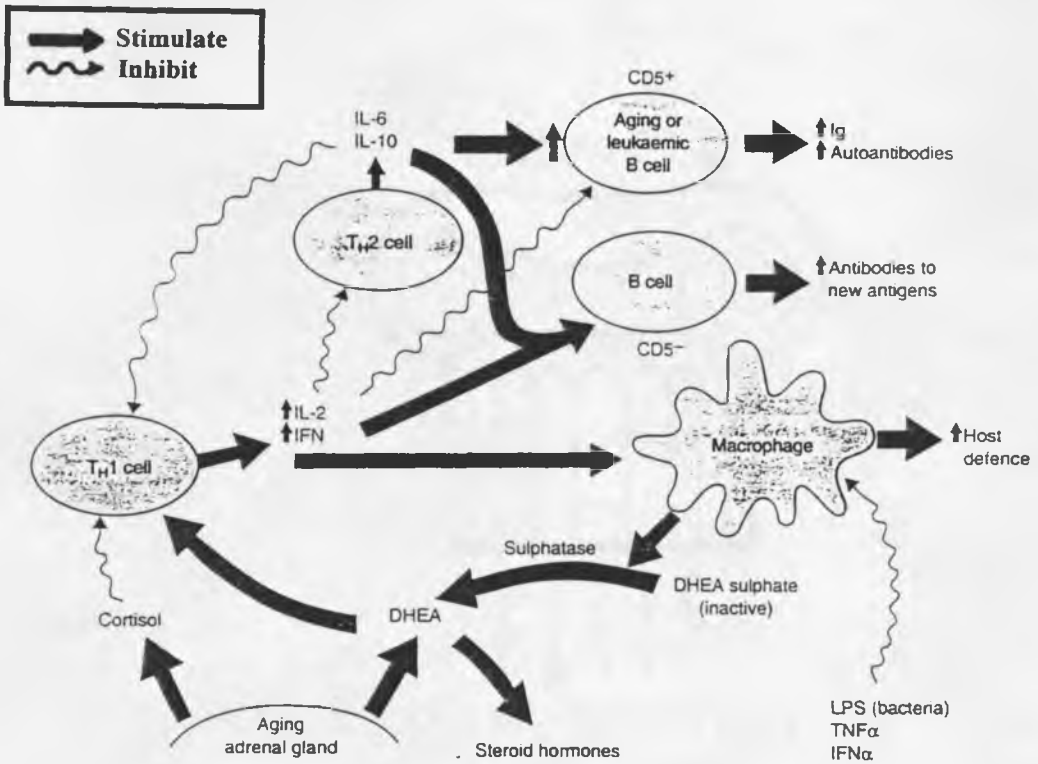
Figure 3. MECHANISMS OF IMMUNOMODULATION BY DHEA IN THE AGED AND PATIENTS WITH LEUKEMIA. The working hypothesis is that: (I) hormones (DHEA, cortisol, and testosterone) influence the immune system; and (II) DHEA deficiency leads to increase IL-6 production, which in turn stimulates malignant or aging B cell to proliferate and secrete greater quantities of autoantibodies. In addition, the increased levels of IL-6 suppress the activity of Th1 cells, which is necessary to stimulate B cells to produce immunoglobins (Ig).

DHEA(S) METABOLISM



DHEA(S) : POSSIBLE MECHANISMS OF ACTION




Stimulate
Inhibit
CD5+
IL-6
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Aging or leukaemic B cell
↑Ig
↑Autoantibodies
TH2 cell
B cell
↑Antibodies to new antigens
CD5−
↑IL-2
↑IFN
TH1 cell
Macrophage
↑Host defence
Sulphatase
DHEA sulphate (inactive)
DHEA
Cortisol
Aging adrenal gland
Steroid hormones
LPS (bacteria)
TNFα
IFNα

Mini-Review

Melatonin, Immune Modulation and Aging

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Melatonin is a hormone secreted by the pineal gland in response to photoperiods and influences many important biological processes. For one, Melatonin has been shown to produce resistance to cancer and infectious diseases in aged animals. Studies in animals have demonstrated melatonin-related mechanisms of action on immunoregulation. Additionally, melatonin has been successfully used in humans, along with interleukin-2, as a treatment of solid tumors. *In vivo* and *in vitro* studies show melatonin enhances both natural and acquired immunity in animals. Despite all of this intriguing evidence, melatonin's mechanism of action on the immune system is only partially defined. It does, however, appear to act through lymphocyte receptors, and perhaps, receptors on other immune tissues, to modulate immune cells. In order to understand immunomodulation and anti-cancer effects, information on melatonin and it's interactions with other endocrine hormones are summarized.

Keywords: Melatonin, Immunoregulation, Immunity, Cancer, AIDS

INTRODUCTION

Melatonin (MLT) has enjoyed much attention from the scientific community, as well as the general public and particularly the elderly for its ability to induce sleep. The production of MLT declines with age (Figure 1), while susceptibility to cancer, infectious disease, oxidative damage and difficulty sleeping increases. It has been reported that MLT has immunomodulatory properties and may counteract the immunodepression following aging, cancer, viral diseases, acute stress, and drug treatment. In order to establish the role of MLT in relation to cancer, infectious diseases, and oxidative damage, the effect of MLT supplementation on the immune system and it relationship to other endocrine hormones will be reviewed.

MECHANISMS OF CELLULAR ACTIVATION

MLT, the main neuro-hormone of the pineal gland, has many immunomodulatory properties. MLT's mechanism of action on the immune system requires one to consider its targets. Studies indicate a wide spectrum of immune system targets for

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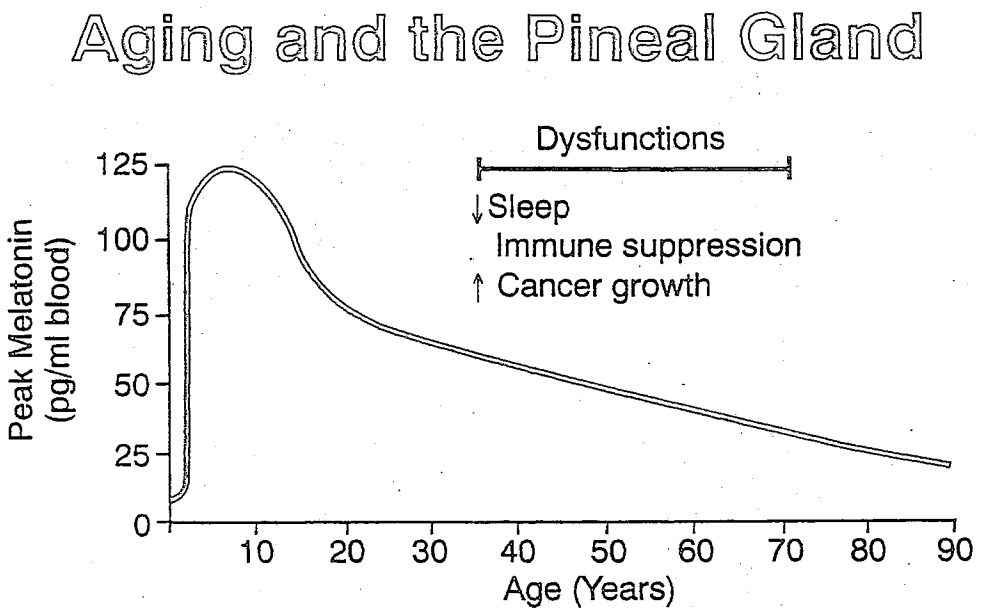

FIGURE 1

MLT. Both immature (thymocytes) and mature (spleen lymphocytes) cells seem sensitive to MLT treatment. The epithelial component of the thymus, which is responsible for synthesize and release of thymic peptides, is also affected by MLT.[1] Active thymulin has chemotaxis properties used for homing prethymic cells into the thymus where they develop and proliferate.[2] Interestingly, MLT rexceptors, as well as receptors for other hormones/neuropeotides are present on these cell lineages, suggesting MLT modulates their action. It has therefore been suggested MLT's immunomodulatory effects are mediated through the hypothalamic-pituitary-adrenal axis,[3] hypothalamic thyrotropin-releasing hormone (TRH),[4] as well as the opioid system.[5]

There is no doubt that receptors for glucocorticoid and pituitary hormone are present on all cell lineages mentioned above.[6,7] MLT increases the affinity and decreases the density of thymic adrenal steroid receptors, suggesting thymic hormone steroid receptors may be the site where MLT and adrenal steroids interact.[8,9] Murine thymic involution, secondary to anterior hypothalamic damage, was restored by both TRH and MLT replacement. TRH receptors have been found on peripheral lymphocytes,[10] their presence on thymocytes and thymic epithelial cells is currently under investigation. Furthermore, it has been suggested that MLT is a promoter of TRH.[11] Both TRH and MLT can restore the thymus of AHA-(anterior hypothalamic area)lesioned mice.[10] A high density of TRH and MLT receptors exists on the "thyrotropic hypothalamic area" of the brain. The anterior hypothalamus is the site where photoperiodic changes are detected and where MLT induced hormone secretion occurs.[10] Since the anterior hypothalamus contains large quantities of IL-1β,[12,13] MLT and TRH these factors may be players in the alpha regulatory loop of delayed-type thymus-mediated immunity.

Opioid receptors, such as enkephalin receptors, are present on thymocytes[14] and peripheral lymphocytes.[15] Physiological concentrations of MLT provide circadian signals which stimulate murine and human activated T helper cells to secrete

opioid agonists. These MLT-induced and immunoderived opioid agonists bind specific receptors on thymus a spleen cells and counteract the depression induced by stress-associated corticosteroids.[16,17] These agonists seem to mediate the immunoenhancing and anti-stress effects of MLT.[18]

Specific MLT binding sites have been described in human and rodent lymphocytes, granulocytes, thymocytes, splenocytes and bone marrow cells. Additionally, cells isolated from the Bursa of Fabricius in birds also have MLT binding sites.[19] The dissociation constant values (Kd) are in the 0.1–1 nM range, suggesting MLT may play a role in lymphocyte regulation by binding immunocompetent cells.[19] MLT receptors are coupled to guanine nucleotide binding proteins which modulate guanine nucleotides in human lymphocytes.[19] MLT increases cGMP production in human lymphocytes by binding specific receptors stimulating vasoactive intestinal peptides to activate cAMP.[20] These peptides are not only potent activators of cAMP,[21,22] but also exhibit inhibitory effects on mitogen-stimulated proliferation,[23] decrease IL-2[24] and modulate IgA and IgM production.[25] MLT may therefore regulate immune function by its acion on vasoactive intestinal peptides.

Human circulating T lymphocytes contain high-affinity binding sites for MLT, however these sites are not present on B lymphocytes.[26] The affinity of these binding sites (kd: 0.27 nM) suggests that they recognize physiological concentrations of MLT. Among the lymphocyte subpopulations studied (CD4+ vs. CD8+), receptors on CD4+ cells have the highest affinity for MLT.[26] Among subtypes of CD4+ cells, T helper 2 (Th2) have the highest affinity (Kd: 0.35 nM) for MLT.[27] Activation of this MLT receptor results in enhanced production of IL-4, which increases endogenous marrow granulocyte/monocyte-colony forming units (GM-CSF) production. GM-CSF may rescue hematopoietic cells from certain toxic therapeutic agents.[28] MLT therefore has important and widespread clinical implications.

MLT may exert its effects on the thymus gland[19] through specific thymocyte MLT receptors.[29] MLT treatment in old mice restored endocrine activity and increased thymic weight. This was also associated with increased thymocyte proliferation.[30]

MLT receptors are present on mouse and rat spleen cells[19,31] and old mice treated with MLT show restored splenocyte numbers and subsets and increased mitogen responsiveness.[30]

There are two subtypes of retinoid Z receptors (RZR α and β) which are part of the nuclear hormone receptor superfamily.[32] Very recently, MLT has been found to be a natural ligand of RZR α and RZR β. A response element on the promoter of 5-lipoxygenase (a key enzyme in the biosynthesis of leukotrienes/inflammatory mediators) binds specifically to RZR α. The activity of the 5-lipoxygenase promoter, as well as the RZR response element, when fused to the heterologous thymidine kinase promoter, could be repressed by MLT. MLT can down-regulate expression of 5-lipoxygenase 5-fold in B lymphocytes which express RZR α.[32]

In addition to MLT's role in regulating normal immune function, it may also restore age-associated immune dysfunction by rejuvenating the zinc pool.[11] With advancing age both MLT and zinc plasma levels decline.[33] Zinc deficiency is associated with thymic atrophy, reduced immune response to T-dependent antigens, deranged cellular immunity, and loss of thymulin activity.[34] MLT modulates zinc plasma levels in rodents[35] and restores zinc levels in old mice.[30] The presise mechanism by which MLT accomplishes this still needs to be explored.

MELATONIN STIMULATION OF CELL MEDIATED IMMUNITY IN OLD ANIMALS

MLT treatment in old mice increased expression of cellular markers on thymocytes, T-cell subset numbers, and ConA mitogen response.[30] Th2 cells produce IL-4 and IL-5, whereas Th1 cells produce IL-2 and IFN-γ.[24] Th2 cells are excessively active in the aged and are targets of MLT. MLT acts on Th2 cells in mice, inducing the production of IL-4[36] which stimulates thymocytes, cytotoxic T cells,

B cells, NK cells, and phagocytes.[37] MLT also increases Th1 cell activity and its production of IL-2 and IFN-γ.[38,39,40] IL-2 plays a critical role in differentiation and proliferation of various effector cells (T helper cells, cytotoxic T cells, B cells, and NK cells)[41,42] and increases IFN-γ production.[43] Th2 cells are sensitive to IFN-γ, which selectively inhibits their proliferation and cytokine synthesis.[24] IL-4 is able to inhibit IFN-γ production[44] while IFN-γ stimulates MLT production and suppresses Th2 cytokines.[45] It is therefore possible to envision a pineal-immune axis in which age-induced secretion of Th2 cytokines suppresses production of Th1 cytokines hence, inhibiting MLT secretion and cellular immunity. Optimal Th1/Th2 responses not only induce a protective mechanism against viruses, bacteria, and parasites, but may also be responsible for alterations seen in some immunopathologic disorders.[24] The existence of a pineal-immune axis which maintains a Th1/Th2 balance is of considerable relevance.[27]

NK cells help lyse cancer cells acting as an early non-T-cell directed defense. MLT increased NK cells and activity of monocytes in young men taking 2 mg of MLT nightly for two months,[46] suggesting MLT has additional effects above normal physiological levels (young men produce MLT). Both antibody production and autologous mixed lymphocyte reactions depend on the number of cells expressing MHC class Ia or II and MLT may exert immunostimulatory effects by increasing the number or activity of Ia+ cells.[47]

Loss of both cellular immune function and MLT could be a cause and effect association and may explain why developing cancer and infectious disease is increased with age. Thus implying that when MLT is present in low levels, cancer and infectious disease are more likely.

MELATONIN AND REGULATION OF HUMORAL IMMUNITY

MLT supplied to young or immunosupressed mice increased splenocyte response to LPS[38] proposing a MLT/B cell interaction. When MLT was administered to young, old and cyclophosphamide-treated mice, significantly increased *in vivo* and *in vitro* antibody production by spleen cells occurred.[38] Inhibiting MLT in mice results in depressed primary antibody response to sheep red blood cells and autologous mixed lymphocyte reactions.[47] MLT induced increases in primary antibody (IgG and IgM) production which is attributed to opioid peptides released by MLT-induced Th cells[5,38] (mediated by increases in IL-2).

MELATONIN AND MECHANISM OF CANCER REGULATION

In addition to declining with age, MLT is also present in decreased amounts in young cancer patients. Since older individuals have lower levels of MLT, along with the associated immune dysfunction, it is not surprising that their risk for developing cancer is much greater. Women with a previous history of breast cancer had significantly lower MLT secretion compared to women without cancer.[48] Although associated, these types of studies do not ascertain whether cancer and MLT are directly related. Furthermore, if related, the diminished secretion of MLT may result either in an inefficient cellular immune response, which facilitates cancer growth, or cancer growth may result in lower MLT levels.[49] Tumors that secrete cytokines like IL-6, which suppresses IFN-γ, could decrease serum IFN-γ levels enough to suppress MLT secretion.[45] Enhanced peripheral metabolism of MLT in breast cancer patients[50] may also account for its decline. Although this is not always seen, a similar decline in serotonin-N-acetyl transferase[50] the rate-limiting enzyme in MLT synthesis, was observed. These results were also replicated in patients with prostate cancer.[51,52] If MLT is related to cancer incidence, the question that still remains is whether a decrease in MLT production is a result of the tumor's ability to block MLT synthesis, or whether a decreased production of MLT predisposes individuals to cancer. In an attempt to answer this question MLT was

shown to exhibit a tumor stage-dependent decreased secretion in breast and prostate cancer.[53] Studies in humans[50] as well as animals[54,55] illustrate an inverse relationship between tumor growth and MLT production. Thus, tumor growth itself, possibly by releasing neurotoxic agents[56] or cytokines.[45] may inhibit MLT production. However, the mechanism by which MLT interacts with tumors is still unclear and must be determined in order for its precise role to be clarified. Still, other unidentified pineal substances can inhibit the growth of a variety of human cancer cell lines, even more effectively than MLT.[56] This suggests that MLT's main physiological effect on tumor growth is not at the cellular level, but rather, that it acts concomitantly with other substances in the immune and endocrine systems to exert its antineoplastic effects. Nevertheless, MLT itself has also been shown to inhibit growth of DMBA-induced breast cancer in rats.[57]

IN VITRO MODELS OF MELATONIN AND CANCER GROWTH

Many studies have been performed using *in vitro* models to determine the effectiveness of MLT at inhibiting human cancer cell lines without assistance from the immune system. MLT was very effective *in vitro* against human breast cancers, but only against estrogen sensitive or estrogen receptor positive ones.[58] Additionally, different estrogen receptor positive cancer cell lines respond differently to MLT. MCF-7 breast cancer cells seem to be the most sensitive to MLT.[59] Tamoxifen, a treatment for estrogen sensitive breast cancer, blocks estrogen receptors preventing estrogen from entering the cell and inducing cell proliferation. MLT can down-regulate steady state levels of mRNA in MCF-7 cell lines by decreasing the transcription rate of estrogen receptor genes.[60] When Tamoxifen was given along with MLT it failed to slow the growth of the tumor.[60] Thus, MLT may bind to estrogen receptors and inhibit tumor growth in a manner similar to Tamoxifen. However, MLT amplified the effects of Tamoxifen *in vitro* on MCF-7 cells, when the cells had been pretreated with MLT.[60] This produced a 100 fold increase in the effectiveness of Tamoxifen.[60] While the mechanism of action is still unclear, MLT seems to sensitize cells to respond to Tamoxifen treatment more effectively.[60] MLT may therefore be useful at priming cancer cells for treatment with Tamoxifen. Furthermore, MLT treatment resulted in a lower effective dose of Tamoxifen.[61] On the other hand, 5-fluorouracil diminished the beneficial effects of MLT[62] on human MCF-7 breast cancer cells. These authors suggest that 5-fluorouracil should not be administered over continuous infusions, as MLT levels rise at night. Perhaps daytime infusions of 5-fluorouracil and nighttime MLT treatment may be the most effective treatment for human breast cancer (MCF-7). Further studies were still needed to determine if MLT actually works by a similar mechanism as Tamoxifen does *in vivo*. Subsequently, MLT was administered to a MCF-7 cell culture via a pulsatile or continuous exposure.[63] This system, although still an *in vitro* model, more closely mimics the physiological state of MLT, and was a more effective means of inhibiting MCF-7 cells. So although additional studies are needed to confirm MLT's mechanism of action at the cellular level on breast cell growth, its role seems to be a significant one worthy of additional investigations.

MELATONIN AND CANCER TREATMENT

In cancer patients with metastatic solid tumors. MLT administration increased the ratio of CD4+ cells to CD8+ cells.[40] There were marked increases in the circulating levels of IL-2, IFN-γ, and TNF-α when 16 patients with advanced solid tumors were treated with MLT (10 mg/day orally for a month).[64] Patients with progressing metastatic renal cell carcinoma were studied for 2 years. They were treated with human lymphoblastoid IFN-γ; 3 mega units injected intramuscularly 3 times per week: and MLT; 10 mg taken orally every day.[65] There were seven remissions (33%), nine patients stabilized and five patients progressed however, all patients exhibited only mild toxicities. These

results compare favorably with the use of IFN-γ and MLT in the treatment of metastatic renal cell carcinoma.[65] In addition, combination therapy aimed at increasing NK cells and stimulating the host immune system has also been done with human lymphoblastoid IFN-γ and MLT. This is consistent with the hypothesis that by triggering endogenous cytokine production, especially IL-2, MLT might restore T cell responses and increase killer cell activity, both of which are frequently depressed in cancer patients.[66]

MLT significantly improved survival rates of non-small cell lung cancer patients who failed more conventional therapies.[67] Since chemotherapy is generally not effective against this type of cancer, other approaches need to be developed to prolong survival. An alternative to chemotherapy would be immunotherapy using cytokines, particularly IL-2, because of its potent ability to activate the cellular immune system. IL-2 treatment, however, was not effective in treating non-small cell lung cancer and was poorly tolerated. Treatment of non-small cell lung cancer using a combination therapy of IL-2 and MLT though, resulted in tumor regression.[68] The advantage of neuroimmunotherapy, as opposed to chemotherapy, is that the host's immune system remains intact and is activated, rather than suppressed. Chemotherapy can be highly toxic to hematopoietic cells while MLT, may in fact, protect bone-marrow cells from toxicity. MLT may act on murine bone-marrow T-cells increasing GM-CSF.[69] Unfortunately, most studies do not look at the effects of IL-2 and MLT individually, so although the data look promising, it cannot be concluded that combination therapy is more effective than IL-2 or MLT alone. When IL-2 plus MLT was compared to chemotherapy using cisplatin,[70] it was more effective than cisplatin alone and may be extremely useful as a first line therapy against non-small cell lung cancer.

MLT's effectiveness is not limited to lung cancer. IL-2 plus MLT was useful in treating human metastatic hepatocellular carcinoma,[71] gastric cancer,[72] colorectal cancer[72] and other cancers of the gastrointestinal tract.[72] MLT effectiveness in this combinational therapy limited the necessary dose, and subsequently, side effects of IL-2. Advanced solid neoplasm's are usually resistant to treatment with IL-2 alone. However, when IL-2 was given along with MLT, not only was tumor regression observed,[72,73] but there was also an increase in lymphocytes and eosinophils.[74] Since MLT, at these doses, had no known side-effects or toxicities, there may be a substantial benefit to adding it to traditional chemotherapy regimes.

Thrombocytopenia is a common complication of cancer. The etiology of this disorder is multifactorial, but includes chemotherapy-induced myelosuppression, bone-marrow infiltration, and disseminated intravascular coagulation.[74] Treatment with low-dose IL-2 plus MLT has been shown to normalize platelet counts in cancer patients with thrombocytopenia.[74] This combinational therapy has been shown to be an effective treatment for renal cell carcinoma and may warrant further clinical studies.[75]

Therefore, although MLT's mechanism of action in cancer chemotherapy and tumor regression is still unclear, it seems to play an important role. MLT may not only have a direct effects on tumor regression, but it may also modulate cytokine function for regulation of cellular immune defenses.

MELATONIN AND INFECTIOUS DISEASE RESISTANCE

The pineal gland and its major hormone MLT are capable of translating environmental information into signals that modulate reproduction, adrenal gland hormone synthesis, immune function, as well as other neuroendocrine interactions.[76,77] Viral infection can increase glucocorticoids levels[78] resulting in thymus and spleen involution and subsequently, immunosuppression.[78] MLT decreased mortality associated with viral infection by suppressing the potentially damaging inflammatory response. The antiviral activity of MLT was evaluated in normal mice inoculated with Semliki Forest virus (SFV) and in stressed mice injected with the attenuated West Nile virus (WNV).[78] MLT

was injected subcutaneously daily beginning 3 days before infection until 10 days after viral inoculation. MLT reduced viremia, significantly postponed the onset of disease and delayed death by 7 to 10 days. Moreover, MLT injection reduced mortality of SFV inoculated mice from 100% to 44%. In mice inoculated with high doses of SFV, MLT postponed death and reduced mortality by 20%. In all of the surviving mice anti-SFV antibodies were detected 22 days after virus inoculation. Infected mice stressed by either isolation or dexamethasone and injected with WNV have mortality rates of 75% and 50%, respectively. This mortality rate was reduced to 31% and 25% when MLT was administered. MLT seems to provide mice with efficient protection from lethal murine viral infections, however, studies in humans are lacking.[78]

MLT also shows promise as a therapy for Acquired Immune Deficiency Syndrome (AIDS). Synthetic IL-2 injected into AIDS patients increased T helper cells, but unfortunately, is extremely toxic.[79] When patients were given 10 mg of MLT each night for a period of one month, there was a 51% increase in IL-2 levels without any toxicity.[79] MLT stimulated production of immune components deficient in HIV patients i.e., T helper cells, NK cells, null cells, macrophages, IL-4, IFN-γ, GM-CSF, IL-10, eosinophils, and red blood cells.[80] Furthermore, MLT may act by repressing 5-lipoxygenase therefore resulting in fewer leukotrienes; which contribute to the inflammatory response in AIDS.[81]

MLT may also help defeat chronic immune dysfunction in AIDS by reducing excessive free radical production.[82] Retrovirus infections leads to an increase in reactive oxygen compounds, together with a decline in antioxidants, which quickly results in oxidative stress. Oxidative stress in AIDS results in damage and death of T helper cells (CD4+), thus further weakening the immune system.[83] A decline in the ratio of CD4+ to CD8+ cells is one sign of progression from HIV infection to AIDS.[84] Therefore, antioxidant therapy may be useful in slowing or preventing AIDS. Decreased glutathione levels have been associated with increased inflammatory cytokines such as TNF, which may be involved in AIDS associated wasting. Increased production of TNF increases free radical production activating nuclear factor kappa-B (NFκB); a element involved in promoting HIV replication, and further increases TNF production.[85] MLT can stimulate the action of a related antioxidant, glutathione peroxidase, which prevents activation and subsequent binding of NFκB. This suggests that MLT has the potential to be an effective antioxidant in the treatment of AIDS.[86]

HORMONAL MODULATION BY MELATONIN: A MECHANISM OF IMMUNOREGULATION

The action of MLT on immune defenses may be due to its effect on other hormones. The pineal gland helps maintain hormone levels and normal cycling patterns by transmitting messages through primary messengers like MLT. Hormones control body temperature, reproduction, blood pressure, kidney function, and to some degree, immune function and cancer resistance. The variety of beneficial effects observed by MLT treatment, as well as the detrimental effects found in individuals with low MLT levels, could be explained by its effect on hormonal regulation.

Stress is known to be immunosuppressive because it induces the adrenal gland to produce stress hormones including corticosteroids. Repeated exposure to high levels of corticosteroids in response to stress, can cause damage to the heart, brain, and immune system. When corticosteroid levels become high, as in adolescence, MLT levels begin to rise. The release of melatonin results in the return of these immune damaging hormones to their normal levels.[6] In the elderly, declined MLT levels could result in an increase in lymphoid organs' exposure to corticosteroids and thus, be responsible for age-induced immune dysfunction. In fact, diseases associated with aging, such as diabetes, heart disease, and cancer, are in part, the result of an imbalance of hormones. By restoring the proper hormonal balance, MLT may help prevent many diseases.

Clearly, modulation of hormones (including locally acting hormones such as, cytokines) could be a mechanism by which MLT effects immune restoration in the aged. Interestingly, the majority of identified binding sites of MLT are on the central nervous system and lymphoid cells.[87,88] However, mechanistically, it is not yet totally clear how and where MLT intervenes to modulate hormones and must be investigated further.

CONCLUSION

In summary, MLT plays an important immunoregulatory role by both direct and indirect action on the immune system. Mechanisms by which MLT affects the immune system are not definitive. MLT binding sites and signals on immunologically active organs and cells, as well as on tumor cells, certainly warrant further studies. Even in some organs where melatonin receptors are well studied e.g., the pars tuberalis of the anterior pituitary gland, the molecular mechanisms of MLT are only partially defined.[89] In fact, MLT can affect large numbers of tissues and organs such as nervous tissue, adrenal and sex glands, and particularly, the thyroid which has an important immunomodulatory role.[89,90] This constitutes a sound rationale for the clinical use of MLT as an immuno-equilibrating agent. Potential uses of exogenous MLT in cancer patients have been described, although the chronobiological aspects of such treatments, as well as optimal doses of MLT, must be clarified. Furthermore, MLT replacement therapy may constitute an important means of improving immune defenses and preventing cancer and infectious diseases in the aged. Clearly, some interesting mechanisms and roles of MLT have been studied and further clarification will shed new light on the precise beneficial role(s) of MLT.

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chapter thirteen

Antioxidants and AIDS

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Introduction

Acquired immunodeficiency syndrome (AIDS) is a result of infection with human immunodeficiency virus (HIV-1 or HIV-2) which eventually destroys subset CD4+ helper T lymphocytes. This results in enhanced susceptibility to opportunistic infection and neoplasms.[1] Oxidative stress plays a major role in the progression of HIV infection to AIDS and has been suggested to contribute to the decline in CD4+ lymphocytes.[2] The existence of oxidative stress in HIV infection and AIDS is exemplified by the excess production of reactive oxygen species (ROS) and a general loss of antioxidant defenses in HIV-infected patients.[3] Therefore, the reduction of oxidative stress by antioxidant treatment may be a desirable therapy during the asymptotic HIV infection as well as advanced AIDS.[4]

Oxidative stress and HIV infection

Oxidative stress is a pathologic phenomenon resulting from an imbalance between the system producing ROS and the antioxidant defense systems which function synergistically to prevent or destroy ROS.[5] An increased production of ROS is caused by infecting agents in neutrophils and macrophages[6] as well as by abnormal production of TNF-α in HIV-infected patients.[7,8] Increased secretion of TNF-α results from direct stimulation by free radicals and the antigens of opportunistic bacteria only in AIDS when severe immunedysfunction permits persistent infection. In the asymptomatic stage, activation of the TNF gene occurs by the viral replication machinery.[5] TNF may play an important role in causing a further increase in the levels of oxidants by providing an "amplification loop" that feeds back to excite further production of ROS from macrophages and neutrophils.[9] It may also

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react with T cells to enhance expression of autocrine cell activators, such as IL-2, and receptors, thereby promoting activation of T cell respiratory activity for greater intracellular ROS.[10,11] The excessive production of oxygen-free-radicals causes the oxidation of circulating or membrane lipids, proteins, and DNA, and functions as a potent inducer of viral activation, DNA damage, and immunosuppression.[12]

Apoptosis, programmed cell death of CD4+ lymphocytes, is of fundamental importance in progression towards AIDS.[3] The cascade of events that results from oxidative stress can initiate apoptosis, a possible pathway of immune cell loss in patients with HIV infection.[3] It includes oxidation of cellular membranes, alteration in metabolic pathways, disruption of electron transport systems, depletion of cellular ATP production, loss of Ca^{2+} homeostasis, endonuclease activation, and DNA/chromatin fragmentation. The DNA damage caused by oxidative stress may be related to HIV-associated malignancies and disease progression.[13] Downstream events secondary to these effects may also play a role in activation of the latent virus and subsequent viral replication.[3] Oxidative stress is a known activator of HIV replication *in vitro* through the activation of a nuclear factor κB (NF-κB). NF-κB in turn stimulates HIV gene expression by acting on the promoter region of the viral long terminal repeat (LTR), a critical region for transcription in the integrated virus.[13] TNF-α is an important activator of HIV by generating ROS which activates NF-κB.[14]

Antioxidants and AIDS

The suggestion that oxidative stress is a feature of HIV infection and AIDS is also supported by multiple nutritional deficiencies and increased metabolism of antioxidants in HIV-infected patients.[3] This results from malabsorption of nutrients, hypermetabolism, and drug-nutrient interactions.[15] The antioxidant status of lymphocytes is important for their functioning, which is closely linked to their redox potential, particularly to their cysteine and glutathione levels.[5] In a weakened antioxidant system, DNA repair capacity of the cells may be altered and lymphocytes may be killed or impaired.[16-18] Since ROS is involved in the signal transduction mechanisms for HIV activation, a possible therapeutic use of antioxidants in preventing HIV activation has been suggested.[19-23]

Glutathione and N-acetylcysteine

Glutathione (GSH), a thiol derived from cysteine, is important in scavenging reactive oxygen intermediates released by activated neutrophils and monocytes.[24] It regulates many lymphocyte functions including their proliferative response to mitogens, responsiveness of cytotoxic T cells to IL-2, and cytotoxicity of lymphokine-activated killer cells.[25-29] Depletion of GSH inhibits proliferation of T lymphocytes, particularly those from HIV-infected

patients.[30] Another important effect of GSH is its ability to inhibit HIV replication when stimulated by TNF or phorbol myristate acetate (PMA) in infected macrophages and lymphoid cells.[31]

HIV-infected patients have greatly decreased levels of GSH in their plasma and peripheral blood lymphocytes.[32,33] The decreased levels of GSH are highly correlated with depressed numbers of CD4+ cells.[24] GSH is a good candidate for clinical investigation, as flow cytometry can measure glutathione levels in T cell subsets and has been used to show GSH changes in such subsets following HIV infection.[18] Since GSH and vitamin E spare each other, vitamin E appears to prevent the drop in GSH levels and thus TNF-α-induced HIV replication.[34]

N-acetylcysteine (NAC) has both a direct and indirect antioxidant role. It is a cysteine precursor which is converted intracellularly into GSH and can also act directly as an antioxidant.[4] By increasing cellular GSH levels and decreasing TNF-α, it can also inhibit TNF-α-induced HIV replication and prevent TNF-α-induced apoptosis of T lymphocytes and other cells in HIV-infected people.[35,36] NAC has been reported to increase antibody-dependent cell mediated cytotoxitity of neutrophils.[35] Early clinical trials have shown that NAC prevents the decline in CD4+ cells in GSH-deficient individuals.[37] Unfortunately, oral and intravenous GSH do not effectively enhance cellular GSH stores.[38] Although aerosolized GSH does increase cellular stores, the most effective means for raising cellular GSH levels is oral or intravenous administration of the GSH precursor NAC.[34,38] Therefore, treating patients with NAC may be a useful strategy in slowing the progression of the disease.

L-2-oxothiazolidine 4-carboxylate (OTC) is another pro-GSH drug that has been proposed for AIDS therapy. Although both NAC and OTC were shown to block cytokine induction of HIV *in vitro*, NAC was far more effective than OTC.[4] In isolated peripheral blood mononuclear cells, NAC fully replenishes depleted intracellular GSH whereas OCT only minimally replenishes GSH.[4] Although NAC is markedly more effective at blocking HIV expression than OCT *in vitro*, both drugs could prove equally effective in the clinical setting.[4] A report studying rats noted that procysteine, also a pro-drug for glutathione, effectively reduced ischemia-induced heart damage by increasing levels of cellular GSH.[39] In what manner procysteine will be effective in people with AIDS remains to be determined.

Vitamin E (tocopherol)

Vitamin E, a fat soluble vitamin, is also a well-known natural antioxidant. It attaches to free radicals and prevents the further generation of free radicals which ultimately prevents membrane lipid peroxidation.[40] Vitamin E functions as an immune enhancer by its antioxidant activity. Deficiencies in vitamin E lead to prooxidant status and have detrimental effects on the immune system.[15]

Plasma vitamin E levels were demonstrated to be lower in HIV-infected patients than in controls.[41] Vitamin E derivatives such as vitamin E acetate, α-tocopheryl succinate and 2,2,5,7,8-Pentamethyl-6-hydroxychromane (PMC) exhibited a concentration dependent inhibition of NF-κB activation by TNF-α.[1] Thus, vitamin E acetate which is a natural, safe compound, and PMC which was demonstrated to be very effective in blocking NF-κB activation, should be considered for possible inclusion in combination therapies for AIDS.[1] The intake of supplementary vitamin E is associated significantly with slower progression to AIDS in HIV-seropositive men.[42] Vitamin E has also been shown to increase the CD4+/CD8+ lymphocyte ratio in AIDS patients by enhancing CD4+ cell counts.[43]

α-Lipoic acid

Recently, α-lipoic acid was found to exert antioxidant action *in vivo* and *in vitro*.[44] In addition, α-lipoic acid has been shown to inhibit HIV-1 replication in infected cells.[45,46] This may be due to the inhibition of NF-κB activation imposed by the antioxidant properties of dihydrolipoic acid (DHLA) generated from α-lipoate.[14] DHLA can cause a complete inhibition of NF-κB activation induced by TNF-α by scavenging free radicals and recycling vitamin E.[14] The inhibitory action of α-lipoic acid was found to be very potent — only 4 mM was needed for a complete inhibition, whereas 20 mM was required for NAC,[45] which indicates that α-lipoic acid may be effective in AIDS therapeutics.

Vitamin C (ascorbic acid)

Vitamin C has a role in moderating the immune system, possibly by affecting natural killer cell, macrophage, and T cell activities.[47] Unfortunately, most of these studies have been done on mice, and mice can synthesize vitamin C internally. In humans, vitamin C properties seem to make it a potential anticancer treatment.[47] Vitamin C also appears to have direct effects on HIV, thus enhancing its importance in treating people with AIDS. *In vitro* vitamin C inhibits the replication of HIV by more than 90% at levels of no toxicity of vitamin C.[48,49] Vitamin C is apparently more efficient than GSH or NAC in reducing the HIV-1 replication in chronically infected T lymphocytes.[49] The effects of vitamin C on HIV can also be increased by the addition of NAC.[67]

Large amounts of vitamin C are consumed by HIV-infected patients.[50] No clinical benefit is associated with ingestion of vitamin C, in spite of the report that vitamin C can improve the clinical situation of patients suffering from different viral diseases and improve their CD4+ cell count with massive doses of vitamin C.[5] A survey of the nutritional status of HIV-seropositive patients[51] showed a nonsignificant decrease in serum vitamin C, and no significant difference in the prevalence of a low status even with an increase in vitamin C intake (10 times the RDA) due to supplementation.

Carotenoids

There is a severe deficit in plasma carotenoid including β-carotene levels in HIV-infected patients.[5] The degree of reduction in carotene levels is secondary to its depletion, given its ability to act as an antioxidant and scavenge the excess active oxygen.[52]

Other vitamins

In a study of micronutrients in HIV-infected patients, there was a decrease in vitamin A and vitamin B_2 (riboflavin) levels.[51] Vitamin B_2 deficiency results in a decreased activity of glutathione reductase, which regenerates oxidized GSH to reduced GSH, enabling it to rejuvenate its antioxidant functions. Mean serum levels of vitamin B_1 (thiamin), vitamin B_6 (pyridoxal), folate, and vitamin B_{12} were unchanged by HIV infection, whereas the prevalence of deficiencies in vitamin A, B_6, B_{12}, and E were significantly increased.[5]

Zinc

Zinc has a very interesting role in HIV infection. Zinc not only functions as an antioxidant, but it also has a more direct effect on the immune system. Zinc increases the secretion of IL-2, the activity of thymulin, and prevents apoptosis.[5] Zinc penetrates cells, enabling regulatory proteins to bind DNA, which results in expression of the IL-2 gene.[53] The addition of zinc to a serum-free culture medium increases the proliferation of T lymphocytes and the synthesis of IL-2 in response to stimulation.[54] In the presence of zinc, thymulin assumes an active cyclic form enabling zinc to be recognized by high affinity receptors on T lymphocytes. This results in the differentiation of T lymphocytes by the induction of antigen B and in response to concanavalin.[56] It is very important to note that Zn^{2+} inhibits the endogenous endonuclease activated by Ca^{2+} which is responsible for the apoptosis of CD4+ cells induced by TNF.[54] Additionally, zinc acts as an antiviral agent by inhibiting the reverse transcriptase.[5]

HIV-infected patients whose status remained stable for two years had normal plasma zinc levels,[56] whereas zinc levels of those who progressed towards AIDS were lower. Thymulin, which is also considered good marker of zinc status, was found to be extremely low in the blood of patients with AIDS.[57]

Faced with this decreased zinc status,[58] the effect of zinc supplementation was investigated in these patients. The most worrisome risk was that of an upsurge of viral activity due to the existence of several zinc-finger proteins in the structure of HIV-1.[59,60] It has been reported that the supplementation of zinc in AIDS patients can increase the CD4+/CD8+ lymphocyte ratio,[61] however, very few studies can confirm these results.

Selenium

Selenium is a cofactor of glutathione peroxidase (GPx). Due to its antiviral effects and its importance in all immunological functions, the administration of selenium is suggested as a supportive therapy in early as well as in advanced stages of HIV infection.[62] A characteristic of the protective effects of selenium against viral pathogens is that its benefits occur at supplemental levels above physiological requirements. This suggests that it may not be associated solely with its function in GPx.[62] Selenium inhibits reverse transcriptase activity in RNA-virus-infected animals, therefore supplemental selenium could also prevent the replication of HIV and retard the development of AIDS in newly HIV-infected subjects. Selenium is required for lymphocyte proliferation, macrophage-initiated tumor cytodestruction, and natural killer-cell activity.[63]

Subnormal serum or plasma selenium levels and erythrocyte GPx activities have been observed in patients with AIDS and AIDS-related complex (ARC).[64] Selenium levels and GPx activity were correlated to the total number of lymphocytes in HIV-infected patients.[64,65]

Selenium supplementation in HIV-infected patients causes symptomatic improvements, especially in appetite and intestinal functions,[66] and possibly slows the course of the disease. During the period of supplementation, CD4 cell numbers still tended to decline; however, this decline was often only slight, or not observed at all. CD8+ cells counts tended to decrease more often than to increase, causing the CD4+/CD8+ lymphocyte ratio to increase.[66]

Copper

It is extremely difficult to study the copper status in patients with inflammations. Cytokines, IL-1, and TNF, cause serum copper to undergo a clear-cut increase, due to the increase in ceruloplasmin, an "acute phase protein", even in copper-deficient subjects.[67]

Serum copper increased in AIDS patients after a decrease in the asymptomatic stage. Low serum zinc levels with high copper levels are predictive of progression towards AIDS, independent of the basal level of CD4+ cell counts.[51,58,68]

The measurement of copper-zinc superoxide dismutase (Cu-Zn SOD) in red cells is a more reliable marker of the zinc status with no variation in the enzyme, regardless of the stage of the disease.[67]

Antioxidant enzymes

The study of antioxidant enzyme activities, in addition to the changes in GPx described above, has shown a progressive and considerable increase in serum catalase,[69] while red cell SOD remains normal.[67]

Serum catalase activity increased progressively with advancing HIV infection (i.e., AIDS > symptomatic infection > asymptomatic infection > controls).[69] This correlates with increases in serum hydrogen peroxide (H_2O_2) scavenging ability, and may reflect or compensate for systemic GSH and other antioxidant deficiencies in HIV-infected individuals.[69]

Manganese-containing superoxide dismutase (Mn-SOD) is the key enzyme in cellular protection from apoptosis induced by TNF[70] via expression of the gene for Mn-SOD and for metallothioneins.[5] A decreased production of Mn-SOD was seen, while mRNA of this enzyme was overproduced in HIV-infected patients.[71] This results from the inhibition of translation of SOD mRNA caused by the binding of HIV tat protein to an RNA hairpin.[5] The sequence on which tat binds presents a sequence homology with a part of viral RNA, which is the biological target of tat, permitting the regulation of viral expression. The anomaly of Mn-SOD production is accompanied by signs of oxidizing stress in cells.[5]

Diethyldithiocarbamate (DDTC)

DDTC has a GPx-like activity. It is the only antioxidant drug that has been extensively studied in clinical trials, although it has not shown any *in vitro* antiviral activity.[72,73] In animals, DDTC increased GSH levels in a variety of tissues.[13] A significant reduction in these rates of new opportunistic infections was reported in AIDS patients receiving DDTC as compared to placebo.[73] However, a subsequent study apparently failed to demonstrate the similar benefit in a cohort study of HIV-infected asymptomatic patients.[13]

Desferrioxamine (DFX)

DFX is an iron chelator with strong antioxidant properties. DFX can inhibit *in vitro* HIV-1 replication in the H-9 T lymphocyte cell line. The rationale for this work is to explain the low rate of symptomatic HIV-1 infection in multiply transfused thalassaemic patients who have been intensively chelated with DFX.[76]

Plant-derived metabolites with synergistic antioxidant activity

Plants experience death due to oxidative stress, which closely parallels the process of apoptosis in humans, particularly as related to the destructive phenomena seen in HIV infection and AIDS. Primary and secondary metabolites found in plants act as synergistic antioxidants and can protect plants from oxidation-induced cell death. Some of these same metabolites can inhibit cell killing by HIV.[3] These metabolites are exemplified by phenolic compounds, nitrogen-containing compounds, enzyme systems and polypeptides, and vitamins. Therefore, use of these antioxidants in patients with HIV/AIDS is proposed as a mechanism by which viral replication and cell killing in HIV infection can be inhibited.[3]

Phenolic compounds (hydroxyl derivatives of aromatic hydrocarbons)

Ubiquinone. Although ubiquinone (coenzyme Q_{10}, CoQ_{10}) is known for its activity as a redox component of transmembrane electron transport in mitochondria, its reduced form, ubiquinol, is an active antioxidant. Ubiquinol scavenges products from the peroxidation of membrane lipids even after the peroxidation process has been initiated. Lipid peroxidation will not occur, in fact, until all ubiquinol is consumed, which spares vitamin E in the process.[75]

Patients with AIDS had significantly lower blood CoQ_{10} levels than healthy controls, while patients with ARC and asymptomatic HIV-seropositive infection had decreased blood levels of CoQ_{10}, but not to the extent of those of AIDS patients.[76] Supplementation with CoQ_{10} retarded the progression from ARC to AIDS and has a positive effect on the T4+/T8+ lymphocyte ratio.[66] However, CoQ_{10} may actually increase the level of free radicals thereby increasing oxidative stress.[66]

Flavonoids. Flavonoids (plant phenolic pigment products, particularly the catechins and quercitin), scavenge peroxyl and hydroxyl free radial.[77] They are protective against lipid peroxidation probably by donating H+ atoms to peroxyl radicals and terminating the chain radical reaction.[78] They can also control the release of reactive oxygen products from macrophages and neutrophils by regulation enzymes such as NADPH oxidase.[79] Quercitin, in particular, can inhibit the PKC-induced phosphorylation of I-κB that can liberate NF-κB to play a role in activation of viral replication.[21,80]

Coumarins (benzopyrones). Coumarins are effective against oxidative stress by acting in a similar manner to flavonoids.[81]

Nitrogen-containing compounds: di- and poly-amines (e.g., spermine, putrescine, cadaverine)

Nitrogen-containing compounds effectively inhibit lipid peroxidation and impede the release of superoxide radicals from senescing membranes. They exert their stabilizing effects by binding with negative charges of both nucleic acids and phospholipids.[82] They inhibit protease and RNAase activity, which is observed as a consequence of oxidative stress in plants.[82] Similar actions of polyamines in humans have been shown to help maintain intracellular Ca^{2+} homeostasis.[3]

Polyamines decline during oxidative stress, particularly when their necessary precursor, arginine, is either deficient or diverted. Arginine is consumed during nitric oxide production in HIV infection.[3]

Enzyme systems and polypeptides

Dismutase, catalases, and peroxidases all exist in plants, and their enhancement can increase resistance to oxidative stress.[85] The reduced form of GSH

(a tri-peptide), so integral to the discussion of oxidative stress in HIV infection, is a scavenger of peroxides in plants and arrests senescence.[86]

Vitamins

Vitamin C and E and the various carotenoids are ubiquitous in plants. As in humans, they do not suffice as protection from superoxidative stress. This is confirmed by the multiplicity of the antioxidant systems that are necessary to synergize with vitamins and provide adequate protection.[85,86]

Conclusion

Oxidizing stress is not merely an epiphenomenon, but is at the heart of the pathogenesis of HIV disease. This has incited researchers to test the effect of antioxidants in cell models, showing the high efficacy of certain micronutrients, but also of some other antioxidants. It is indispensable to combine the return of a deficient antioxidant nutritional status to normal (zine, selenium, carotene, and vitamins C and E) with the supply of high doses of synthetic antioxidant-generating glutathione as N-acetyl cysteins.

Generally, supplementation with antioxidants appears to offer some hope in slowing the progression of HIV infection. Currently there is considerable debate over the use of antioxidant therapies in many illnesses, although more and more mainstream practitioners are considering the potential benefits of such therapies.[87] While the substances discussed here are largely free of toxic effects, caution still must be taken, and the development of adverse effects should be carefully monitored. It is also important for practitioners to stress to their patients the importance of a basic balanced diet as the essential groundwork underlying any additional supplementation or drug treatment. The research into antioxidants is still at an early stage of development, but future studies will likely resolve which of these substances can be used effectively in treating HIV infection.[67]

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APPENDIX B:

ORIGINAL RESEARCH

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.**

[P.S.E.B.M. 1997, Vol 216]

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).

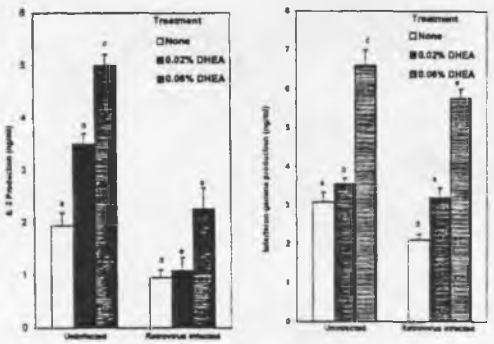

Figure 1. The effects of DHEA administration on Th1 cytokine production by splenocytes. The results are averages from triplicate assays of cells from each mouse in a group and are expressed as mean ± SE; $n = 8$. Immune responses were significantly different ($P < 0.05$) in IL-2 production (A) by splenocytes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

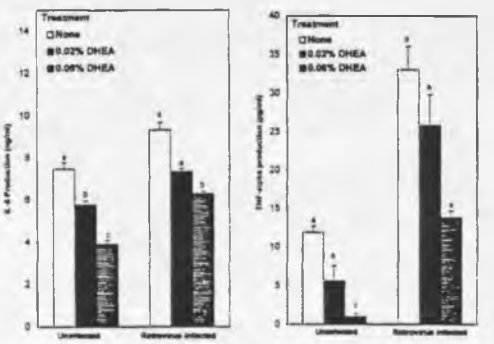

Figure 2. The effects of DHEA administration on Th2 cytokine responses by splenocytes. The results are averages from triplicate assays of cells from each mouse in a group and are expressed as mean ± SEM; $n = 8$. Immune responses were significantly different ($P < 0.05$) in IL-6 production (A) by splenocytes 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.

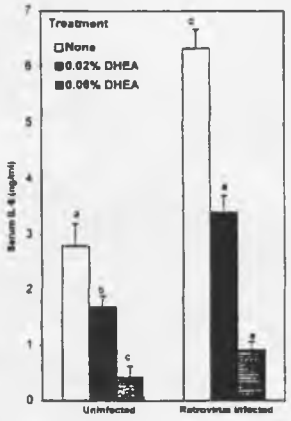

Figure 3. The effects of DHEA administration on serum IL-6. The results are averages from triplicate assays from each mouse in a group and are expressed as mean ± SE; $n = 8$. Immune responses were significantly different ($P < 0.05$) among certain groups: a > b > e > c and d > a.

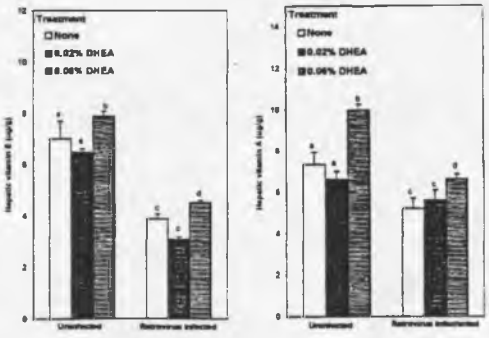

Figure 4. The effects of DHEA administration on hepatic vitamin E (A) and A (B) levels. The results are averages from duplicate assays done for each mouse in a group and are expressed as mean ± SE; $n = 8$. Vitamin levels were significantly different ($P < 0.05$) due to DHEA supplementation among certain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

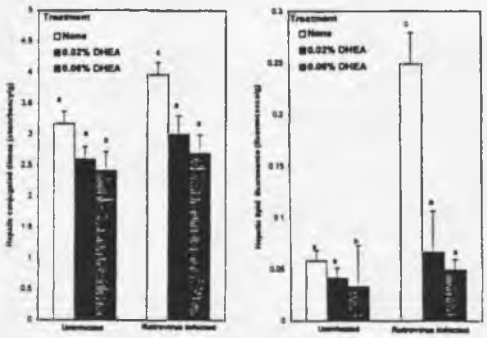

Figure 5. The effects of DHEA administration on hepatic lipid peroxidation (A and B). The results are averages of duplicate assays from each mouse in a group and are expressed as mean ± SE; $n = 8$. Lipid peroxidation responses were significantly ($P < 0.05$) higher in retrovirus-infected mice. Lipid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

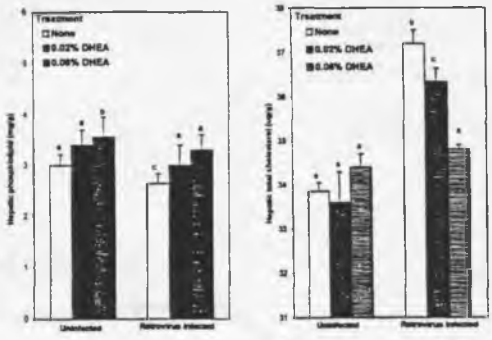

Figure 6. The effects of DHEA administration on hepatic phospholipid (A) and total cholesterol (B) levels. The results are averages from duplicate assays done for each mouse in a group and are expressed as mean ± SE; $n = 8$. Phospholipid (A) changes were significantly lower in retrovirus-infected mice, 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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Prevention of immune dysfunction and vitamin E loss by dehydroepiandrosterone and melatonin supplementation during murine retrovirus infection

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SUMMARY

Female C57BL/6 mice infected with the LP-BM5 leukemia retrovirus developed murine AIDS. Dehydroepiandrosterone (DHEA) and melatonin (MLT) modify immune dysfunction and prevent lipid peroxidation. We investigated whether DHEA and MLT could prevent immune dysfunction, excessive lipid peroxidation, and tissue vitamin E loss induced by retrovirus infection. Retrovirus infection inhibited the release of Th1 cytokines, stimulated secretion of Th2 cytokines, increased hepatic lipid peroxidation, and induced vitamin E deficiency. Treatment with DHEA or MLT alone, as well as together, largely prevented the reduction of B and T cell proliferation as well as of Th1 cytokine secretion caused by retrovirus infection. Supplementation also suppressed the elevated production of Th2 cytokines stimulated by retrovirus infection. DHEA and MLT simultaneously reduced hepatic lipid peroxidation and prevented vitamin E loss. The use of DHEA plus MLT was more effective in preventing retrovirus-induced immune dysfunction than either DHEA or MLT alone. These results suggest that supplementation with DHEA and MLT may prevent cytokine dysregulation, lipid oxidation and tissue vitamin E loss induced by retrovirus infection. Similarly, hormone supplementation also modified immune function and increased tissue vitamin E levels in uninfected mice.

INTRODUCTION

Murine AIDS is induced in genetically susceptible strains of mice inoculated with the LP-BM5 murine leukemia retrovirus mixture (MuLV). It is strikingly similar to human AIDS, even though HIV and murine retrovirus represent different retroviruses.[1] Murine AIDS is characterized by splenomegaly, lymphadenopathy, reduced B and T cell function, loss of disease resistance, dysfunctional cytokine production, and tissue vitamin E deficiency.[1] In retrovirus-infected people and mice, T helper 1 (Th1) cytokine (IL-2 and IFN-γ) production declines, while Th2 cytokine (IL-4, IL-5, IL-6, and IL-10) production increases.[2, 3, 4] The excessive Th2 cytokines suppress Th1 cells, causing anergy of cell mediated immunity, thus allowing the retrovirus as well as normal flora to reproduce and stimulate oxidative radical secretion by macrophages.[5]

Superoxide radicals including lipid peroxides are produced at greater quantities during retroviral infection as breached immune defenses facilitate increased exposure to bacterial mitogens and endotoxins.[6] Oxidation results in damage to lymphocyte DNA that inhibits cell function. Increased oxidants should promote disease progression from retrovirus infection to AIDS by exacerbating nutritional deficiencies and suppressing immune cell function.[7] Tissue levels of vitamin E and immune function were decreased during murine AIDS.[5] In HIV-infected patients, serum vitamin E levels were reduced while supplementary vitamin E slowed the progression to AIDS.[8]

DHEA and DHEA sulfate (DHEAS) are major secretory products of the adrenal gland

in humans and function as precursors for androgenic hormones. Serum DHEAS levels declined as HIV-1 infection progressed.[9] DHEA supplementation could reduce the immune dysfunction of murine AIDS.[10] DHEA moderated human immune dysfunction by overcoming cytokine dysregulation[11] and preventing cell damage caused by free radicals.[12]

Melatonin (MLT), the principal pineal hormone secreted in response to photoperiod, influences many biological functions. MLT production declines with age when susceptibility to immune dysfunction, cancer, infectious disease, and oxidative damage increases. MLT enhances immunity and resistance to cancer in aged animals.[13] Additionally, as a powerful free radical scavenger,[14] it protects against lipid peroxidation. Although MLT has immunomodulatory properties, its actions on immune dysfunction induced by murine retrovirus infection have not been studied.

Since both MLT and DHEA are antioxidant immunomodulators they would be expected to slow immune dysfunction and oxidation during AIDS. The current study investigated individual and synergistic effects of DHEA and MLT in preventing immune dysfunction, excessive lipid peroxidation, and vitamin E loss during murine retrovirus infection.

MATERIAL AND METHODS

Animals and murine retrovirus infection

Female C57BL\6 mice, 6 wk old, were obtained from the Charles River Laboratories

Inc.(Wilmington, DE). They were housed in transparent plastic cages with stainless steel wire lids (4 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 wk housing and consuming AIN 93A (control) diet, these mice were randomly assigned to one of the following treatments with eight mice per group: uninfected and LP-BM5 infected mice given control diet and drinking water containing 0.05% ethanol; uninfected and infected mice given 0.02% DHEA supplemented diet for the first 3 wk (0.9 mg DHEA/mouse/day) and then 0.06% DHEA supplemented diet for the following 9 wk (2.7 mg DHEA/mouse/day) with 0.05% ethanol containing drinking water; uninfected and infected mice given control diet with 10.0 μg/ml MLT dissolved in 0.05% ethanol containing drinking water (49.8 μg MLT/mouse/day); uninfected and infected mice given 0.02% DHEA supplemented diet for the first 3 wk and then 0.06% DHEA supplemented diet for the following 9 wk with 10.0 μg/ml MLT in 0.05% ethanol containing drinking water. The time and dose of DHEA and MLT treatment were derived from previous work by Araghi-Niknam[10] and Mocchegiani[15] respectively.

LP-BM5 retrovirus was administered i.p. to mice in 0.1 ml with an esotropic titer (XC) of 4.5 $\log_{10}$ plaque-forming units (PFU)/ml, which induces disease with a time course comparable to that previously published.[16] Uninfected mice were injected with complete culture medium [CM, RPMI 1640 containing 10% fetal calf serum (FCS), 2 mM glutamine, 100 units/ml penicillin and streptomycin] as controls. Infection of adult female

C57BL/6 mice with LP-BM5 murine leukemia leads to the rapid induction of clinical symptoms with virtually no latent phase. Supplementation with DHEA and MLT was begun 2 wk after LP-BM5 infection. DHEA was a kind gift from Edenland, Inc. (Baybush, Kildare, Ireland). The 0.02% DHEA supplemented diet and 0.06% DHEA supplemented diet were prepared by Diets Inc.(Bethlehem, PA) using the same pelleted AIN 93A diet. MLT (Sigma, St. Louis, MO) was dissolved in 95% ethanol and then diluted with distilled water. The final concentration of MLT in the drinking water containing 0.05% ethanol was 10.0 μg/ml. The treatment period was 12 wk for all groups.

ELISA for cytokines

IL-2, IFN-γ, IL-4, IL-6, IL-10 and TNF-α were produced by splenocytes as described previously.[17] Rat anti-murine IL-2, IFN-γ, IL-4, IL-6, IL-10 and TNF-α mAb, biotin- rat anti-murine IL-2, IFN-γ, IL-4, IL-6, IL-10 and TNF-α mAb, and standard murine rIL-2, rIFN-γ, rIL-4, rIL-6, rIL-10 and rTNF-α were obtained from Pharmingen (San Diego, CA).

Briefly, spleens were collected after sacrifice under ether anesthesia. Mononuclear cells were obtained by gently teasing with forceps in CM, producing a single cell suspension of spleen cells. RBC were lysed by 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. The 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 Con A (0.1 ml/well; 10 μg/ml; Sigma, St. Louis, MO) for induction of IL-2, IL-4 and IL-10 with 24 hr incubation, and for induction of IFN-γ with 72 hr incubation at 37°C in a 5% CO_2 incubator. Splenocytes were also stimulated by LPS (0.1 ml/well; 10 μg/ml; Gebco, Grand Island, NY) for 24 hr to induce 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 as described previously.[17]

Mitogenesis of splenocytes

Splenic B and T 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 culture plates (Falcon) with LPS (0.1 ml/will; 10 μg/ml) and Con A (0.1 ml/well; 10 μg/ml). They were incubated at 37°C in a 5% CO_2 incubator for 44 h for LPS-induced B cell and Con A-induced T cell proliferation, and then pulsed with ^{3}H-thymidine (0.5 μCi/well; New England Nuclear, Boston, MA). After 4 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 min (CPM).

Determination of vitamin E

Vitamin E levels in liver were measured by HPLC as described previously.[19] Briefly, about 0.2 g of tissue was homogenized in 1 ml of water. Butylated hydroxytoluene was added to prevent oxidation of α-tocopherol. Pentane, ethanol and sodium dodecyl sulfate were used to extract α-tocopherol from the homogenate. Extracts were evaporated under steady flow of nitrogen gas at 20°C and then redissolved in 0.5 ml of methanol injection onto a C18 column (3.9×150 mm NovaPak, Millipore, Bedford, MA). A mobile phase composed of methanol:sodium acetate (1 mol/l) in the ratio of 98:2 (v/v) at a flow rate of 1.5 ml/min was used. α-Tocopherol, eluting at 6.5 min, was monitored by a fluorescence detector (Millipore) at 290 nm excitation and 320 nm emission wavelength.

Determination of conjugated dienes

Approximately 0.5 g of liver tissue was homogenized in 10 ml of Folch solution (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 and used to determine conjugated dienes as previously described.[20] The hepatic levels of conjugated dienes were determined by obtaining absorbency of the solution at 237 nm in a Shimdzo UV 160 UV recording spectrophotometer (Tokyo, Japan) using an appropriate blank.

Determination of phospholipid

The phospholipid content of liver was determined by the method of Raheja et al.[21] This

method does not require predigestion of the phospholipid. Briefly, 0.5 ml chloroform was added then followed by 0.2 ml of a coloring reagent and 3.0 ml of carbon tetrachloride. Phospholipid was determined by obtaining absorbency of the chloroform solution at 710 nm in a Shimadzo UV 160 UV recording spectrophotometer (Tokyo, Japan). Dipalmitoyl phosphatidylcholine was used as a standard.

Determination of total cholesterol

The total cholesterol content of liver was determined by the method of Zak.[22] Briefly 0.3 ml of Folch extract was dried under air at 70°C. Then 3 ml Zak's reagent was added followed by 2 ml of sulfuric acid. Total cholesterol was determined by obtaining the absorbency of the solution at 570 nm in a Zhimadzo UV 170 UV recording spectrophotometer (Yokyo, Japan) using cholesterol standards (Sigma, St. Louis, MO).

Statistics

The statistic 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

Body weight

There was no change in diet and water consumption due to murine retrovirus infection or treatment with DHEA or MLT (data not shown). Body weights were not affected by DHEA or MLT treatment post retrovirus infection (data not shown). Spleen and lymph node weights (data not shown) were significantly ($p<0.05$) elevated in infected mice, indicating that infection had progressed to murine AIDS as shown previously.[1,6] However, neither DHEA nor MLT significantly reduced the elevated spleen weights.

Proliferation of splenocytes

Proliferation of LPS- and Con A-induced splenocytes was significantly ($p<0.05$) reduced by murine retrovirus infection (Fig. 1). This was significantly ($p<0.05$) prevented by DHEA, MLT, and DHEA+MLT supplementation as compared to untreated, infected mice (Fig. 1). DHEA, MLT, and DHEA+MLT similarly significantly ($p<0.05$) increased B cell proliferation in uninfected mice. MLT, DHEA+MLT, but not DHEA increased proliferation of T cells from uninfected mice (Fig. 1). Infected mice treated with DHEA+MLT had significantly ($p<0.05$) higher T cell proliferation than infected mice given either DHEA or MLT alone did.

Cytokine production by splenocytes

In vitro production of Th1 cytokines (IL-2 and IFN-γ) by Con A-stimulated splenocytes was significantly ($p<0.05$) suppressed in retrovirus-infected mice (Fig. 2). Treatment with DHEA, MLT, and DHEA+MLT significantly ($p<0.05$) stimulated IL-2 and IFN-γ release

by mitogen-stimulated splenocytes from both uninfected and infected mice as compared with their untreated controls (Fig. 2). Infected mice treated with DHEA+MLT had significantly ($p<0.05$) higher IL-2 production than mice treated with DHEA alone did. In addition, these mice produced significantly ($p<0.05$) more IFN-γ than infected mice given either DHEA or MLT did.

Release of Th2 cytokines (IL-4, IL-6, and IL-10) (Fig. 3, data not shown for IL-4) as well as TNF-α (data not shown), from mitogen-stimulated splenocytes was significantly ($p<0.05$) elevated in retrovirus-infected mice. Treatment with DHEA, MLT, and DHEA+MLT significantly ($p<0.05$) inhibited production of Th2 cytokines and TNF-α in both uninfected and infected mice (Fig. 3). However, DHEA had no significant effect on IL-10 production in uninfected mice. Cells from infected mice treated with DHEA+MLT produced significantly ($p<0.05$) less IL-4, IL-6, and IL-10 than those from DHEA-treated infected mice.

Hepatic vitamin E and lipid peroxidation

Hepatic vitamin E concentrations were significantly ($p<0.05$) decreased by retrovirus infection (Fig. 4). Treatment with MLT and DHEA+MLT significantly ($p<0.05$) increased vitamin E levels in both uninfected and infected mice as compared with untreated controls (Fig. 4). DHEA+MLT was more effective than DHEA at increasing vitamin E given to uninfected and infected mice, and it was also more effective than MLT fed to uninfected mice.

Retrovirus infection significantly ($p<0.05$) increased hepatic diene conjugates, a major

product of lipid peroxidation (Fig. 4). Treatment of infected mice with DHEA and DHEA+MLT significantly ($p<0.05$) normalized hepatic diene conjugates to levels near those of uninfected mice (Fig. 4). The supplements had no significant effect on diene conjugate levels in uninfected mice.

Hepatic phospholipids and total cholesterol

Retrovirus infection significantly ($p<0.05$) reduced hepatic phospholipids (Fig. 5). Treatment with MLT and DHEA+MLT significantly ($p<0.05$) increased phospholipid levels in uninfected and infected mice compared to their respective untreated controls. DHEA treatment significantly ($p<0.05$) elevated phospholipid levels only in infected mice (Fig. 5).

Significantly ($p<0.05$) higher hepatic total cholesterol was induced by retrovirus infection (Fig. 5). Treatment of infected mice with DHEA and DHEA+MLT maintained near-normal cholesterol levels that were significantly ($p<0.05$) lower than those in infected, untreated mice (Fig. 5). The treatments had no effect on cholesterol levels in uninfected mice, nor did MLT alone suppress the retrovirus-induced increase.

DISCUSSION

The current study showed that retrovirus infection induced immune dysfunction, lipid peroxidation, and vitamin E loss. Murine retrovirus infection inhibited proliferation of B and T cells, repressed release of Th1 cytokines, and stimulated secretion of Th2 cytokines

as well as TNF-α. Retrovirus infection also increased hepatic lipid peroxidation and induced tissue vitamin E deficiency. DHEA and MLT treatment significantly prevented immune dysfunction, excessive lipid peroxidation, and loss of tissue vitamin E induced by retrovirus infection. DHEA and MLT also significantly increased B cell proliferation, Th1 cytokine production, and decreased Th2 cytokine production in uninfected mice. While DHEA and MLT increased hepatic vitamin E and phospholipids in uninfected mice also, they had little effect on conjugated dienes or cholesterol.

DHEA modestly downregulated HIV-1 expression in infected human cells.[23] DHEA blocks cytokine-induced NF-κB activation by inhibiting NADPH-dependent oxidative intermediates, thus preventing HIV-gene expression.[24] As HIV infection progresses, the mean DHEAS levels fall and the cortisol/DHEAS ratio rises,[25] predicting disease progression from HIV-1-infection to AIDS.[26] Since DHEA may have a direct antiglucocorticoid activity, reduced DHEA could allow cortisol to act more effectively, enhancing immunosuppression.[27] Retrovirus infection may shift precursor adrenal hormones from androgenic pathways into cortisol pathways, excessively stimulating cortisol by reducing DHEA production. There was a clear positive relationship between $CD4^+$ lymphocytes and DHEAS values in AIDS patients.[25] DHEA and DHEAS may be natural mediators of T cell responses.[28] High-affinity DHEA binding sites in human and murine T-cells are regulated by DHEA during the process of signal-induced activation.[29] DHEA binding to its receptor is directly related to stimulation of IL-2 production.[30] Lymphocytes from DHEA-treated tolerant mice produced more IFN-γ and less IL-4 and

IL-6 than those from untreated mice did.[31] Thus, DHEA supplementation was expected to stimulate Th1 cells in both uninfected and infected mice.

DHEA prevented suppression of hepatic phospholipids and increase of total cholesterol during murine AIDS. Since membrane fluidity is a necessary component for the function of signal transduction, DHEA may work by improving membrane fluidity which should facilitate lymphocyte function.[32]

We found that MLT treatment prevented retrovirus-induced reduction in B and T cell proliferation and in Th1 cytokine secretion, as well as overproduction of Th2 cytokines and TNF-α. MLT also significantly increased Th1 cytokine production and decreased Th2 cytokine production in uninfected mice. These results support the hypotheses of MLT's role in cytokine regulation. MLT increased immune components deficient in HIV-infected patients including lymphoid cells and Th1 cytokines.[33] Human circulating $CD4^+$ lymphocytes contain specific[34] and high-affinity[35] binding sites for MLT, and MLT has been shown to enhance IL-2 and IFN-γ production.[36] MLT also affects adrenal and sexual glands with immunomodulatory roles.[37]

MLT was more effective than vitamin E in scavenging peroxyl radicals.[14] MLT supplementation significantly increased hepatic vitamin E and phospholipid levels in uninfected mice as well as their suppressed quantities in infected mice. Thus, MLT may protect against lipid peroxidation and chronic immune dysfunction in murine AIDS as well as in uninfected mice by reducing the production of free radicals.

We also observed that infected mice treated with DHEA+MLT had significantly higher splenic T cell proliferation and IFN-γ production than infected mice given either DHEA

or MLT alone. DHEA+MLT treatment was more effective at increasing IL-2 production and decreasing Th2 cytokine production in infected mice than DHEA alone. In addition, DHEA+MLT was more effective at increasing tissue vitamin E levels than either DHEA or MLT alone. Although both DHEA and MLT decline with age,[13,27] this is the first study where their combined treatment and actions were examined in retrovirus-infected mice and uninfected young mice. Lymphocytes from uninfected and infected mice respond similarly to these hormones. Thus, these cells are not unresponsive due to infection. In addition, not only do DHEA and MLT overcome the damage done by infection but also they can further stimulate cells from uninfected young mice who are at the peak of their immune function. Nevertheless these hormones do not totally overcome this damage, as the activities of cells from infected mice do not fully reach those of cells from uninfected mice.

Our results also suggest an association between immune dysfunction, lipid peroxidation, and tissue vitamin E levels during retrovirus infection. Immune dysfunction caused by retrovirus infection decreases host resistance to opportunistic pathogens.[38] Increased infections stimulate phagocytes to release more free radicals, explaining the increased lipid peroxidation.[1] Free radicals react with antioxidant vitamins and also increase TNF-α production which further excites oxidative stress by activating macrophage and neutrophils.[39] The antioxidant, vitamin E, is an important immune modulator particularly in murine AIDS.[5] Vitamin E deficiency could accentuate retroviral immunosuppression since vitamin E supplementation partially restored immune function in retrovirus-infected mice.[5] Oxidative stress stimulates of HIV replication in vitro through activation of NF-κB

which stimulates HIV gene expression by acting on the promoter region of the viral long terminal repeats.[40] In addition, oxidative stress in AIDS may lead to the damage and death of Th cells, thus weakening the immune system. In murine AIDS, increased lipid peroxidation, together with decreased phospholipids and elevated cholesterol levels may be responsible for reduced membrane fluidity and elevated membrane viscosity,[32] interfering with the signal transduction processes.[41] Such changes in HIV-infected people could help explain their increased risk for heart disease.

Increased hepatic lipid peroxidation and decreased vitamin E may be due to the retrovirus-altered cytokine production.[5] Th1 cells secrete IL-2 and IFN-γ, which are involved in stimulating cell-medicated responses. Th2 cells, in contrast, secrete IL-4, IL-6, and IL-10 which activate antibody production and suppress Th1 cells. In vivo, activated B cells and macrophages from HIV-infected patients, produced high levels of IL-6 and TNF-α,[42] as do LPS-stimulated splenocytes and peritoneal macrophages in MuLV retrovirus-infected mice.[43] When IL-4-deficient (IL-4 gene knockout) mice were infected with LP-BM5 retrovirus, there was no lethality and development of T cell abnormalities was delayed.[44] Excessive Th2 cytokines could be responsible for B cell dysfunction during murine AIDS. Elevated levels of IL-6 have also been associated with stimulating HIV replication in macrophages and T cells.[45] IFN-γ administration significantly retarded murine AIDS by preventing activation of Th2 cytokines.[43] Thus, the reduction of Th2 cytokines by hormone supplementation could be a potential treatment for AIDS.[27] Our data demonstrated that T cell proliferation and Th1 cytokines declined

while Th2 cytokines increased during retrovirus infection. Additionally we showed that these effects could be attenuated by hormone supplementation. These results are in accordance with the hypothesis of Clerici et al.[2] which implicates a cytokine imbalance in the pathogenesis of human AIDS.

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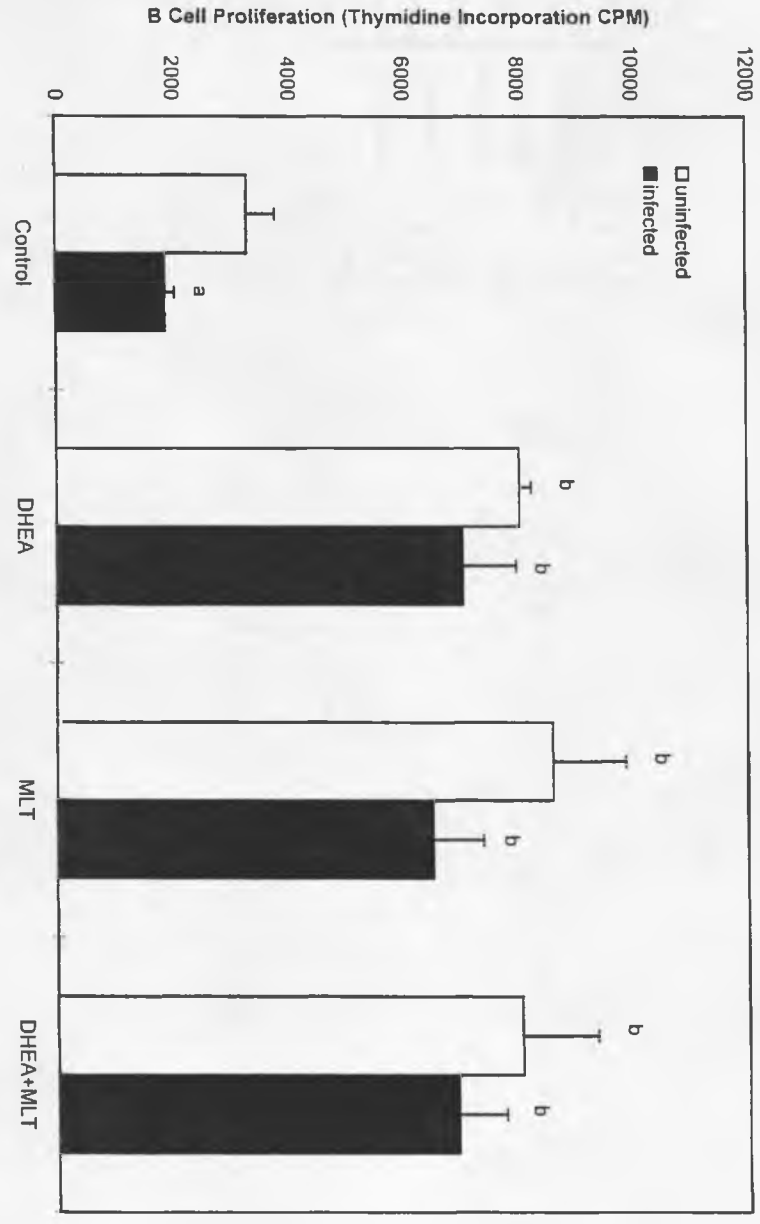
Figure 1. Effects of DHEA, MLT and DHEA+MLT on LPS-stimulated B cell and Con A-stimulated T cell proliferation of splenocytes. Every sample from each mouse was measured in triplicate. The bars are the mean ± SE for eight mice. Letters indicate significant differences at $p<0.05$: a, compared with uninfected mice receiving the same treatment; b, compared with their respective untreated controls; c, compared with their respective DHEA-treated mice; d, compared with their respective MLT-treated mice.

Figure 2. Effects of DHEA, MLT and DHEA+MLT on Th1 cytokine production by splenocytes. Every sample from each mouse was measured in triplicate. The bars are the mean ± SE for eight mice. Letters indicate significant differences at $p<0.05$: a, compared with uninfected mice receiving the same treatment; b, compared with their respective untreated controls; c, compared with their respective DHEA-treated mice; d, compared with their respective MLT-treated mice.

Figure 3. Effects of DHEA, MLT and DHEA+MLT on Th2 cytokine production by splenocytes. Every sample from each mouse was measured in triplicate. The bars are the mean ± SE for eight mice. Letters indicate significant differences at $p<0.05$: a, compared with uninfected mice receiving the same treatment; b, compared with their respective untreated controls; c, compared with their respective DHEA-treated mice.

Figure 4. Effects of DHEA, MLT and DHEA+MLT on hepatic vitamin E concentrations and conjugated diene production. Every sample from each mouse was measured in triplicate. The bars are the mean ± SE for eight mice. Letters indicate significant differences at $p<0.05$: a, compared with uninfected mice receiving the same treatment; b, compared with their respective untreated controls; c, compared with their respective DHEA-treated mice; d, compared with their respective MLT-treated mice.

Figure 5. Effects of DHEA, MLT and DHEA+MLT on hepatic phospholipid content and total cholesterol levels. Every sample from each mouse was measured in triplicate. The bars are the mean ± SE for eight mice. Letters indicate significant differences at $p<0.05$: a, compared with uninfected mice receiving the same treatment; b, compared with their respective untreated controls; c, compared with their respective DHEA-treated mice; d, compared with their respective MLT-treated mice.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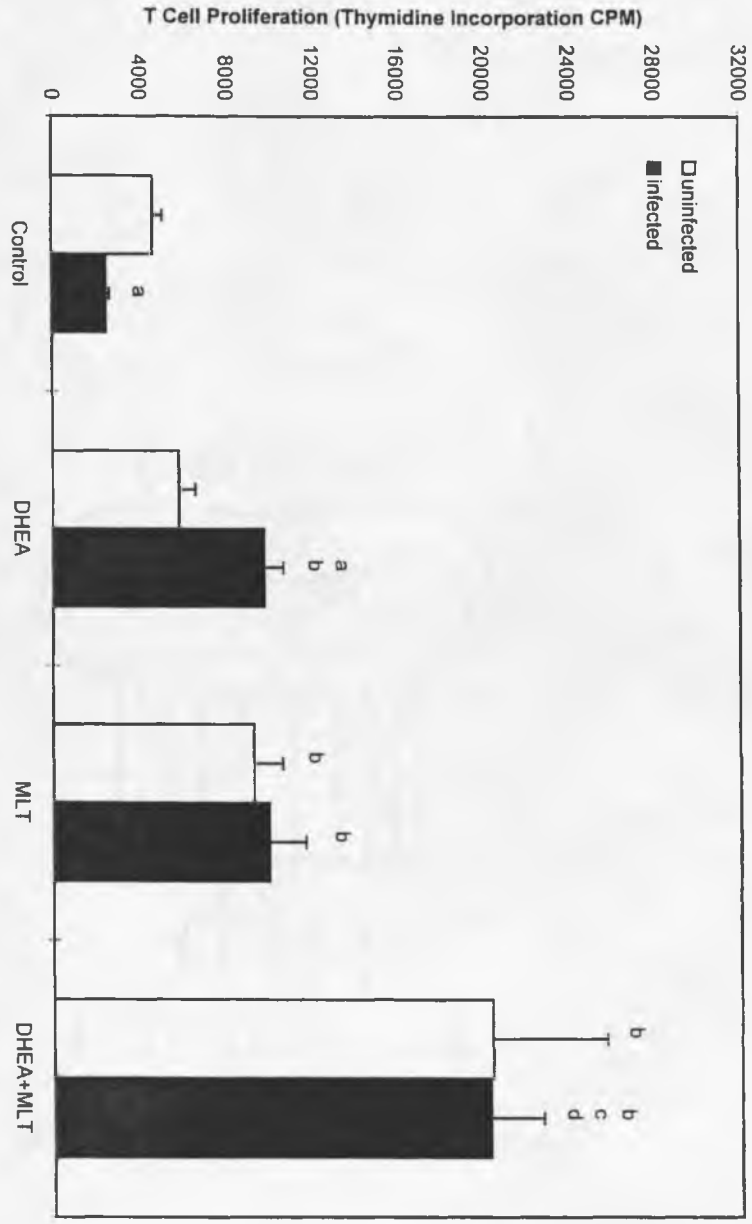
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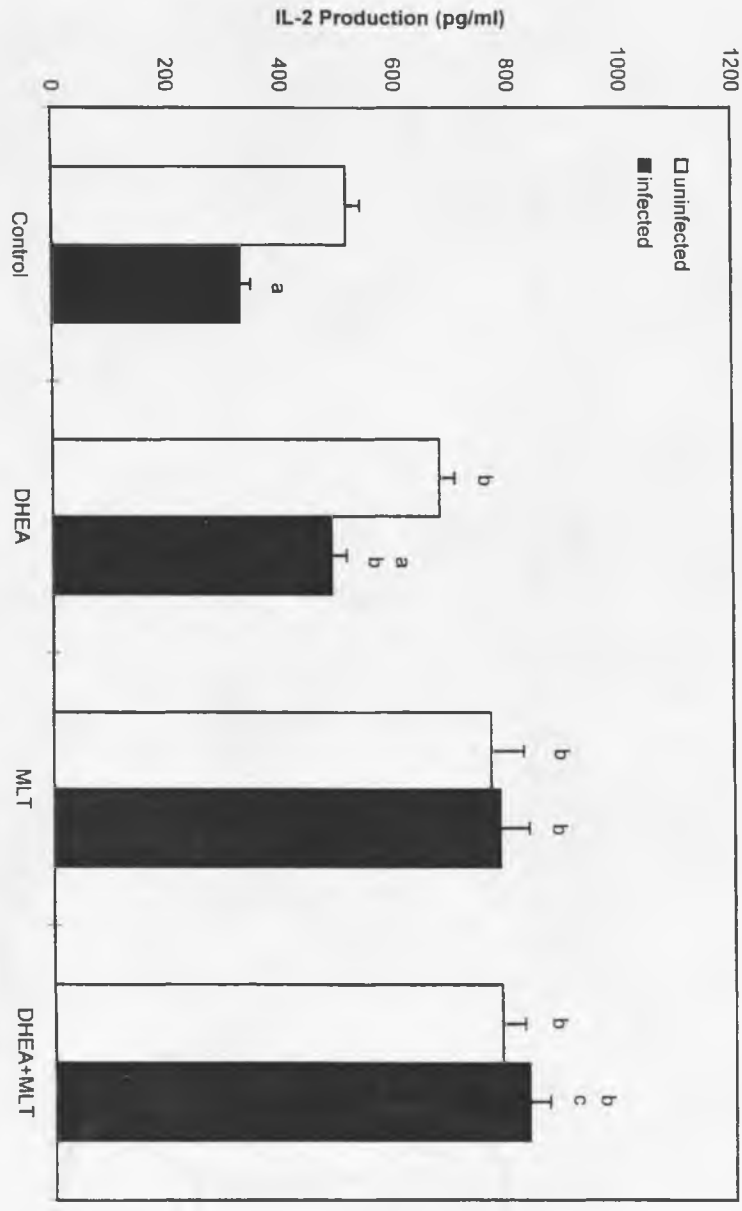
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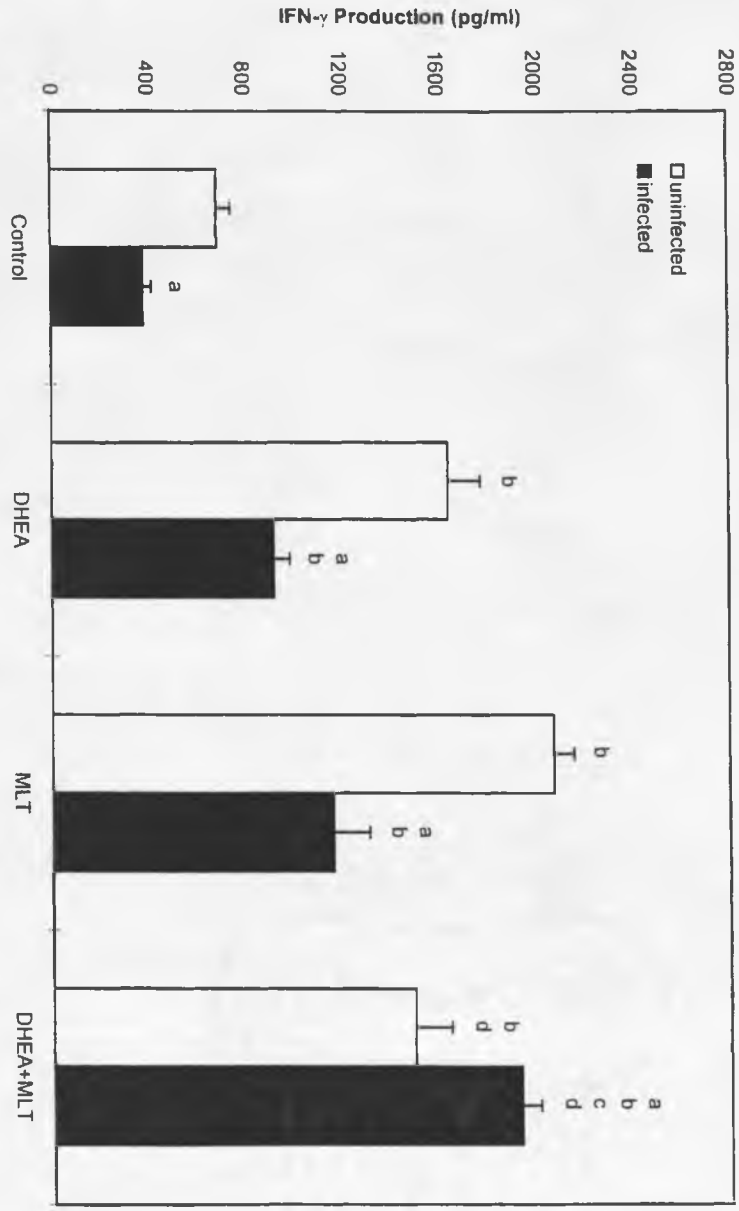
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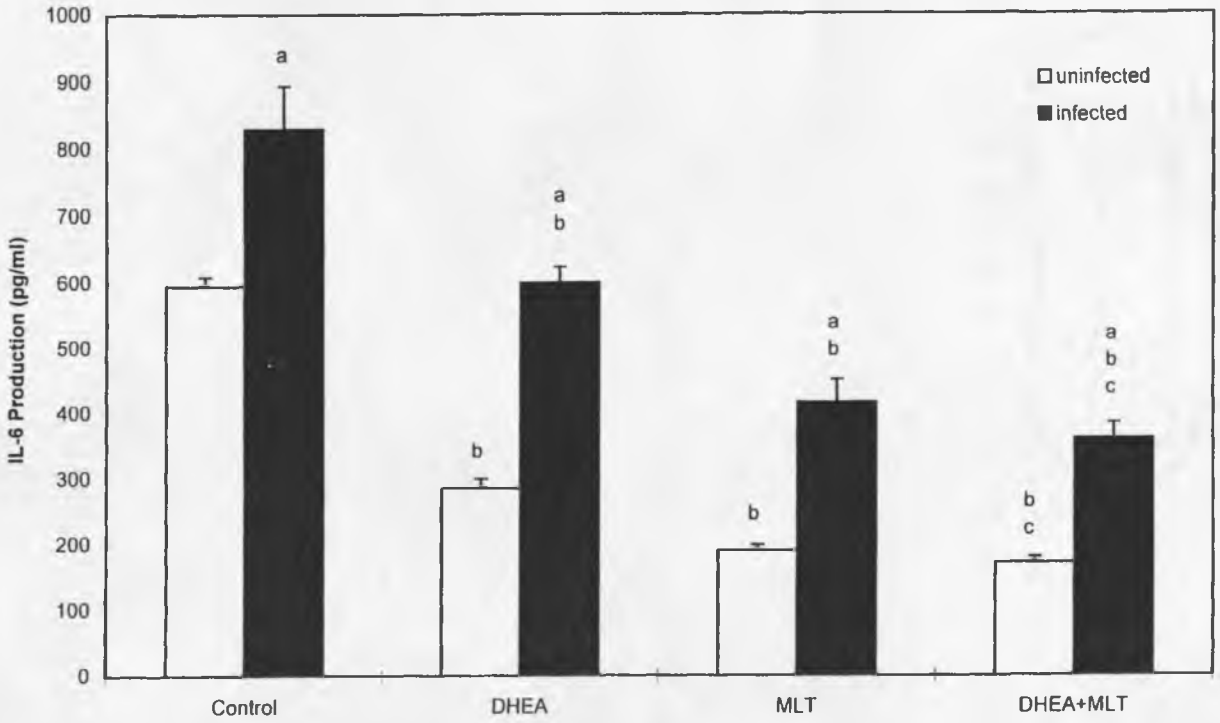
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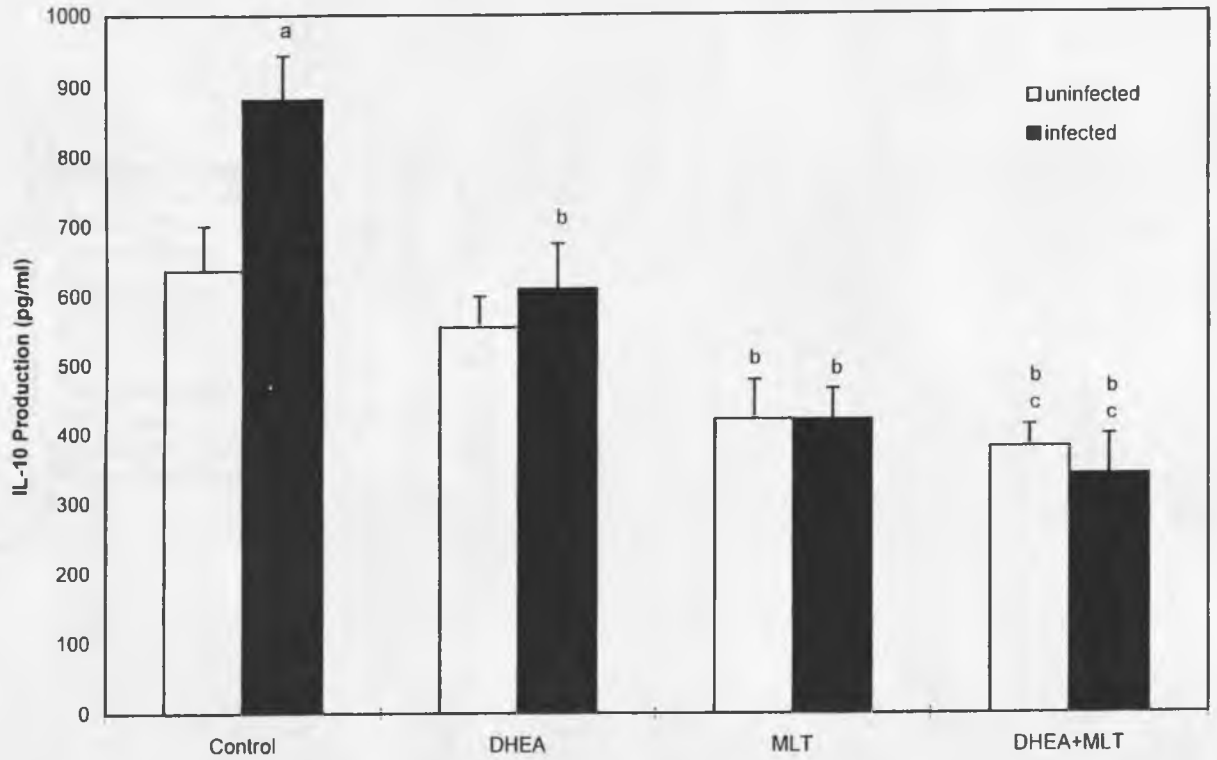
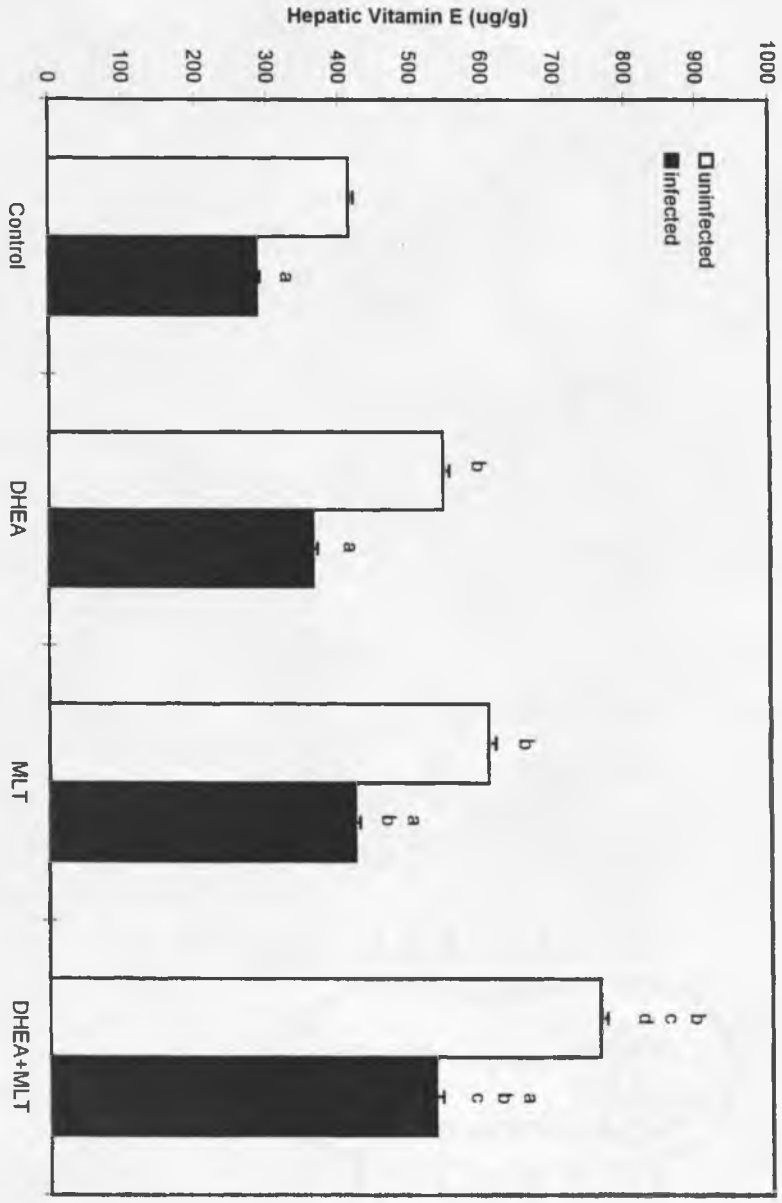
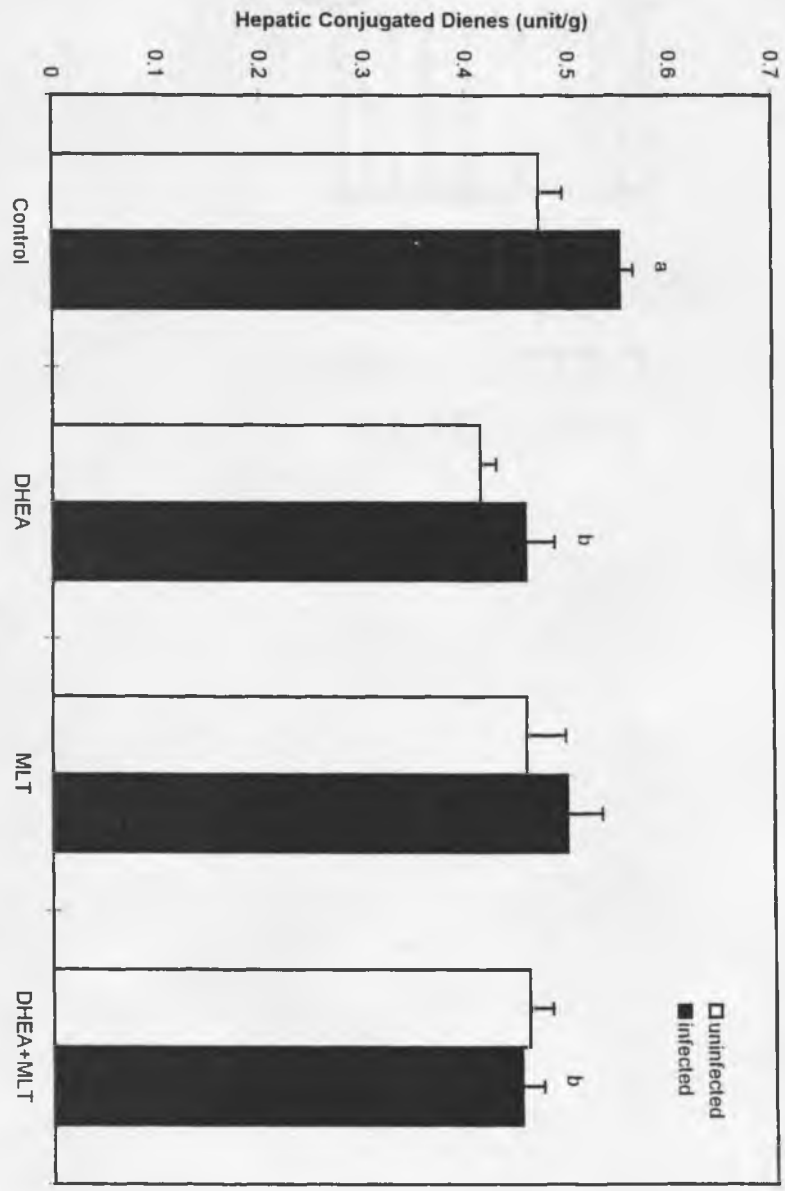
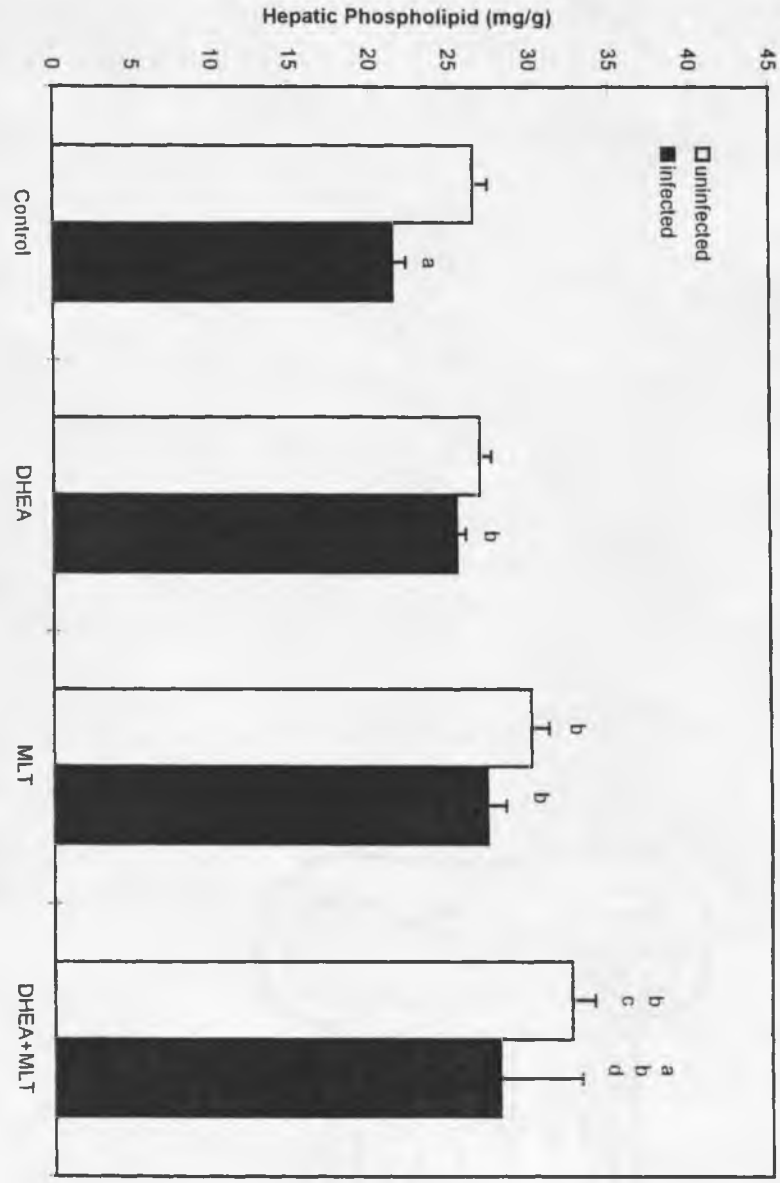
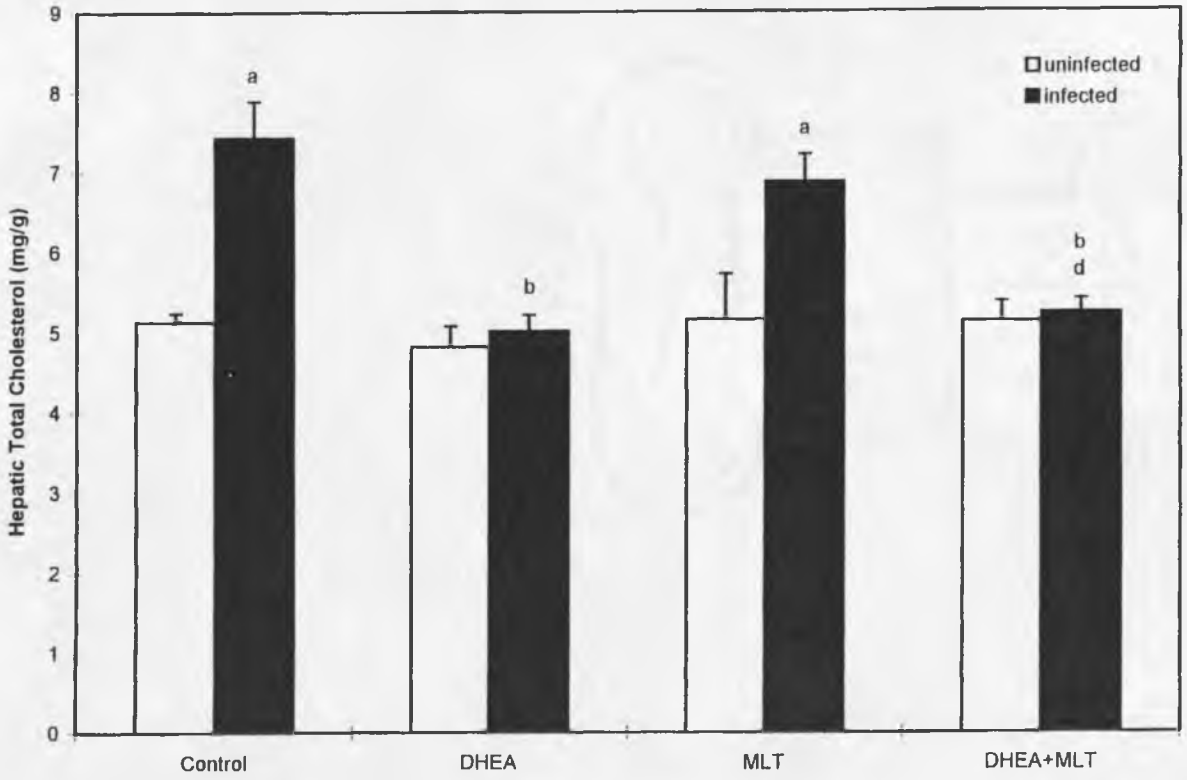
200
100
0
uninfected
infected
a
a
b
b
a
b
b
a
b
c
b
c
Control
DHEA
MLT
DHEA+MLT


IL-10 Production (pg/ml)
1000
900
800
700
600
500
400
300
200
100
0
a
b
b
b
b
c
b
c
uninfected
infected
Control
DHEA
MLT
DHEA+MLT


Hepatic Vitamin E (ug/g)
0
100
200
300
400
500
600
700
800
900
1000
uninfected
infected
Control
DHEA
MLT
DHEA+MLT
a
b
a
b
b
a
b
b
c
d
a
b
c


Hepatic Conjugated Dienes (unit/g)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Control
DHEA
MLT
DHEA+MLT
a
b
b
uninfected
infected


Hepatic Phospholipid (mg/g)
0
5
10
15
20
25
30
35
40
45
uninfected
infected
Control
DHEA
MLT
DHEA+MLT
a
b
b
b
b
c
a
b
d


Hepatic Total Cholesterol (mg/g)
9
8
7
6
5
4
3
2
1
0
uninfected
infected
a
b
a
b
d
Control
DHEA
MLT
DHEA+MLT

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

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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-

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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.

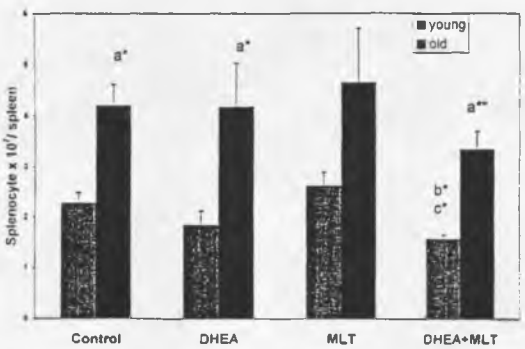

Figure 1. Effect of DHEA, MLT, and DHEA + MLT on splenocyte numbers in old and young mice. The values are mean ± SE. The data represent eight young mice per group and four old mice per group. (a) *P* value compares old mice with young mice receiving the same treatment. (b) *P* value compares DHEA + MLT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

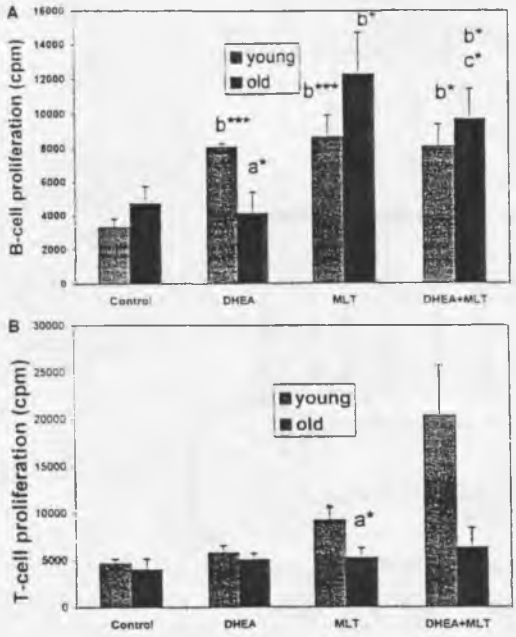

Figure 2. Effect of DHEA, MLT, and DHEA + MLT on (A) B cell proliferation by LPS-stimulated and (B) T-cell proliferation by ConA-stimulated splenocytes from old and young mice. Each sample was done in triplicate and averaged. The values are mean ± SE. The data represent eight young mice per group and 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. $^{*}P < 0.05$, $^{**}P < 0.005$.

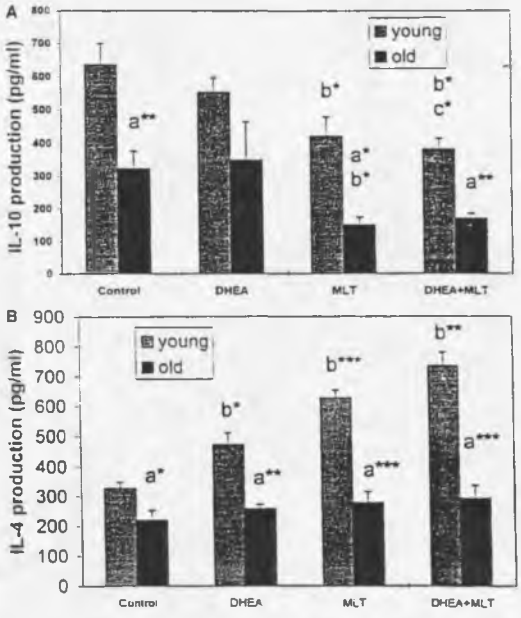

Figure 3. Effect of DHEA, MLT, and DHEA + MLT on (A) IL-10 and (B) IL-4 production by ConA-stimulated splenocytes from old and young mice. Each sample was done in triplicate and averaged. The values are mean ± SE. The data represent eight young mice per group and four old mice per group. (a) *P* value 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

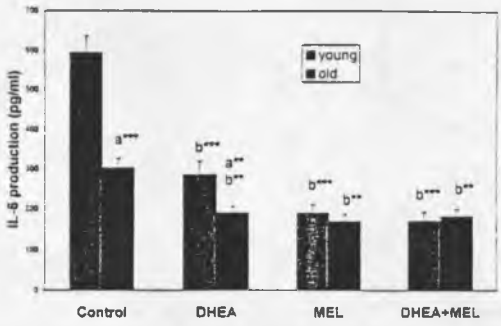

Figure 4. Effect of DHEA, MLT, and DHEA + MLT on IL-6 production by ConA-stimulated splenocytes from old and young mice. Each sample was done in triplicate and averaged. The values are mean ± SE. The data represent eight young mice per group and four old mice per group. (a) *P* value compares old mice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

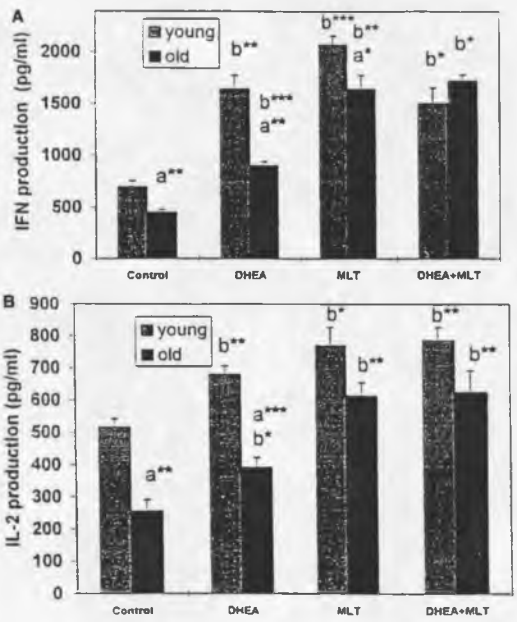

Figure 5. Effect of DHEA, MLT, and DHEA + MLT (A) IFN-γ and (B) IL-2 production by ConA-stimulated splenocytes from old and young mice. Each sample was done in triplicate and averaged. The values are mean ± SE. The data represent eight young mice per group and four old mice per group. (a) *P* value compares 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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Prevention of Retrovirus-Induced Aberrant Cytokine Secretion, Excessive Lipid Peroxidation, and Tissue Vitamin E Deficiency by T Cell Receptor Peptide Treatments in C57BL/6 Mice (44074)

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Abstract. **To test whether T cell receptor (TCR) peptide treatment can prevent immune dysfunction, excessive lipid peroxidation, and malnutrition caused by retrovirus infection, female C57BL/6 mice were infected with LP-BM5 retrovirus. Infection with retrovirus inhibited lymphocyte proliferation, cytokine release T helper 1 cells, stimulated cytokine secretion by T helper 2 cells, induced abnormal hepatic and cardiac lipid profiles, and produced excessive tissue lipid peroxidation with hepatic and cardiac vitamin E deficiency. Two weeks after infection, TCR peptides Vβ5.2, Vβ8.1, Vβ8.1 + Vβ5.2, Vβ8.1(N), and Vβ8.1° were injected to the mice at dose of 200 μg/mouse. Vβ8.1 and Vβ5.2 treatments largely maintained lymphocyte proliferation and IL-2 and IFN-γ release, and prevented excessive IL-6, IL-10, and TNF-α secretion. Concomitantly, these treatments normalized hepatic and cardiac lipid profiles, reduced tissue lipid peroxidation, and thereby significantly maintained vitamin E in the liver and heart. Vβ8.1 segments treatment did not prevent the immune dysfunction, abnormal lipid profile and lipid peroxidation, and vitamin E deficiency caused by the retrovirus infection. In conclusion, injection of intact TCR peptides during murine retrovirus infection largely prevented immune dysfunction by blocking the excessive stimulation of a T cell subset caused by retroviral superantigens. It also ameliorated malnutrition status by normalizing lipid profile, lipid peroxidation, and vitamin E deficiency. T cell immune dysfunction and its prevention by TCR peptide treatment is important in the therapy of vitamin E deficiency induced by retrovirus infection.**
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Murine acquired immune deficiency syndrome (AIDS) is induced by infection with the LP-BM5 murine leukemia retrovirus mixture. It shares many similarities to the pathogenesis of human AIDS, even though human immunodeficiency virus (HIV) and LP-BM5 murine leukemia virus (MuLV) represent different types of retrovirus (1). Murine AIDS is characterized by lymphadenopathy, splenomegaly, hypergammaglobulinemia, deficient B cell response to T-independent antigens *in vitro*, reduced T cell functions, loss of disease resistance, impaired cytokine production, and tissue vitamin E deficiency (1).

Anorexia, weight loss, and complications of recurrent infections in AIDS patients frequently progress to multiple nutrient deficiencies and protein energy malnutrition (2), which could accelerate immunosuppression. Superoxide radicals including hydrogen peroxides, hydroxyl radicals, and lipid peroxides are produced at high levels when immune defenses are breached with increased exposure to bacterial mitogens and endotoxins. These highly reactive oxygen-containing molecules may facilitate disease progression from HIV infection

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to AIDS (3) by their reaction with antioxidant vitamins, exacerbating nutritional deficiency as well as directly inducing immunosuppression.

Vitamin E (4) may be an important immune modulator as tissue levels of vitamin E are reduced by immune dysfunction during murine AIDS (5–7). In uninfected mice, vitamin E supplementation (15 times of basal content 500 IU/g) increased the $CD4^+/CD8^+$ ratio and total lymphocytes, and stimulated activity of cytotoxic cells, natural killer (NK) cell activity, T- and B-cell mitogen responsiveness, and phagocytosis by macrophages (4, 8). Vitamin E deficiency could accentuate the immunosuppression of the retrovirus infection as supplementation of vitamin E partially restored immune function in retrovirus-infected mice (5–7). The loss of vitamin E may be due to murine retrovirus-induced immune dysfunction (5), resulting in increased production of free radicals and lipid peroxides, which are immunosuppressive and could accelerate development of murine AIDS.

Immunological methods preventing retrovirus-induced immune dysfunction have been studied to assert their inhibiting effects on the excessive lipid peroxidation and loss of vitamin E during infection. T-cell receptor (TCR) peptide treatment largely prevented the loss of immune function during retroviral infection by blocking the interaction of retroviral superantigens with a subset of T cells (9). This treatment stopped the T-cell subset from being stimulated to become activated T-helper 2 (Th2) cells whose excessive cytokine production suppresses T-helper 1 (Th1) cells and cellular immunity. The response of autoantibodies to the TCR peptides, elevated to regulate the activated T cells subsets, was stimulated by the murine retrovirus (10). Infected mice made high levels of antibodies against two human TCR peptides to suppress T cells bearing homologous murine Vβ peptides. Thus these two human peptides were identified as important factors in the mouse's attempts to regulate and suppress specific T-cell subsets, excessively stimulated by retroviral superantigen (10). By stopping stimulation of this subset by retroviral superantigens, TCR peptide treatment prevented T-helper 0 (Th0) cells' conversion to Th2 cells and excessive production of Th2 cytokines (9). Hyperproduction of Th2 cytokines suppresses neighboring Th0 and Th1 cells and induces immune suppression. While several TCR peptides are good immunogens, the peptides used to treat murine AIDS were not, even when used with adjuvants (9). Thus, additional antibodies did not develop against this Vβ peptides after their injection into the infected mice even in the presence of adjuvant. Therefore, the TCR peptide prevents excessive stimulation of the Th2 cells by retroviral superantigens by an unknown mechanism and their production of large amounts of IL-4, -6, and -10 (9). Th2 cytokines suppress Th1 cells, causing anergy of cell mediated immunity, allowing the retrovirus to continue to reproduce and stimulate oxidative radical secretion by macrophages (11).

The current study tested whether treatment with different TCR Vβ CDR1 peptides or fragments of the peptides would balance cytokine production, reduce oxidative damage, lipid peroxidation, and thus prevent the loss of tissue vitamin E during the LP-BM5 retrovirus infection.

Methods and Materials

Animals and Murine AIDS. Female C57BL/6 mice, 4 weeks old, were obtained from the Charles River Laboratories Inc. (Wilmington, DE) and housed in transparent plastic cages with stainless steel wire lids (four mice per cage) in the animal facility of the Arizona Health Science Center. 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 12:12-hr light:dark cycle. Water and semipurified diet (4% mouse diet, #7001; Teklad, Madison, WI) were freely available. After 2 weeks of housing, the mice in the dose and adjuvant studies were randomly assigned to the following treatments with eight mice per group: uninfected, normal mice injected with saline (pyrogen free); LP-BM5–infected, normal mice injected with saline (pyrogen free); LP-BM5–infected mice injected with 200 μg TCR internal Vβ 5.1 control peptide; LP-BM5–infected mice injected with 200 μg TCRVβ8.1 pep β3 CDR1 peptide; LP-BM5–infected mice injected with 200 μg TCRVβ5.2 CDR1 peptide; LP-BM5–infected mice injected with 200 μg TCRVβ8.1 pep β3 and 200 μg Vβ5.2 CDR1 peptide; LP-BM5–infected mice injected with 200 μg TCR pep β3(N) CDR1 peptide segment; LP-BM5–infected mice injected with 200 μg TCR pep β3© CDR1 peptide segment.

LP-BM5 retrovirus was administered intraperitoneal to mice in 0.1 ml minimum essential medium (MEM) medium with an ecotropic titer (XC) of 4.5 $\log_{10}$ plaque forming units $\times 10^{-3}$/l, which induces disease with a time course comparable to that previously published (1). Administration of peptides (dissolved in saline) and adjuvants was performed 2 weeks after LP-BM5 infection. Uninfected mice were injected with MEM used for LP-BM5 virus growth as controls. Infection of adult female C57BL/6 mice with LP-BM5 MuLV leads to the rapid induction of clinical symptoms with virtually no latent phase (1).

The infection and treatment period was 14 weeks for all groups. Mice were sacrificed while under ether anesthesia. Spleens and lymph nodes were then dissected, removed, and kept at 4°C. Livers and hearts for nutritional analysis were collected and stored at −70°C until assayed.

Peptides. A set of overlapping 16-mer peptides that duplicate covalent structure of the TCR β product (12, 13) predicted from the human JURKAT sequence (14) has been produced (Table I). An effective immunomodulatory peptide has the sequence C K P I S G H N S L F W Y R Q T, which corresponds to the complete CDR1 and N-terminal five residues of Fr2 (12, 13) of the human Vβ8.1 gene

Table I. T-Cell Receptor Vβ Synthetic Peptides Used in the Study

Description	Sequence	Designation
CDR1 of Vβ8.1	C K P I S G H N S L F W Y R Q T	Vβ8.1
N terminus of Vβ8.1	C K P I S G H N S L F	Vβ8.1(N)
C terminus of Vβ8.1	S G H N S L F W Y R Q T	Vβ8.1(C)
CDR1 of Vβ5.2	C S P K S G H D T V S W Y Q Q A	Vβ5.2
Internal Vβ5.1	S P R S G D L S V Y	INT 5.1

product (14). Two segments of Vβ8.1 were used in this study. They were the N-terminal of the Vβ8.1 and C-terminal of the Vβ8.1 (15). A peptide corresponding to the sequence of the 16 mer of the Vβ5.2 peptide gene product, C S P K S G H D T V S W Y Q Q A, was synthesized as a homolog often recognized by autoantibodies. Normal polyclonal IgG pools contain natural AAbs against peptide segments correspond to CDR1, Fr3 and to a constant region "loop" peptide (12). Untreated mice also have natural IgG antibodies directed against the same peptide segments; in particular, there is strong reactivity to the human CDR1 test peptides (15). A computer comparison of human and murine Vβ sequences (Marchalonis, unpublished analysis) using the progressive alignment algorithm of Feng and Doolittle (16) showed that certain human and murine Vβ sequences could be grouped into families (e.g., human Vβ6 and Vβ8 correspond to murine Vβ11, and human and murine Vβ5 are in the same clusters).

Determination of Conjugated Dienes and Lipid Fluorescence. Approximately 0.5 g of tissue was homogenized in 10 ml of Folch solution (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 and used to determine conjugated dienes and lipid fluorescence as previously described (17). The residue was redissolved in methylene chloride and washed twice with water. To the methylene chloride solution was added 0.5 ml methanol to clarify the emulsion. Conjugated diene fatty acids were determined by obtaining absorbency of the solution at 237 nm in a Beckman DU-7 recording spectrophotometer (Fullerton, CA) using an appropriate blank. Lipid fluorescence of the homogenate was measured in a Hitachi F-2000 fluorescence spectrophotometer (Hitachi Ltd, Tokyo) with a setting of fluorescence maximum at 470 nm and an activation wavelength of 395 nm. Details of the methods used have been previously described (18).

Determination of Phospholipid. The phospholipid content of the livers and hearts was determined by the method of Raheja *et al.* (19). This method does not required the predigestion of the phospholipid. Dipalmitoyl phosphatidylcholine was used as a standard.

Determination of Vitamin E. Vitamin E levels in liver and heart tissues were measured by high-pressure liquid chromatography as described previously (20). Briefly, approximately 0.1 g of tissue was homogenized in 1 ml of water. Butylated hydroxytoluene was added to prevent oxidation of α-tocopherol. Pentane, ethanol, and sodium dodecyl sulfate were used to extract α-tocopherol from the homogenate. Extracts was evaporated under steady flow of nitrogen gas at 20°C and then redissolved in 0.5 ml methanol injection onto a C18 column (3.9 × 150 mm NovaPak; Millipore, Bedford, MA). A mobile phase composed of methanol and sodium acetate in the ratio of 98:2 (by volume) at a flow rate of 1.5 ml/min was used. α-Tocopherol, eluted at 6.5 min, was monitored by a fluorescence detector (Millipore, Bedford, MA) at 290 nm excitation and 320 nm emission wavelength.

Standard Cytokines and Their Antibodies. Rat anti-murine IFN-γ, IL-2, IL-6, and IL-10 purified antibodies; rat anti-murine IFN-γ, IL-2, IL-6, and IL-10 biotinylated antibodies; and recombinant murine IFN-γ, IL-2, IL-6, and IL-10 were obtained from Pharmingen (San Diego, CA).

ELISA for Cytokines. The production of IFN-γ, IL-2, IL-6, and IL-10 from mitogens-stimulated splenocytes was determined as described previously (21). Briefly, spleens were gently teased with forceps in culture medium (CM, RPMI 1640 containing 10% fetal calf serum, 2 m*M* glutamine, 1×10^5 units/l penicillin and streptomycin), producing a 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 concentrations were counted and adjusted to 1×10^{10} cells/l. Splenocyte viability was more than 95% as determined by trypan blue exclusion. Cultured in triplicate on 96-well flat-bottom culture plates (Falcon 3072, Lincoln Park, NJ) with CM were 0.1 ml/well of splenocytes (1×10^{10} cell/l). The splenocytes were then stimulated with concanavalin A (Con A, 1×10^{-2} g/l, 0.1 ml/well, Sigma Chemical Co., St. Louis, MO) to determine their production of IL-2 and IL-10 after 24 hr of incubation, IFN-γ after 72 hr of incubation in a 37°C, 5% CO_2 incubator. Splenocytes were also incubated for 24 hr after the addition of lipopolysaccharide (LPS, 1×10^{-2} g/l; Gibco, Grand Island, NY) to induce IL-6 and TNF-α production. After incubation, the plates were centrifuged for 10 min at 800 *g*. Supernatants were collected and stored at −70°C until analysis. The cytokines were determined by sandwich enzyme-linked immunosorbent assay (ELISA) as described previously (21).

Statistics. All parameters were compared using a one-way analysis of variance (ANOVA), followed by a *t*

test (two-sample assuming unequal variances) for comparison between any two groups. $P < 0.05$ was considered to be significantly different between two groups.

Results

Body Weight. Body weights were not affected by various TCR Vβ CDR1 peptide treatments post retrovirus infection (data not shown). There was no significant change in food consumption due to infection or peptide treatments (data not shown). The spleen and lymph node weights (14 weeks postinfection) were significantly ($P < 0.05$) elevated in the infected mice (data not shown), which indicated that infection had progressed to murine AIDS (1). However, none of the peptides significantly prevented the increase in spleen weight.

Hepatic and Cardiac Vitamin E. The liver and heart are the major organs which have been studied for tissue vitamin E deficiency in murine AIDS (5–7). Hepatic and cardiac vitamin E was significantly ($P < 0.05$) reduced by retrovirus infection (Fig. 1 and Table II). TCR Vβ8.1 and Vβ5.2 peptides treatment significantly ($P < 0.05$) retarded the loss of tissue vitamin E during infection (Fig. 1 and Table II). Infected mice administered TCR Vβ8.1, Vβ5.2, and Vβ8.1 + Vβ5.2 peptides had significantly ($P < 0.05$) higher hepatic and cardiac vitamin E levels than infected mice without TCR peptide treatment. Retrovirus-infected mice that received TCR Vβ8.1(N) and Vβ8.1© peptide segments treatment had significantly lower ($P < 0.05$) hepatic and cardiac vitamin E levels than uninfected mice as well as infected mice injected with TCR Vβ8.1 and Vβ5.2 peptides, alone and combined. Mice treated with the combination of Vβ8.1 + Vβ5.2 peptides treatment maintained hepatic and cardiac vitamin E levels near those of uninfected mice.

Hepatic and Cardiac Lipid Peroxidation. Diene conjugates and lipid fluorescence are the major products of lipid peroxidation. Significantly ($P < 0.05$) higher hepatic and cardiac diene conjugates and lipid fluorescence levels were induced by retrovirus infection (Fig. 2, A and B; Table II). Treatment with TCR Vβ8.1 and Vβ5.2 significantly ($P > 0.05$) retarded the excessive production of diene conjugates and lipid fluorenscence in the liver and heart during infection (Fig. 2 A and B; Table II). Infected mice administered TCR Vβ8.1, Vβ5.2, and Vβ8.1 + Vβ5.2 peptides had significant ($P < 0.05$) lower hepatic and cardiac diene conjugates and lipid fluorescence levels than that of infected mice without TCR peptide treatment. Retrovirus-infected mice that received TCR Vβ8.1(N) and Vβ8.1© peptide segments treatment had significantly higher ($P < 0.05$) hepatic and cardiac diene conjugates and lipid fluorescence levels than uninfected mice as well as infected mice treated with TCR Vβ8.1 and Vβ5.2. The mice given the combination of Vβ8.1 + Vβ5.2 peptides maintained hepatic and cardiac diene conjugates and lipid fluorescence levels near those of uninfected mice.

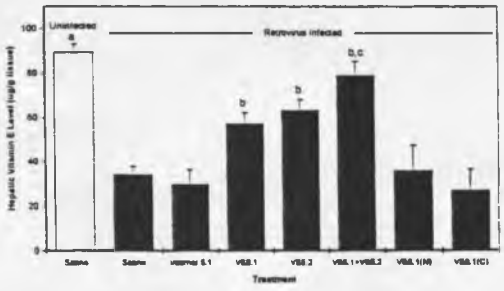

Figure 1. Effect of different T cell receptor peptide treatments on hepatic vitamin E level. Values are mean ± SD; $n = 8$. Letters indicate significant differences at $P < 0.05$: [a]compared with all of the retrovirus-infected groups; [b]compared with the retrovirus-infected groups injected with saline, internal 5.1 peptide, Vβ8.1(N), and Vβ8.1(C) peptide; [c]compared with the retrovirus-infected groups injected with Vβ8.1 and Vβ5.2 peptide alone.

Hepatic Lipid Profiles—Phospholipid, Triacylglycerol, and Cholesterol Levels. Lipid profiles significantly affect fat-soluble vitamin E levels in tissue. Significantly ($P < 0.05$) higher hepatic phospholipid, triacylglycerol, and cholesterol levels were caused by retrovirus infection (Fig. 3, A–C). TCR Vβ8.1 and Vβ5.2 peptides treatment significantly ($P < 0.05$) retarded the excessive accumulation or synthesis of phospholipid, triacylglycerol, and cholesterol in the liver during infection (Fig. 3, A–C). Infected mice administered TCR Vβ8.1, Vβ5.2, and Vβ8.1 + Vβ5.2 peptides had significant ($P < 0.05$) lower hepatic phospholipid, triacylglycerol, and cholesterol levels than that of infected mice without TCR peptide treatment. Retrovirus-infected mice that received TCR Vβ8.1(N) and Vβ8.1© peptide segments had significantly higher ($P < 0.05$) hepatic phospholipid, triacylglycerol, and cholesterol levels than uninfected mice as well as infected mice injected with TCR Vβ8.1 and Vβ5.2. The mice treated with the combination of Vβ8.1 + Vβ5.2 peptides maintained hepatic phospholipid, triacylglycerol, and cholesterol levels near those of uninfected mice.

Immune Function Analysis. IL-2 and IFN-γ are secreted by Th1 lymphocytes and module cell-mediated immunity. IL-6, IL-10, and TNF-α are produced by Th2 lymphocytes and regulate humoral responses while suppressing Th1 cells (22). Significantly ($P < 0.05$) lower Th1 cytokines levels (Fig. 4A; data not shown for IFN-γ) and excessive Th2 cytokines levels (Fig. 4B; data not shown for IL-6 and IL-10) were induced by retrovirus infection. TCR Vβ8.1 and Vβ5.2 peptides treatment significantly ($P < 0.05$) normalized cytokine production by Th1 cells and retarded the excessive production of cytokine by Th2 cells during infection. Infected mice administered TCR Vβ8.1, Vβ5.2, and Vβ8.1 + Vβ5.2 peptides had significant ($P < 0.05$) higher Th1 cytokines production and lower Th2 cytokines production than did infected mice without TCR peptide treatment. Retrovirus-infected mice that received TCR Vβ8.1(N) and Vβ8.1© peptide segments treatment had significantly ($P < 0.05$) lower Th1 cytokines production and higher Th1 cytokines production than uninfected mice as well as infected

Table II. Cardiac Vitamin E, Diene Conjugates, and Lipid Fluorescence Levels

Treatment	Vitamin E (μg/g tissue)	Diene conjugates (absorbance units/mg phospholipid)	Lipid fluorescence (Fluorescence units/mg phospholipid)
Uninfected + saline	69.2 ± 3.1^{a}	1.9 ± 0.6^{a}	12.7 ± 2.8^{a}
Infected + saline	12.9 ± 3.5	6.7 ± 0.9	36.8 ± 2.3
Infected + internal 5.1	15.7 ± 5.7	6.1 ± 1.5	39.9 ± 5.9
Infected + Vβ8.1	38.3 ± 6.1^{b}	3.5 ± 1.1^{b}	29.1 ± 4.3^{b}
Infected + Vβ5.2	45.1 ± 5.8^{b}	3.2 ± 1.3^{b}	26.4 ± 4.1^{b}
Infected + Vβ8.1 + Vβ5.2	$62.8 \pm 7.3^{b,c}$	$2.1 \pm 0.6^{b,c}$	$18.3 \pm 5.4^{b,c}$
Infected + Vβ8.1 (N)	21.5 ± 12.8	5.4 ± 1.7	35.5 ± 9.7
Infected + Vβ8.1 (C)	16.3 ± 10.6	7.5 ± 1.9	31.0 ± 10.2

Note. Every sample from each mouse was measured in triplicate. Values are mean ± SD; $n = 8$. Letters indicate significant differences at $P < 0.05$: [a]compared with all of the retrovirus-infected groups; [b]compared with the retrovirus-infected groups with TCR Vβ8.1, Vβ5.2, and Vβ8.1 + Vβ5.2 peptide treatment; [c]compared with the retrovirus-infected groups with single TCR Vβ8.1 and TCR Vβ5.2 peptide treatment.

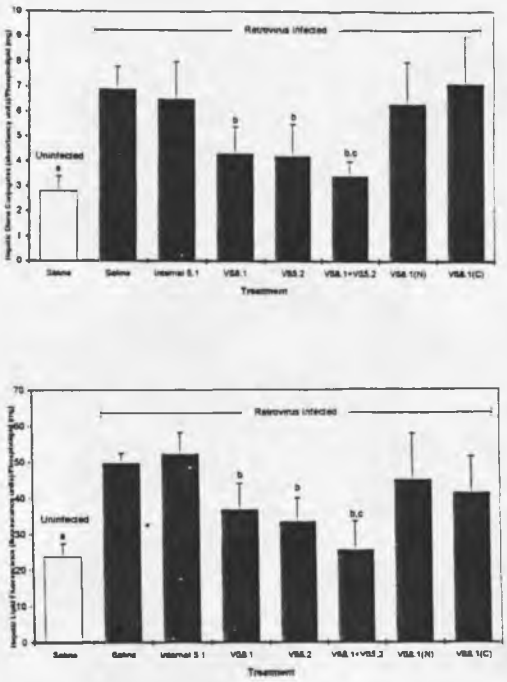

Figure 2. Effect of different T cell receptor peptide treatments on hepatic diene conjugates level (A) and on hepatic lipid fluorescence level (B). Every sample from each mouse was determined in triplicate. Values are mean ± SD; $n = 8$. Letters indicate significant differences at $P < 0.05$: [a]compared with all of the retrovirus-infected groups; [b]compared with the retrovirus infected groups injected with saline, internal 5.1 peptide, Vβ8.1(N), and Vβ8.1(C) peptide; [c]compared with the retrovirus infected groups injected with Vβ8.1 and Vβ5.2 peptide alone.

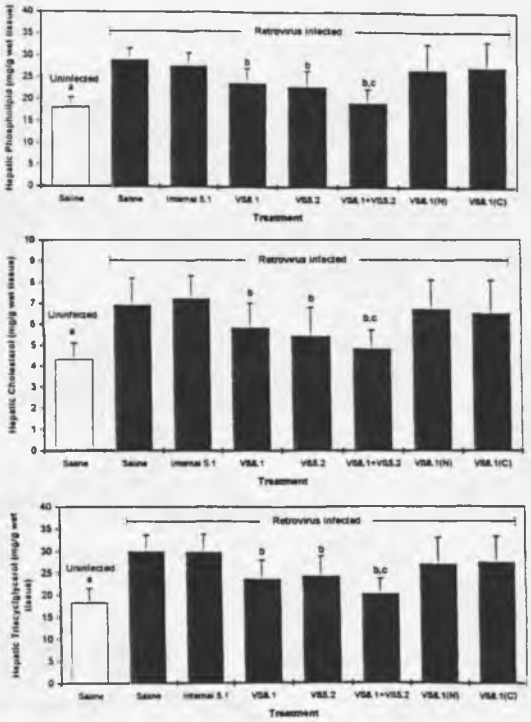

Figure 3. Effect of different T cell receptor peptide treatments on hepatic phospholipid level (A), tnacyclglycerol level (B), and cholesterol level (C). Every sample from each mouse was measured in triplicate. Values are mean ± SD; $n = 8$. Letters indicate significant differences at $P < 0.05$: [a]compared with all of the retrovirus-infected groups; [b]compared with the retrovirus-infected groups injected with saline, internal 5.1 peptide, Vβ8.1(N), and Vβ8.1(C) peptide; [c]compared with the retrovirus-infected groups injected with Vβ8.1 and Vβ5.2 peptide alone.

mice injected with TCR Vβ8.1 or Vβ5.2. The mice given the combination of Vβ8.1 + Vβ5.2 peptides treatment maintained Th1 and Th2 cytokines production near those of uninfected mice.

Discussion

Our studies help clarify the relationship between loss of vitamin E, increased lipid peroxidation, and immune dysfunction caused by murine retrovirus infection. TCR pep-

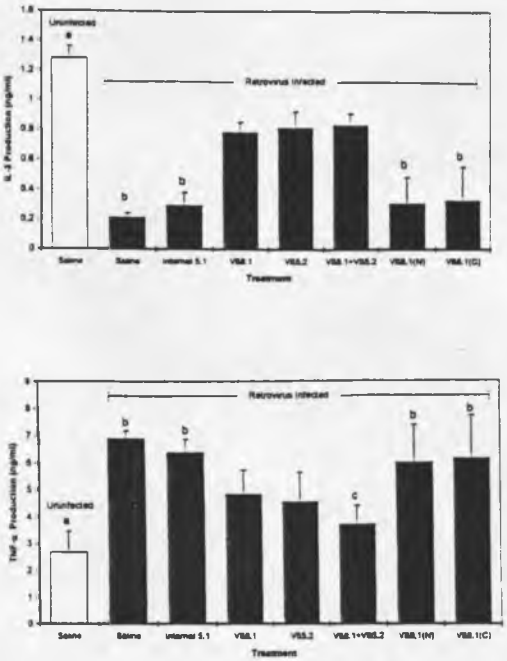

Figure 4. Effect of different T cell receptor peptides treatment on interleukin-2 (A) and tumor necrosis factor–α (B) production by splenocytes from *in vitro*. Every sample from each mouse was measured in triplicate. Values are mean ± SD; $n = 8$. Letters indicate significant differences at $P < 0.05$: [a]compared with all of the retrovirus-infected groups; [b]compared with retrovirus-infected groups with TCR Vβ8.1, Vβ5.2, and Vβ8.1 + Vβ5.2 peptide treatment; [c]compared with the retrovirus infected groups with single TCR Vβ8.1 and TCR Vβ5.2 peptide treatment.

tide treatments simultaneously prevented immune dysfunction and the loss of tissue vitamin E (11). The current studies demonstrated that treatment with TCR Vβ CDR1 peptides significantly decreased the evidence of oxidative stress associated with murine retrovirus infection. TCR peptide treatment significantly reduced immune dysfunction, oxidative damage in tissue, and loss of tissue vitamin E. Although the interaction between immune function and nutritional status still needs further elucidation, maintenance of immune function and tissue vitamin E levels occurred concomitantly with prevention of the stimulation of a T cell clone induced by murine retrovirus superantigens (9). However, fragments representing 10 amino acids of a 16–amino acid sequence from either the N terminus or C terminus of the active peptide Vβ8.1 did not retain activity in preventing immune dysfunction or loss of vitamin E. Thus, fragments of the CDR1 segment or the reading frame segment only did not maintain activity. Both the CDR1 portion and the reading frame component of the peptide are necessary for activity, or the peptide must be longer than 10 amino acid to be functional.

Most antigens are recognized through their interaction with the variable V portions of the TCR α- and β-chains. T cells also recognize superantigens, which interact with the Vβ region alone, independently from other variable TCR components. $CD4^+$ T-cell expansion or depletion requires the stimulation of T-cell subgroups by chronic or superretroviral antigens. Over time, this results in excessive activation of $CD4^+$ T cells bearing superantigen selected Vβ's followed by a general anergy. Autoantibodies against Vβ peptides, found in high levels in infected mice (10), defined the TCR epitopes used to select the two peptides for our studies. Similarly autoantibodies against some TCR Vβ peptides were high in AIDS patients. Our previous data (9) showed that TCR Vβ8.1 peptide treatment prevented immune dysfunction, indicating that this peptide could be considered an immunoregulatory element in the complex network of interactions between components of the immune response.

Our data suggest an association among immune dysfunction, lipid peroxidation, and tissue vitamin E. Immune dysfunction during murine retrovirus infection decreases the host resistance to opportunistic pathogens, facilitating infection and pathogen reproduction. The resulting increased antigen levels stimulate phagocytes to release more free radicals and increase lipid peroxidation. Prevention of immune dysregulation by the various TCR peptides simultaneously eliminates excessive lipid peroxidation and reduces the loss of tissue vitamin E. Similarly maintenance of immune function in retrovirus-infected mice occurred when early treatment with large doses of the TCR Vβ8.1 peptide was conducted, which prevented development of high level of lipid peroxidation while retaining tissue vitamin E (11).

HIV^+ patients have reduced serum vitamin E levels at various stages of the disease (23). Most patients who had AIDS (50%), who had AIDS-related complex (58%), and/or who were HIV-infected (38%) had a vitamin E intake of less than 50% of the Recommended Daily Allowance (24). However, reduced tissue levels of vitamin E were not due to the lower intake during murine retrovirus infection as deficiencies of vitamin E occurred in the liver, spleen, and thymus (6, 7), even though the mice consumed the recommended amount of vitamin E. Vitamin E–deficient rats have depressed antibody-dependent cell cytotoxicity (25), lymphocytes blastogenesis in response to mitogens (26), and natural killer cell–mediated cytotoxicity (27). Thus, immunological defects related to retrovirus infections could be exacerbated by retrovirus-induced vitamin E deficiency. Supplementation with high levels of vitamin E during murine retrovirus infection restored tissue vitamin E levels, while it partially normalized immune dysfunction (5).

Vitamin E inhibits oxidation of cellular components by free radicals and singlet oxygen, and is the most effective antioxidant at higher partial pressures (28). Vitamin E is an immune enhancer associated with a reduced risk of atherosclerosis, cancer, and tissue damage (8). In chicks, vitamin E supplementation alleviated the effects of lipid peroxida-

tion during zinc deficiency (29). Prevention of vitamin E loss may delay development of debilitating diseases and conditions directly affected by retrovirus infection. Low vitamin E levels were seen in patients infected with HIV (30). Prevention of lipid peroxidation of cell membrane by vitamin E is part of its immune enhancing response (31). The rapidly proliferating cells of the immune system are particularly susceptible to oxidative damage by free radicals. The antioxidants also modulate the biosynthesis and activity of important cell regulators, prostaglandin, thromboxanes, and leukotrienes (32).

In the current studies, there was evidence of increased hepatic and cardiac lipid peroxidation in retrovirus infected mice concomitantly with decreased hepatic and cardiac vitamin E levels. This may be due to immune dysfunction induced by the retrovirus altering cytokine production toward that seen in inflammatory diseases (5), which should increase oxidative stress and decrease tissue antioxidant levels. Similarly, plasma lipid peroxidation was increased in AIDS patients (33). Oxidative stress may be a second messenger as TNF-α levels are elevated in the serum of HIV-infected patients (34) and murine AIDS (35). TNF-α and IL-6, produced in excessive quantities during murine and human AIDS, are potent enhancing factors in the spreading of HIV to new target cells (22). Vitamin E supplementation restored a less oxidative environment in murine retrovirus–infected mice while decreasing the excessive IL-6 production (5), much as occurred with TCR peptide therapy which simultaneously prevented vitamin E losses.

Free radicals can induce the expression of HIV in human T cell lines by activating transcription of NF-κB (36). Vitamin E may block NF-κB activation by reducing oxidative stress and IL-6 levels, thereby inhibiting HIV replication and retarding progression of infection. A unique feature of HIV infection is its persistence in a quiescent state, prior to activation, without production of either viral mRNA or proteins. As free radical stimulus seems important to HIV multiplication, vitamin E may retard murine retrovirus replication by lowering the oxidative stress, keeping retrovirus in a quiescent state, and inhibiting progression to murine AIDS. Reduced levels of antioxidants, vitamin E, glutathione, and other acid soluble thiols correlated well with the accelerated progression to human AIDS (37). Oxidative stress may also be a potent inducer of viral activation by causing DNA damage in infected cells, inducing certain alterations in the cells necessary for HIV reproduction, and producing a long-term consequence of HIV infection, immunosuppression (38). If there were reduced levels of superoxide dismutase in early murine retrovirus infection (39), it should result in increased persistence of hydrogen peroxide with more oxidative damage, lipid peroxidation, and loss of vitamin E *via* its reaction with free radicals. The evidence of increased free radical or oxidative activity and greater lipid peroxidation products fit well with a loss of tissue vitamin E during T cell immune dysfunction in murine AIDS. Our data in these studies further support this concept with prevention of immune dysfunction by immunological regulation. TCR peptide treatment largely prevented loss of vitamin E. Low doses or delayed treatment of a TCR peptide Vβ8.1 did not correct the immune dysfunction nor prevented loss of vitamin E (11). T and B cell dysfunction should permit greater bacterial infections, yielding more bacterial lipopolysaccharides for macrophage activation. This would cause release of highly reactive free radicals, altering cellular function and enzymic activity (40). As we have found that vitamin E supplementation partial normalized the immune functions during murine AIDS, tissue vitamin E levels appear to be a critical component in maintaining immune functions against free radical damage. Vitamin E prevented much of the oxidative damage of alcohol alone and during retrovirus infection, retarding esophageal tumor growth induced by a carcinogen (41). Therefore, antioxidant activity is important in preventing tumor growth in murine AIDS, perhaps by immune modulation *via* maintenance of tissue vitamin E (40).

TCR peptides that were effective in preventing immune dysfunction in murine AIDS prevented excessive lipid peroxidation and loss of vitamin E. However, the TCR peptide fragments that did not prevent immune dysfunction also did not prevent oxidative damage.

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Immunology 1998 93 462–468

Injection of T-cell receptor peptide reduces immunosenescence in aged C57BL/6 mice

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SUMMARY

Previous studies established that retrovirally infected young mice produced large amounts of autoantibodies to certain T-cell receptor (TCR) peptides whose administration diminished retrovirus-induced immune abnormalities. C57BL/6 young (4 weeks) and old (16 months) female mice were injected with these same synthetic human TCR Vβ8.1 or 5.2 peptides. Administration of these autoantigenic peptides to old mice prevent immunosenesence, such as age-related reduction in splenocyte proliferation and interleukin-2 (IL-2) secretion. TCR Vβ peptide injection into young mice had no effect on T- or B-cell mitogenesis and IL-4 production while modifying tumour necrosis factor-α (TNF-α), IL-6, and interferon-γ (IFN-γ) secreted by mitogen-stimulated spleen cells. TCR Vβ injection also retarded the excessive production of IL-4, IL-6 and TNF-α induced by ageing. These data suggest that immune dysfunction and abnormal cytokine production, induced by the ageing process, were largely prevented by injection of selected TCR Vβ CDR1 peptides.

INTRODUCTION

Ageing is a progressive decline in physiological homeostasis with an increasing vulnerability to a myriad of infections and cancers caused, in part, by immunosenescence. A progressive decline in overall immune competence and regulation is a common, unifying factor contributing to the susceptibility of the elderly to diseases. T-cell proliferation in response to a primary signal through the T-cell receptor (TCR) is impaired in healthy, aged mice.[1] Chronic stimulation of T helper 2 (Th2) cells' cytokines by pathogens suppresses T helper 1 (Th1) cells' cytokine production and thus suppresses cellular immunity. Autoimmune diseases increase with ageing, when many elderly have monoclonal gammopathies and high levels of circulating autoantibodies (AAbs).[2] AAbs binding peptide determinants of the CDR1 region in the TCR Vβ domain were elevated during murine retrovirus infection.[3] Injection of the TCR Vβ peptides identified by elevated levels of AAbs slowed the progressive loss of T- and B-cell mitogenesis and cytokine dysregulation.[4] Blocking the retroviral antigen stimulation of Th2 subset with TCR Vβ peptides prevented immune dysfunction and loss of *Cryptosporidium* resistance in retrovirus infected young mice.[4]

The current studies characterized the role of these TCR peptides in preventing age-related immune dysfunction. The effects of the TCR Vβ peptides, previously identified by AAbs as present in larger quantities in retrovirus infected young mice,[4] on T- and B-cell responses and cytokine regulation in old and young mice were investigated. AAbs to these peptides were elevated also in uninfected old mice. Therefore the effectiveness of the peptides in preventing age-related immune dysfunction was determined. The TCR peptide injection significantly reduced the age-related depression of the immune response, alleviated immunosenescence, and restored normal cytokine secretion by splenocytes.

MATERIALS AND METHODS

Animals and treatment

Female C57BL/6 mice, 4 weeks (young) and 16 months (old) of age from Charles River Laboratories Inc. (Wilmington, DE) were cared for as required by the University of Arizona Committee on Animal Research. After 2 weeks, they were randomly assigned to one of the following treatments with 6 mice per group: young mice injected intraperitoneally with saline (pyrogen free); young mice injected with 200 μg TCR Vβ8.2 peptide; old mice injected with saline (pyrogen free); old mice injected with 200 μg TCR Vβ8.2 peptide; old mice injected with 200 μg TCR Vβ5.2 peptide; old mice injected with 200 μg Vβ8.2 and 200 μg Vβ5.2 peptides. The animals were killed 14 weeks after injection. The dose of the peptides

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was based upon dose–response studies in young, retrovirally infected mice.[4]

Peptides

A set of overlapping 16-mer peptides that duplicate covalent structure of the TCR β product[5,6] predicted from the human JURKAT sequence[7] has been produced. An effective immunomodulatory peptide has the sequence, C K P I S G H N S L F W Y R Q T, that corresponds to the complete CDR1 and N-terminal five residues of Fr2[5,6] of the human Vβ8.1 gene product.[7] A peptide corresponding to the sequence of the 16-mer of the Vβ5.2 peptide gene product, C S P K S G H D T V S W Y Q Q A, was synthesized as a homologue often recognized by AAbs. Normal human[5] and mouse[3] polyclonal immunoglobulin G (IgG) pools contain natural AAbs against peptide segments correspond to the human CDR1, Fr3 and to a constant region 'loop' peptide.[5] A computer comparison of human and murine Vβ sequences (Marchalonis, unpublished analysis) using the progressive alignment algorithm of Feng and Doolittle[8] showed that certain human and murine Vβ sequences could be grouped into families: human Vβ6 and Vβ8 correspond to murine Vβ11; human and murine Vβ5 are also in the same clusters. We have previously[4] shown that these peptides were lipopolysaccharide (LPS) (endotoxin) free. A control peptide also had no effect on immune functions when injected previously,[4] so saline was used.

Enzyme-linked immunosorbent assay (ELISA) for cytokines

Interferon-γ (IFN-γ), interleukin (IL)-2, IL-6, IL-10, and tumour necrosis factor-α (TNF-α) were produced by splenocytes as described previously.[9] Briefly, spleens were gently teased with forceps in culture medium, producing a single cell suspension of spleen cells. 0·1 ml/well of splenocytes (1×10^7 cells/ml) was cultured in triplicate on 96-well flat-bottom culture plates (Falcon 3072, Lincoln Park, NJ), and then stimulated with concanavalin A (Con A; 10 μg/ml, 0·1 ml/well, Sigma) for induction of IL-2 and IL-10 with 24 hr incubation, IFN-γ with 72 hr incubation at 37°, 5% CO_2 incubator. Splenocytes were also stimulated by LPS (10 μg/ml, Gibco, Grand Island, NY) for 24 hr induction for IL-6 and TNF-α production. Cytokines in supernatant fluids were determined by sandwich ELISA as described previously.[9]

Mitogenesis of splenocytes

Splenic T- and B-cell proliferation was determined by [^{3}H]thymidine incorporation as described previously.[9]

Statistics

The statistic 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. $P < 0{\cdot}05$ was considered significant difference between two groups.

RESULTS

Body weight

There was no change in food consumption, body weight, spleen and lymph node weight of the mice from the various TCR peptide treatments (data not shown).

Mitogenesis of splenocytes

Proliferation of ConA- and LPS-induced splenocytes from aged mice was significantly increased ($P < 0{\cdot}05$) by TCR peptide injection (Figs 1 and 2), which had no significant effect on splenocytes from young mice. TCR Vβ8.1, Vβ5.2, and Vβ8.2+Vβ5.2 peptide injection maintained T-and B-cell proliferation which was significantly ($P < 0{\cdot}05$) higher than that of saline injected old mice. The combination, Vβ8.1+Vβ5.2, induced changes yielding significantly ($P < 0{\cdot}05$) higher T-cell proliferation than injection of either peptide alone.

Cytokine production of splenocytes

In vitro production of a Th1 cell cytokine, IL-2, by ConA-stimulated splenocytes from old mice was significantly ($P < 0{\cdot}05$) lower than that produced by splenocytes from young mice (Fig. 3). TCR Vβ peptides injection significantly ($P < 0{\cdot}05$) prevented suppression of IL-2 secretion by mitogen-stimulated splenocytes in old mice (Fig. 3). Cells from old mice injected with the combination of Vβ8.2+Vβ5.2 peptides had significantly ($P < 0{\cdot}05$) higher IL-2 production than cells from mice given either peptide alone.

Release of Th2 cells' cytokines, IL-6, IL-10, and TNF-α, by mitogen-stimulated splenocytes was significantly ($P < 0{\cdot}05$) increased in old mice (Figs 4–6). TCR Vβ peptide injection significantly ($P < 0{\cdot}05$) prevented elevation of IL-6, IL-10, and TNF-α release by mitogen-stimulated splenocytes from young and old mice (Figs 4–6). Cells from old mice injected with the combination of Vβ8.2+Vβ5.2 peptides produced significantly ($P < 0{\cdot}05$) less IL-6 than cells from old mice given either peptide alone. The combined peptide injection lowered IL-6 production in old mice to the level of young mice, a 55% reduction. TCR peptide injection also significantly ($P < 0{\cdot}05$) reduced IL-6 and TNF-α production in young mice, but not as dramatically as in old mice.

Production of AAbs to TCR Vβ8.1

Comparison of mean titres of serum AAbs binding TCR Vβ CDR1 peptides indicates that aged C57BL/6 mice have significant higher specific AAbs titres than young mice (Fig. 7). The anti-TCR AAb profile of aged mice is comparable to that following LP-BM5 retrovirus infection in young mice which has been previously characterized.[4] Treatment of both young and aged mice with TCR Vβ CDR1 peptides did not result in a significant change in mean AAb titres compared with age-matched, untreated mice.

DISCUSSION

In the present study, administration of TCR Vβ CDR1 peptides significantly prevented age-related depression of immune responses while largely maintaining normal cytokine production. The 16-mer TCR Vβ peptides constitute an intact immunoregulatory element in the complex network of interactions among its components in mice. Both of Vβ8.2 and Vβ5.2 peptides used to prevent development of immunosenescence were identified by increased AAbs production in murine retrovirus infected mice[9] and, in our study with old mice. Both peptides were effective in preventing most, but not all, immune dysfunction caused by ageing. The combined

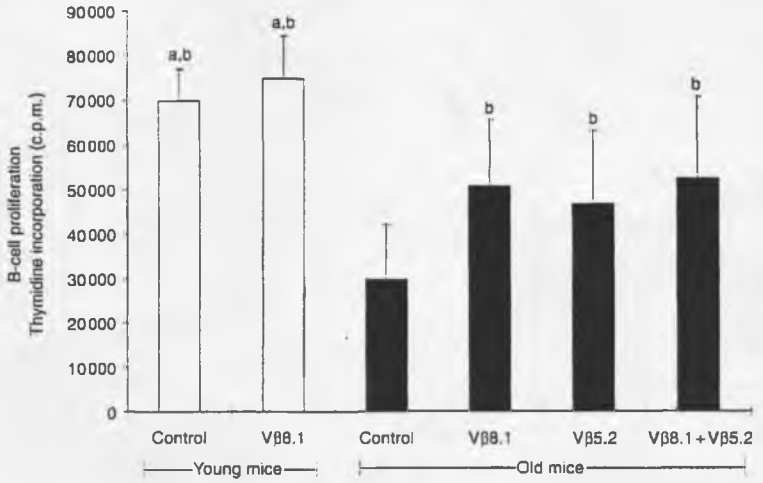

Figure 1. Effect of T-cell receptor peptide injection on Con A-stimulated B-cell proliferation. Every sample from each mouse was determined in triplicate. Values are mean ± standard deviation, $n = 6$. Letters indicate significant differences at $P < 0{\cdot}05$: a, compared with old mice groups except the one given both peptides; b, compared with control old mice group.

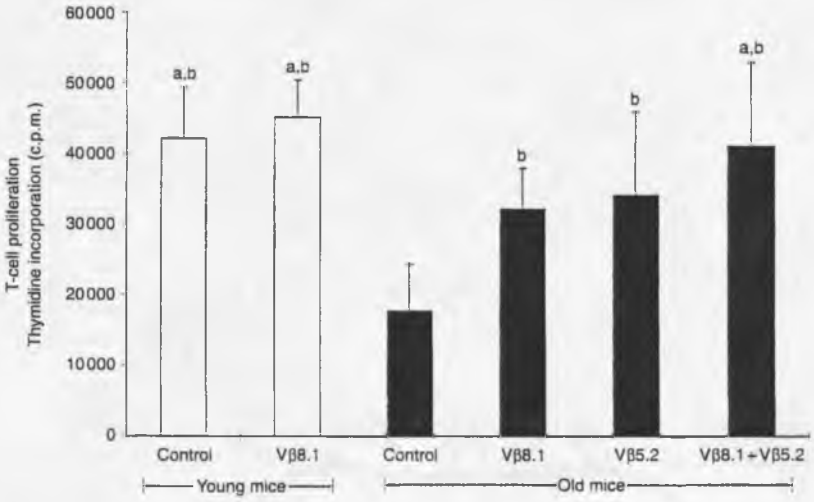

Figure 2. Effect of T-cell receptor peptides injection on LPS-stimulated T-cell proliferation. Every sample from each mouse was determined in triplicate. Values are mean ± standard deviation, $n = 6$. Letters indicate significant differences at $P < 0{\cdot}05$: a, compared with old mice groups except the one given both peptides; b, compared with control old mice group.

use of both peptides was modestly more effective in preventing immune dysfunction than either alone. Thus it appears that preventing chronic stimulation of T cells bearing one TCR by a TCR peptide largely stops excessive activity of other T-cell subsets, as the use of TCR peptides only slightly improved immune functions in the present study. Therefore it may not be necessary to identify all individual TCR Vβ T-cell subsets excessively stimulated by chronic or super antigen exposure to use TCR Vβ peptides to maintain immune function. TCR peptide injection was much less effective in modulating immune functions in young mice and did not affect T- or B-cell mitogenesis. TCR peptide injection significantly decreases IL-6 and IFN-α production while having no significant effect on IL-2 and IL-4 production in young mice.

TCR peptide injection may stimulate a natural immunoregulatory network that induces tolerance of T cells expressing the targeted TCR Vβ gene product.[10] Early treatment, prior

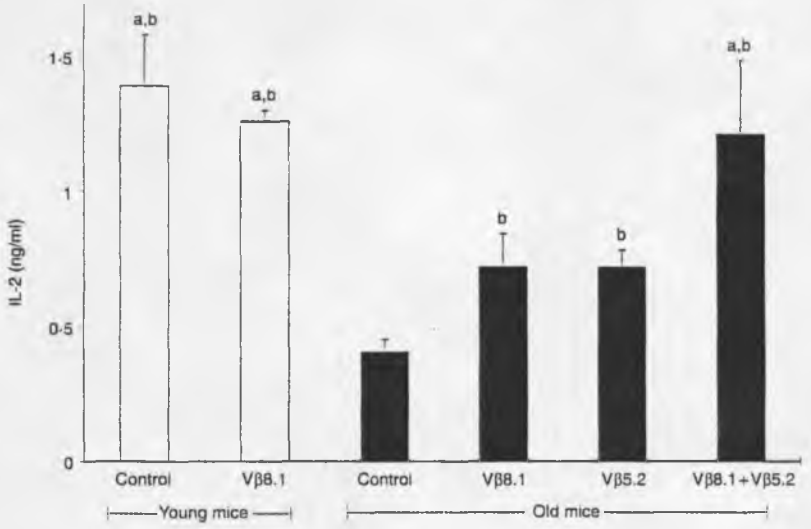

Figure 3. Effect of T-cell receptor peptide injection on interleukin-2 production by splenocytes *in vitro*. Every sample from each mouse was measured in triplicate. Values are mean ± standard deviation, $n = 6$. Letters indicate significant differences at $P < 0{\cdot}05$: a, compared with old mice groups except the one given both peptides; b, compared with control old mice group.

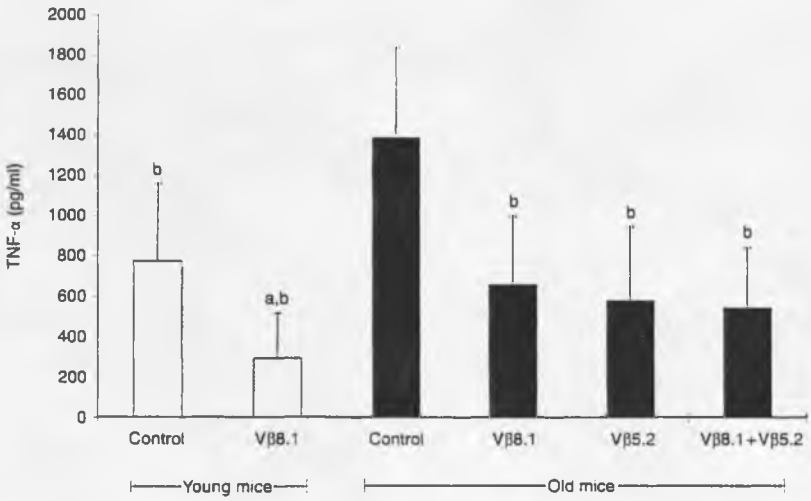

Figure 4. Effect of T-cell receptor peptide injection on TNF production by splenocytes *in vitro*. Every sample from each mouse was measured in triplicate. Values are mean ± standard deviation, $n = 6$. Letters indicate significant differences at $P < 0{\cdot}05$: a, compared with control young mice group and all the old mice groups; b, compared with control old mice group; c, compared with control old mice group and the old mice groups given either peptide alone.

to significant immune dysfunction, with a significant amount of TCR antigen was critical to prevent immune dysfunction during retrovirus infection in young mice.[9] The addition of adjuvant had a variable effect expanding the efficacy of very low (otherwise ineffective) doses of TCR antigen.[9] Thus TCR Vβ CDR1 peptide functions as an immunoregulatory element in the complex networks of interactions among the components of the immune system.

T-cell proliferation is the component of cell-mediated immunity that most consistently shows defects associated with ageing. Lymphocytes obtained from aged persons show a markedly decreased mitogenic response to plant lectins such

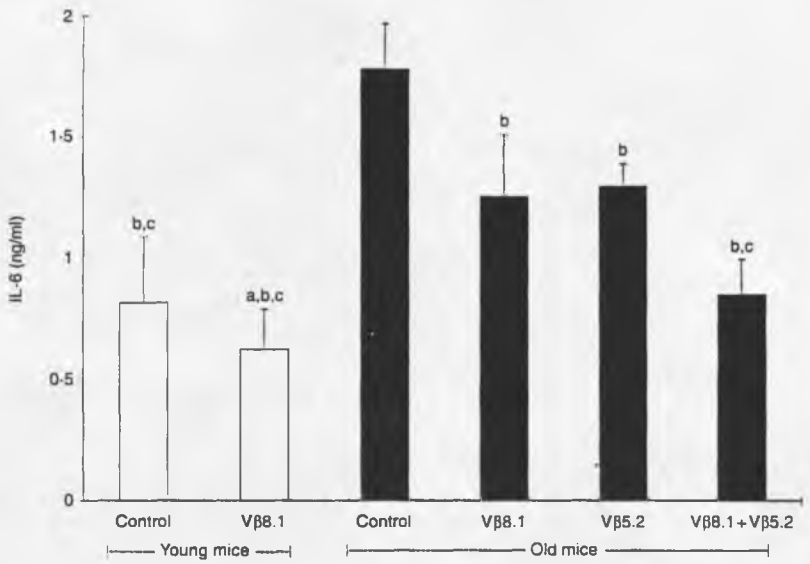

Figure 5. Effect of T-cell receptor peptide injection on IL-6 production by splenocytes *in vitro*. Every sample from each mouse was measured in triplicate. Values are mean ± standard deviation, $n=6$. Letters indicate significant differences at $P<0{\cdot}05$: a, compared with control young mice group and all the old mice groups; b, compared with control old mice group.

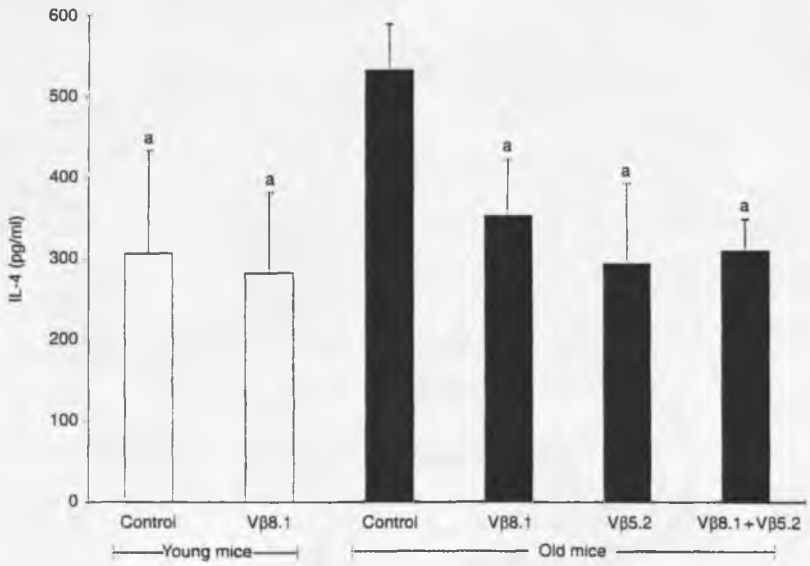

Figure 6. Effect of T-cell receptor peptide injection on interleukin-4 production by splenocytes from *in vitro*. Every sample from each mouse was measured in triplicate. Values are mean ± standard deviation, $n=6$. Letters indicate significant differences at $P<0{\cdot}05$: a, compared with control old mice group.

as phytohaemagglutinin (PHA).[11] The elderly have fewer responsive non-adherent cells, and even responsive cells fail to divide normally after stimulation. The number of cells progressing throughout the cell cycle following PHA stimulation was decreased when cells from elderly donors were compared with cells from young donors.[1] Preservation of T-cell function after TCR Vβ peptide injection in ageing mice may have occurred by maintenance of IL-2 production, necessary for

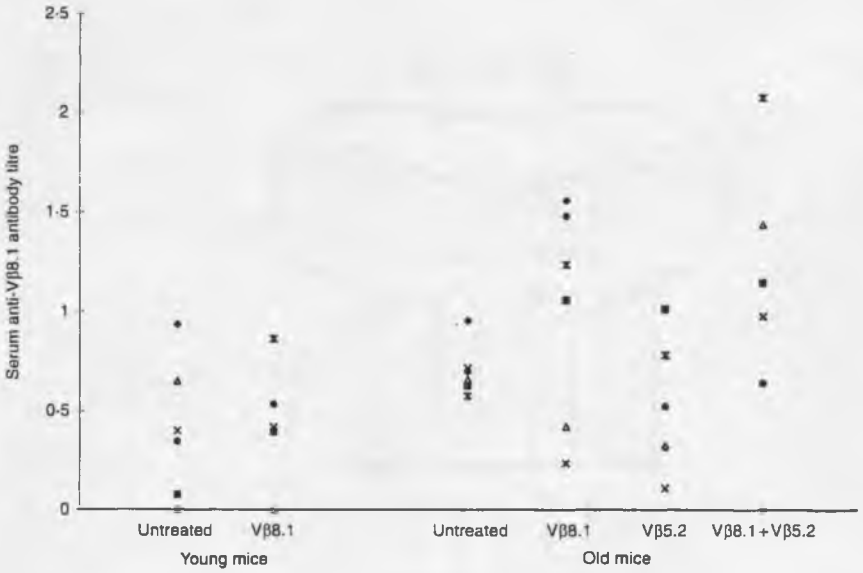

Figure 7. Distribution of individual ELISA titres of AAb against the target TCR Vβ8.2peptide. Every sample from each mouse was measured in triplicate. Values are mean ± standard deviation, $n = 6$.

T-cell division, while preventing excessive secretion of inhibitory Th2 cells' cytokines.

From sexual maturity to old age, the peripheral T-cell pool undergoes progressive changes which include accumulation of memory T cells. They have different activation requirements than do naive T cells[12] and produce a different pattern of cytokines,[13] including less IL-2, and more IL-4, consistent with age-related changes in T cell cytokine production. The IL-10 level was ≈10-fold higher in old mice than that in young mice.[14] In our previous studies,[4,9] TCR peptide injection significantly normalized retroviral-induced suppressed Th1 cells' cytokine production and elevated Th2 cells' cytokine production in murine acquired immune deficiency syndrome (AIDS). In the current study, similar cytokine release patterns have been observed. Saline injection had no effect on lymphocyte functions. It is important to note that previous studies using a control peptide[4] from the light chain of IgG had no effect either on T- or B-cell function.

The level of some immunoglobulins and their avidity decline during the ageing process.[15] However, some AAbs increase with age, particularly in persons over the age of 80,[11] reflecting a loss of immune regulation with emergence of forbidden clones. The highest incidence of AAbs is in those patients with decreased lymphocyte responses to PHA stimulation, implying loss of normal T cell function.[11] In our study, there were increased AAbs in the aged mice, however, the lack of suppression of AAbs by TCR Vβ peptide injection, which normalized cytokine production, may suggest that the production of AAbs by B cells was unaffected. Similarly, TCR Vβ peptide treated retrovirus-infected mice did not reduce their AAbs. Thus TCR Vβ peptide injection modified T-cell functions but did not affect specific B cells.

Specific immune responses appear to be the most vulnerable to age-associated impairment. T lymphocyte proliferative responses are markedly decreased, as is the production of and response to intercellular mediators. When T cells bind to a pathogen antigen, the attached TCR Vβ subset is expanded or deleted in the thymus, affecting the resulting immune response to the pathogen.[16] In human immunodeficiency virus (HIV)-infected cells there was a deletion of Vβ subsets that caused the resulting immune dysregulation.[17] An antagonistic peptide, one with a single amino acid change, inhibited both T-cell proliferation and cytokine production by Th1 and Th2 cells.[16] Prevention of immunosenescence by TCR Vβ peptide injection could have been caused by a deletion or inhibition of the corresponding Th2 subset. Differential conformational changes in the TCR complex in response to distinct major histocompatibility complex (MHC)-peptide ligands or variations in affinities of the TCR for different MHC-peptide ligands should alter T-cell activity and cytokine production.[18] Optimal affinity for the TCR would promote positive selection whereas high affinity should favour programmed cell death.[18] Short lived occupany with rapid dissociation should result in only certain signaling reactions through the TCR–DC3 complex, insufficient for completion of the entire sequence of signaling necessary for full activation of the T-cell subset.[18] Thus, our TCR Vβ peptides, as incomplete proteins, may switch off some or all aspects of activation of a specific T-cell subset. TCR peptides that inhibit signalling directed through the TCR-initiating signalling pathways, leading to proliferation and cytokine secretion, could potentially be powerful modulators of immune function.[16]

ACKNOWLEDGMENTS

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VITAMIN E SUPPLEMENTATION PREVENTS LUNG DYSFUNCTION AND LIPID PEROXIDATION IN NUDE MICE EXPOSED TO SIDE-STREAM CIGARETTE SMOKE[1]

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ABSTRACT

Sidestream cigarette smoke (SSCS) is a major component of environmental tobacco smoke. The purpose of this study was to investigate development of lung injury and lipid peroxidation in the lung and liver of immunodeficient (Nude) mice exposed to a small amount of SSCS (a total 5 hours of exposure). The effects of vitamin E supplementation of the mice were also determined. SSCS increased pulmonary resistance and lipid peroxidation in these mice. Dietary vitamin E supplementation increased vitamin E levels in lung and liver. In addition, vitamin E attenuated SSCS-mediated pulmonary injury and lipid peroxidation. It appears that the enhanced resistance against SSCS-induced lung injury and lipid peroxidation may be primarily due to the increased antioxidant property of vitamin E in vitamin E-supplemented mice.

Key Words: Vitamin E, Tobacco smoke, Sidestream cigarette smoke (SSCS), Lung function, Lipid peroxidation, Nude mice.

INTRODUCTION

Tobacco smoke is a significant contributing factor in the etiology of respiratory and cardiovascular disorders. The effects of constant exposure to sidestream cigarette smoke (SSCS), a major component of environmental tobacco smoke, are poorly understood. Ironically, SSCS may be more toxic than inhaled smoke due to its lower combustion temperature (1). Tobacco smoke contains a large variety of oxidants and free radicals. *In vitro* studies show that cigarette smoke can promote oxidative damage inducing lipid peroxidation. Free radical-induced oxidative damage is related to the development of smoking-related disorders ranging from chronic inflammation to cancer. Numerous studies show that susceptibility to lipid peroxidation is greatly influenced by

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tissue content of vitamin E (2). Depletion of vitamin E and other antioxidants by increased free radical production caused by smoke exposure, may facilitate lipid peroxidation in the lung.

SSCS, containing powerful oxidants and toxins, may stimulate macrophages to produce oxidants which damage lung cells. Responding to foreign antigens in SSCS, T cell-activated macrophages may also secrete large quantities of oxidants and free radicals. When free radicals interact with cellular components, carcinogenesis and cell dysfunction are increased. Since Nude mice lack a thymus and therefore functional T cells, they produce less oxidants and consequently have less oxidative damage than conventional mice with fully functional immune systems. We therefore have begun to develop them as a model to study the direct SSCS damage without accentuation by T lymphocytes, using lipid peroxides as indicators of oxidative damage.

The present study investigated the development of lung injury and lipid peroxidation in the lung and liver of Nude mice briefly exposed to SSCS. The effects of vitamin E supplementation to prevent smoking-induced lung damage and tissue lipid peroxidation in the absence of T cells were examined.

MATERIAL AND METHODS

Animals and treatment

Young female C57BL/6 Nude mice (nu/nu), weighing about 30 g, were exposed to SSCS alone with dietary vitamin E supplementation for 10 days. They were housed in transparent plastic cages with stainless steel wire lids (4 mice per cage) as required by the University of Arizona Animal Care and Use Committee. The housing facility was maintained at 20 °C and 60-80% relative humidity, with a 12 h light:dark cycle. Diet and water were freely available. Body weight, food, and water were measured every 3 days. After one week of housing and consuming the control diet, the mice were randomly assigned to one of the 4 following treatments with eight mice per group: nonsmoking Nude mice given a unsupplemented control diet; nonsmoking Nude mice given vitamin E-supplemented diet; SSCS-exposed Nude mice given control diet; SSCS-exposed Nude mice given vitamin E-supplemented diet.

Diets and vitamin E supplementation

Both control diet (AIN 93 M) and vitamin E-supplemented diet were prepared by Diets Inc. (Bethlehem, PA). The vitamin E-supplemented diet had 117.70 mg RRR-alpha-tocopherol acetate per kg (176.55 IU/kg diet), while the control diet contained 7.70 mg RRR-alpha-tocopherol per kg (11.55 IU/kg diet). There was a 15-fold increase in vitamin E content in vitamin E-supplemented diet compared to in unsupplemented diet. Mice in all groups were provided with drinking tap water. The nutritional treatment period was 10 days for all groups.

Sidestream cigarette smoke (SSCS) exposure

The mice were exposed to SSCS for 30 min/day, 5 days/week for 10 days utilizing an IN-TOX (Albuquerque, NM) vacuum-drawn (15 L/min) exposure system modified for cigarette smoke exposure. The total mass concentration of SSCS particulate matter delivered to the mice was 2 mg/exposure, as measured by a seven-stage multi-jet cascade impactor (IN-TOX Products, Inc.).

The SSCS-air mixture was drawn from the top of the funnel by a plastic hose connected to an inlet port of the IN-TOX exposure chamber system. Total particulates over 10 day SSCS exposure were 0.002 ± 0.000014 g (mean ± SEM). The SSCS particles collected by the cascade impactor were found to have a mass median aerodynamic diameter (MMAD) of 0.34 μm with a geometric standard deviation of 0.46. This SSCS particle size will gain access to the alveolar septal area of the lungs.

The SSCS-exposed mice were placed in the IN-TOX exposure system for a 30-min exposure period. SSCS was generated in the following manner: the first cigarette was lit by a modified syringe device and one puff of cigarette smoke was drawn from the lit cigarette. The lit cigarette was placed upright in a clamp 2.5 cm below the bottom edge of an inverted 220 cm^3 funnel and allowed to burn for 7.5 minutes. A second cigarette was lit at the 7 min timepoint of the 30 min exposure period and replaced the first cigarette in the clamp at the 7.5 min timepoint. After burning for another 7.5 min the second was replaced by a third one at the 15 min timepoint. Then the third was followed by a fourth one at 22.5 timepoint, which completed the 30 min SSCS exposure period. The IN-TOX exposure system was then thoroughly cleaned before the next exposure trial to prevent the accumulation of cigarette tars and other material in the exposure ports. Control mice were treated in a similar manner, except that the cigarettes were not lit before being placed in the clamp.

Pulmonary function assays

On the day of the experiment, the mice were anesthetized intramuscularly with a 1.5 ml/kg mixture of ketamine HCL (50 mg/kg), xylazine (8 mg/kg), and acepromazine maleate (1 mg/kg). The mice were then paralyzed with 6 ml/kg of intraperitoneal gallamine triethiodide and ventilated with a Kent Scientific Co. (Litchfield, CT) pressure-controlled ventilator. Airway pressure was measured with a polyethylene catheter placed at the proximal end of the endotracheal tube. The endotracheal tube for the mice was a specially modified 20-gauge catheter. The airway and esophageal pressure transducers were connected to opposite sides of a differential pressure transducer for measurement of transpulmonary pressure (Ptp). Airflow (V) was calculated with a calibrated heated pneumotachograph (Fleish #0000, Instrumentation Associates, New York, NY) coupled to a Validyne pressure transducer. The Ptp and V signals were input to a PEDS-LAB® computerized pulmonary function system (Medical Associated Services, Hatfield, PA) adapted for mouse pulmonary functions. This system can measure 24 different respiratory variables including pulmonary resistance as determined by the method of Rodarte (3) on a continual basis. After baseline pulmonary functions had been recorded, the mice were administered a 0.1 ml bolus of 100 microCuries of technetium-labeled diethylenetriamine pentaacetate (^{99m}TC-DTPA, MW=492 amu, physical half-life = 6.02 h) through the endotracheal tube with five tidal volume (0.5 ml) air flushes to disperse the radioactive tracer evenly throughout the lungs. The mice were then again placed on the mechanical ventilator. Pulmonary epithelial clearance of ^{99m}TC-DTPA is a measure of lung permeability and was determined over 10 min with a Ludlum (Model 44-62, Sweetwater, TX) gamma counter probe placed centrally over the lungs. The gamma probe was connected to a Ludlum (Model 2200) scintillation center. After that the mice were killed by withdrawing blood from the vena cava, and the lungs and livers of these mice were taken and stored at -70°C until analysis.

Measurement of vitamin E

Vitamin E was measured by HPLC as described previously (4). Briefly, about 0.2 g of lung or liver tissue was homogenized in 1.0 ml of water. Butylated hydroxytoluene was added to prevent oxidation of α-tocopherol. Pentane, ethanol and sodium dodecyl sulfate were used to extract α-tocopherol from the homogenate. Extracts were evaporated under steady flow of nitrogen gas at 20 °C and then redissolved in 0.5 ml of methanol injection onto a C18 column (3.9×150 mm NovaPak, Millipore, Bedford, MA). A mobile phase composed of methannol:1 mol/L sodium acetate in the ratio of 98:2 (by volume) at a flow rate of 1.5 ml/min was used. α-Tocopherol, eluting at 6.5 min, was monitored by a fluorescence detector (Millipore) at 290 nm excitation and 320 nm emission wavelength. A set of α-tocopherol solutions with different concentrations was analyzed to make a standard curve and to verify calibration.

Determination of conjugated dienes and lipid fluorescence

Approximately 0.2 g of lung or liver tissue was homogenized in 5.0 ml of Folch solution (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 and used to determine conjugated dienes as previously described (5). Conjugated diene fatty acids were determined by obtaining absorbency of the solution at 237 nm in a Shimdzo UV 160 UV recording spectrophotometer (Tokyo, Japan) using an appropriate blank. Lipid fluorescence was measured in an Aminco Bowman fluorescence spectrophotometer (Rochester, NY). Maximum fluorescence at 470 nm was measured. The activation wavelength was at 395 nm.

Determination of phospholipids

The phospholipid contents of lung and liver were determined by the method of Raheja et al. (6). This method does not require predigestion of phospholipids. Briefly, 0.5 ml chloroform was added followed by 0.2 ml of a coloring reagent and 3.0 ml of carbon tetra chloride. Phospholipid concentration was determined by obtaining absorbency of the chloroform solution at 710 nm in a Shimadzo UV 160 UV recording spectrophotometer (Tokyo, Japan). Dipalmitoyl phosphatidylcholine was used as a standard.

Determination of total cholesterol and triglycerides

The total cholesterol levels of lung and liver were determined by the method of Zak (7). Briefly 0.3 ml of Folch extract was dried under air at 70°C. Then 3.0 ml Zak's reagent was added followed by 2.0 ml of sulfuric acid. Total cholesterol was determined by obtaining the absorbency of the solution at 570 nm in a Zhimadzo UV 170 UV recording spectrophotometer (Yokyo, Japan) using cholesterol standards (Sigma, St. Louis, MO). Triglycerides were determined colorimetrically (8).

Statistics

Mean (SEM) data were calculated for each group of mice. The statistical tests for comparison among groups were done by using analysis of variance (ANOVA) for the lung function data. Vitamin E and lipid peroxidation data were analyzed in NCSS program (Kaysville, UT) using Friedman's Block/Treatment test, followed by Duncan's Multiple Range Test between any two groups. $P<0.05$ were considered significant differences between two groups.

RESULTS

Diet intake and Body weight

The average diet intake was 4.11 g/mouse/day. No significant differences were observed in food consumption between groups (data not shown). Vitamin E intake in vitamin E-supplemented mice was 503.17 μg/mouse/day, while in unsupplemented mice vitamin E consumption was 30.32 μg/ml/day. SSCS exposure did not affect the amount of vitamin E consumed in the diet. Body weight was not effected by SSCS exposure or vitamin E supplementation (data not shown).

^{99m}TC-DTPA lung clearance

Lung clearance of particulate is an indicator of pulmonary injury. ^{99m} TC-DTPA lung clearance tended to be increased by SSCS exposure and reduced by vitamin E supplementation without any significant differences caused by SSCS or vitamin E supplementation (Table 1).

Pulmonary resistance

Pulmonary resistance is a measure of lung function. SSCS exposure significantly ($p<0.05$) increased pulmonary resistance in vitamin E-supplemented mice, but did not cause a significant change in unsupplemented group (Table 1). Vitamin E supplementation significantly ($p<0.05$) decreased pulmonary resistance in non-smoking mice, but not in smoking mice (Table 1).

TABLE 1

^{99m}TC-DTPA Lung Clearance and Pulmonary Resistance

Treatment		^{99m}TC-DTPA Lung Clearance	Pulmonary Resistance
SSCS	vitamin E	(%/min)	
–	–	0.88±0.52	15365±1133
–	+	0.72±0.51	11738±923*
+	–	2.29±0.41	14239±1083
+	+	1.55±0.36	15604±521#

Values are presented as mean ± SEM (n=8)

* Significantly different ($p<0.05$) from untreated mice which were not SSCS exposed or vitamin E supplemented

Significantly different ($p<0.05$) from vitamin E supplemented but not SSCS exposed

Vitamin E concentrations of lung and liver

SSCS exposure decreased vitamin E levels of lung and liver in vitamin E-supplemented mice, but not in unsupplemented mice (Fig.1). Vitamin E supplementation caused two-fold significant ($p<0.05$) and 56.8 % (non significant) increase in lung vitamin E levels of non-smoking mice and smoking mice respectively (Fig.1). Hepatic vitamin E content was increased about eight-

fold in both non-smoking ($p<0.05$) and smoking mice (Fig.1) after vitamin E supplementation for 10 days.

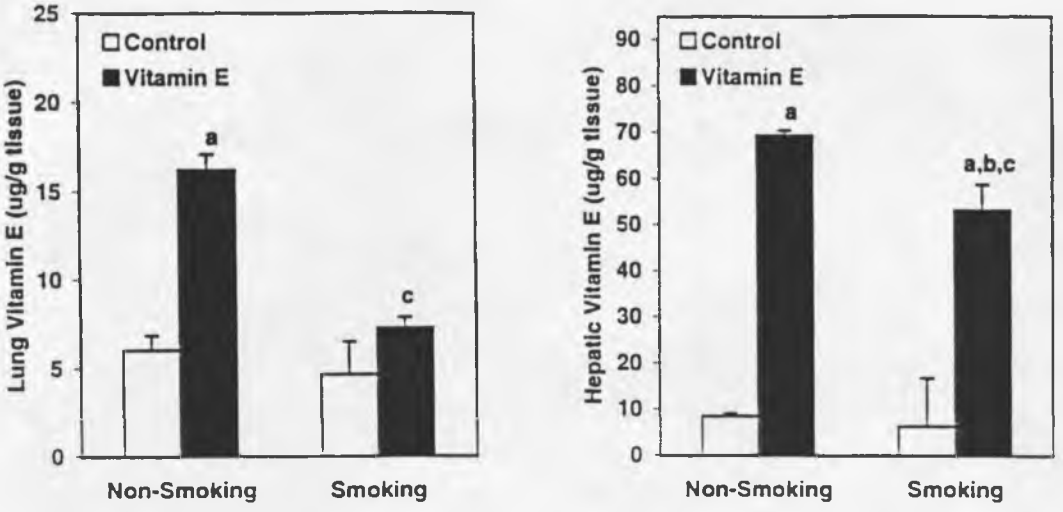

Fig. 1. Effect of SSCS exposure and vitamin E supplementation on lung and hepatic vitamin E content of nude mice. Every sample from each mouse was measured in triplicate. Values are mean ± stand error; n=8. Letters indicate significant differences at $p<0.05$: a, compared with non-smoking mice group without vitamin E supplementation; b, compared with smoking mice group without vitamin E supplementation; c, compared with non-smoking group with vitamin E supplementation.

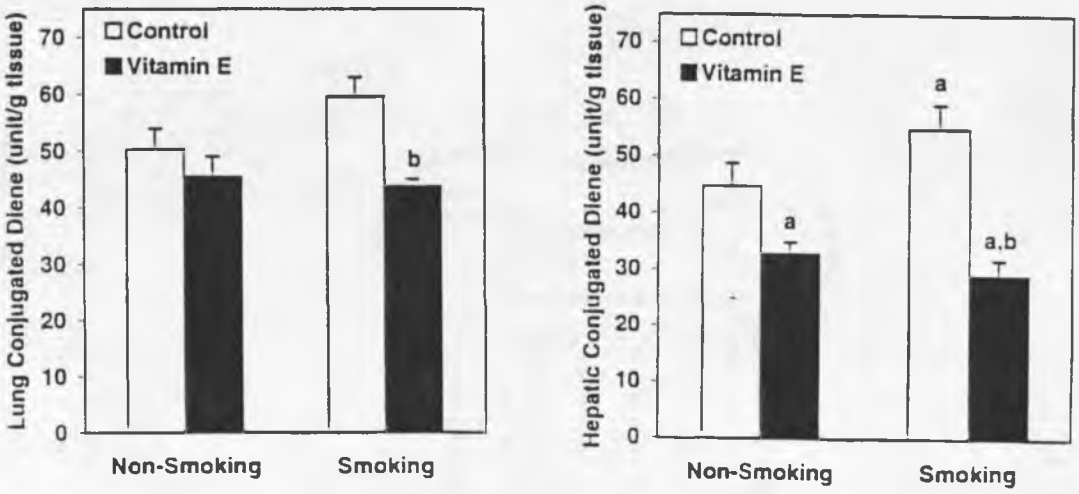

Fig. 2. Effect of SSCS exposure and vitamin E supplementation on lung and hepatic conjugated diene content of nude mice. Every sample from each mouse was measured in triplicate. Values are mean ± stand error; n=8. Letters indicate significant differences at $p<0.05$: a, compared with non-smoking mice group without vitamin E supplementation; b, compared with smoking mice group without vitamin E supplementation.

Conjugated diene levels in lung and liver

Conjugated dienes are major products of lipid peroxidation. SSCS exposure did not significantly increase lung conjugated dienes (Fig.2). However SSCS caused a significant ($p<0.05$) increase in hepatic conjugated dienes (Fig.2). Vitamin E supplementation significantly ($p<0.05$) decreased lung conjugated dienes in smoking mice and hepatic conjugated dienes in both non-smoking and smoking mice (Fig.2).

Lipid fluorescence content in lung and liver

Lipid fluorescence is another indicator of lipid peroxidation. SSCS exposure significantly ($p<0.05$) increased lipid fluorescence in lung and liver (Fig.3). Vitamin E supplementation significantly ($p<0.05$) prevented the increased lipid fluorescence levels of lung and liver in smoking mice, but not in non-smoking mice (Fig. 3).

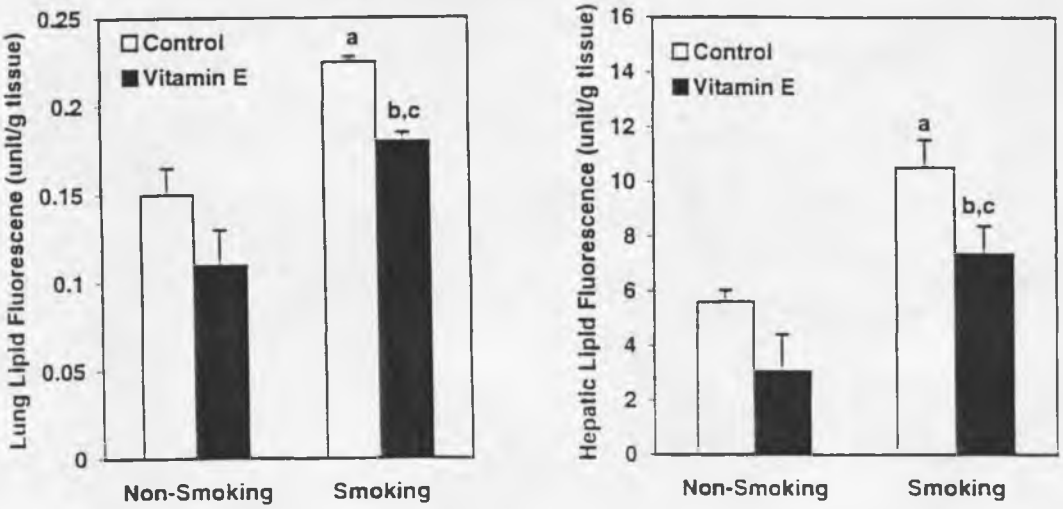

Fig. 3. Effect of SSCS exposure and vitamin E supplementation on lung and hepatic lipid fluorescene content of nude mice. Every sample from each mouse was measured in triplicate. Values are mean ± stand error; n=8. Letters indicate significant differences at $p<0.05$: a, compared with non-smoking mice group without vitamin E supplementation; a, compared with non-smoking mice group without vitamin E supplementation; b, compared with smoking mice group without vitamin E supplementation; c, compared with non-smoking mice group with vitamin E supplementation.

Phospholipids, total cholesterol and triglycerides of lung and liver

There was no significant difference in phospholipid, total cholesterol or triglyceride levels of lung or liver caused by SSCS exposure or vitamin E supplementation (data not shown).

DISCUSSION

Environmental tobacco smoke is a complex mixture of smokes that include SSCS, smoke coming through the cigarette (both filtered and unfiltered), and cigarette smoke exhaled from the smokers' lungs. Cigarette smoke affects various organ systems, especially the pulmonary system. SSCS causes an increase in alveolar epithelial permeability to small solutes as indicated by the changes we observed in ^{99m} TC-DTPA lung clearance. Our experiment demonstrated that SSCS increased pulmonary resistance only in vitamin E-supplemented mice. Vitamin E supplementation decreased pulmonary resistance only in non-smoking mice. On the other hand, SSCS exposure increased conjugated dienes and fluorescent units in lung and liver. This effect was significantly lower in the tissues of vitamin E-supplemented mice than in those of unsupplemented mice. All these changes came after very short term exposure to SSCS for a total of 5 hours or to vitamin E supplement for 10 days.

Cigarette smoke exposure recruits neutrophils (9) and macrophages (1) to the lungs. These activated cells release increased amounts of myeloperoxidase (10) and superoxide anion (1), causing lung cell damage. Similarly, mainstream and sidestream cigarette smoke increases the activities of cytochrome P450 A1 and 2B1 in the lungs of adult rats (11) as well as oxidative stress (12). In addition, cigarette smoke contains a large amount of oxidants and free radicals that directly initiate and promote oxidative damage in the lungs. Oxidative-induced lung injury also results indirectly from reactive oxygen species generated by the increased number and activity of pulmonary alveolar macrophages and neutrophils. This was likely very limited in Nude mice as they lack T cells to assist in activation of macrophages. We found that SSCS significantly increased oxidation damage even after a short exposure. Oxidation damage is a powerful agent, which may suppress and damage the immune system locally in lung and also systemically, which increases cancer risk and other smoking-related disorders.

Vitamin E is a scavenger of different free radicals by working as an antioxidant (13, 14). Since the oxidative damage is a major factor involved in the toxicity of cigarette smoke, the level of tissue vitamin E plays a role in mediating the development of smoking-related disorders. There is increased utilization of vitamin E which may be associated with cigarette smoke as we showed here for SSCS. This was demonstrated by the fact that tissue and serum vitamin E levels were significantly lower in male (15), and female (16) smokers as compared to nonsmokers with normal dietary vitamin E intake. This trend is also associated with higher levels of lipid peroxidation products. Our experiments demonstrated that SSCS exposure significantly decreased vitamin E levels of lung and liver only in vitamin E-supplemented mice but not in unsupplemented mice. This observation may mean that vitamin E-supplemented mice may be more sensitive and susceptible to SSCS damage, which maybe explain why SSCS exposure could significantly increase pulmonary resistance in vitamin E-supplemented mice, but not in unsupplemented group. Pacht *et al.*(17), also showed that vitamin E levels were significantly lower in alveolar fluid of smokers than in those of nonsmokers. They also suggested that smokers had a faster rate of vitamin E utilization and smoking might predispose them to enhanced oxidant attack on their lung parenchymal cells.

Supplementation of Nude mice with vitamin E caused a significant increase in lung vitamin E levels in non-smoking mice and hepatic vitamin E levels in both non-smoking and smoking mice. This suggests the antioxidant capacities of the lung and liver of vitamin E-supplemented mice is

higher than those of the controls. Supplementation dose in our experiment may have been insufficient to compensate for the damaged vitamin E content by free radicals in smoking-exposed lung during the 5 hours of SSCS treatment. In addition, vitamin E supplementation significantly decreased pulmonary resistance in non-smoking mice and attenuated the increased conjugated dienes and fluorescence levels of lung and liver in SSCS-exposed mice.

Conjugated dienes and alpha-tocopheryl quinone were significantly higher in the lung tissues of rats exposed to cigarette smoke (12). These rats had an increased sensitivity to ischemia-reperfusion injury due to the increased levels of free radicals generated by cigarette smoke exposure (18). Supplementation with antioxidant vitamin E resulted in significantly less mitochondrial oxidative damage in rats (19) as well as mice (20). The protective effect of vitamin E supplementation on mitochondrial activity is a function of vitamin E's free radical scavenging ability. However, the exact mechanism of how vitamin E acts on improving cigarette smoke-induced lung injury is largely unexplored. The increased antioxidant capacity of the mice tissues in our experiment seems to be the major effect of supplementation with vitamin E. The enhanced resistance of vitamin E-supplemented Nude mice against SSCS-mediated lung injury and lipid peroxidation may be due to this effect.

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