EFFECT OF ANTIOXIDANT HORMONES AND NUTRIENTS ON IMMUNE FUNCTION IN RELATION TO AGING

by

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DEDICATION

To my parents Alfred and Helen and my fiance, Brent. Your love, encouragement, and guidance will never be forgotten.
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ABSTRACT

Aging results in a progressive decline in immune function and increased oxidative stress which leads to immunosenescence. Immunosenescence is a consequence of an imbalanced immune response (cellular immune function is suppressed and humoral immune function is enhanced) which is demonstrated by suppression of Th1 cytokines and elevation of Th2 cytokines. Oxidative stress contributes to immunosenescence, as immune cells are highly susceptible to free radical damage. This work describes how supplementation with antioxidant hormones and nutrients in the aged can improve immune function.

Elderly subjects were supplemented with fruit and vegetable extracts, containing multiple antioxidants, for 80 days. The aim of this study was to determine the effect fruit and vegetable extract supplementation has on immune function in the aged. The following serum antioxidants increased; Lutein/zeaxanthin (p<0.005), α-carotene (p<0.0001), β-carotene (p<0.0001), lycopene (p<0.05) and α-tocopherol (p<0.005). In non-smokers significant increases in lipopolysaccharide (LPS) and spontaneous cell proliferation (p<0.0001), IL-2 (p<0.0001), and natural killer (NK) cell cytotoxicity (p<0.005) were observed. Furthermore, IL-2 levels significantly increased in smokers. Supplementation with fruit and vegetable extracts appear to significantly improve various measures of immune function.

The antioxidant hormones, dehydroepiandosterone (DHEA) and melatonin (MLT) decline steadily with age. The age-related decline in hormone levels is also
associated with the development of immune dysfunction. 16-month old C57BL/6 aged mice were supplemented with DHEA, MLT, DHEA+MLT or control diets in order to determine their effects on immune function. DHEA, MLT and DHEA+MLT significantly increased Th1 cytokines (IL-2, IFN-γ) (p<0.05), and decreased Th2 cytokines (IL-6, IL-10) (p<0.05). Additionally, MLT and DHEA + MLT increased B-cell proliferation (p<0.05). Supplementation with either, DHEA or MLT appear to modulate immune function in aged mice.

Dysregulated immune function is common in aging. Antioxidant compounds are effective regulators of immune function by increasing Th1 and decreasing Th2 cytokines, increasing in vitro lymphocyte proliferation, increasing NK cell cytotoxicity, and increasing antioxidant stores. Replacement therapy may be an effective approach to “treat” the aging immune system. Improved immune function should make older individuals less susceptible to infections and possibly lower their risk of developing cancer and heart disease.
INTRODUCTION
EXPLANATION OF THE PROBLEM

Free radicals, or reactive oxygen species (ROS), are formed as a normal part of metabolism, particularly during energy deriving reactions. ROS are characterized by the presence of an unpaired electron in their outer orbital. Once a free radical is formed, it can react with stable compounds forming new ROS initiating a chain reaction where free radicals are continually being produced. ROS can therefore react with, and destroy cellular components such as, proteins, membrane lipids, and even DNA, ultimately leading to the initiation of cancer cells. Immune cells are particularly sensitive to free radical damage since their membranes contain high levels of polyunsaturated fatty acids (PUFA). PUFA contain numerous double bonds which are highly susceptible to free radical attack. Additionally, immune cells are capable of generating an oxidative burst to destroy pathogens. ROS are also used for the activation of T cells and in signaling cells to induce cytokine production. Since reactive oxygen species are produced via normal, *in vivo* processes, antioxidant defense mechanisms such as antioxidant enzymes, hormones and nutrients are present and help the body avoid excess damage. Unfortunately, as one ages there is a decrease in antioxidant enzymes and hormones. Furthermore, free radical damage occurs gradually, and accumulates over time. The combination of accumulated free radical damage, and depletion of certain antioxidants, contributes to immunosenescence and a higher risk of
developing cancer. Preventing free radical damage could therefore improve immune function and possibly lower cancer risk in the elderly. Supplementing with antioxidant hormones and nutrients could be a potential way to prevent further development of free radical damage. This work describes how antioxidant hormone and nutrient supplementation can be an effective way to treat immunosenescence.
LITERATURE REVIEW

The review of literature for this study is presented in the manuscripts, book chapters, and papers appended to this dissertation.
EXPLANATION OF FORMAT

Relationship between the papers

The original research papers are included in Appendix B of this document. Briefly, these papers describe how supplemental antioxidant hormones and/or nutrients can regulate immune function in aged mice or people, respectively.

Aging results in a dramatic decline in the antioxidant hormones DHEA and MLT. The aging process is also associated with the development of immune dysfunction and an increase in oxidative stress. These events may be related as an increase in oxidative stress could occur as a result of declining levels of antioxidant hormones. Furthermore, free radical damage could effect immune cell function as immune cells are highly susceptible to the effects of oxidation. The aim of these papers is to determine whether supplementation with the antioxidant hormones, DHEA and MLT or with antioxidant nutrients in the form of fruit and vegetable extracts could improve immune function in the aged.

Aged C57BL/6 mice were used as a model for human aging. This model is very useful as mice develop a similar decline in immune function with age as humans. Some of the major effects of an aging immune system are decreased \textit{in vitro} proliferation of lymphocytes, cytokine imbalances, and decreased NK cell activity. In the experiment where mice were supplemented with DHEA and MLT we found improvements in cytokine production. In humans supplemented with
fruit and vegetable extracts, we not only found improvements in antioxidant nutrient stores, but we also found major improvements in immune function. Additionally, improved immune function as measured by IL-2 levels was also seen in elderly smokers whose level of oxidative stress should be significantly higher.

In conclusion both of these papers studied the effects of supplemental antioxidant hormones or nutrients in aged mice or older humans. They demonstrate improvements in antioxidant vitamin status and immune function after supplementation.

**Contribution to the works**

**Paper 1**: I directed and coordinated this study and supervised the recruiting and screening of potential subjects. I prepared and allocated the supplements for distribution to the subjects. I prepared all the blood samples by collecting serum and isolating the peripheral blood lymphocytes. I performed all the immunological assays and cell culture including T- and B- cell proliferation, cytokine determination, lipid peroxidation and NK cell cytotoxicity. I wrote and prepared the manuscript for publication including performing the statistical analysis and preparing the tables and figures.

**Paper 2**: I assisted in sacrificing the animals and preparing the spleen cells and tissue samples for use in the subsequent experiments. I also assisted in doing the following assays and cell culture experiments; T- and B- cell proliferation,
lymphocyte subpopulation measurements, and NK cell cytotoxicity. In addition I wrote and prepared the manuscript for publication and assisted in the statistical analysis and preparation of the figures.
Summary of Findings

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

**Paper 1:** Epidemiologically, fruit and vegetable consumption is associated with reduced risk for cardiovascular disease (CVD) and cancer. It is thought that the observed reduction in risk is related to antioxidants present in the fruits and vegetables. Antioxidants may directly reduce CVD and cancer risk by preventing free radical damage to low density lipoprotein (LDL) cholesterol and DNA. Indirectly, antioxidants may prevent free radical damage to immune cells, thereby improving immune function. Improved immune function may prevent excessive inflammation and preserve NK cell function. Since immune function and antioxidant nutrient stores decline with age as CVD and cancer risk rises, preventing free radical damage may preserve immune function and both directly and indirectly lower CVD and cancer risk. The aim of this study was to determine the effects fruit and vegetable extracts, containing multiple antioxidants and phytonutrients, have on immune function in the elderly. Subjects (n=40; ages 60-86: mean age 68) had 2 baseline blood samples drawn and consumed the extracts for 80 days. Two additional blood samples were taken at day 40 and again at day
80. The following serum antioxidants significantly increased; Lutein/zeaxanthin (p<0.005), α-carotene (p<0.0001), β-carotene (p<0.0001), and α-tocopherol (p<0.005). Peripheral blood mononuclear cells stimulated with LPS (20μg/ml) and spontaneous cell proliferation significantly increased (p<0.0001). NK cell cytotoxicity significantly increased at effector:target cell ratios of 100:1 (p<0.0001), 50:1 (p<0.0005), and 25:1 (p<0.005). Supernatant from PBM cells stimulated with PHA (10μg/ml) or LPS (20ng/ml) resulted in significant increases in IL-2 (p<0.0001) and IL-6 (p<0.005) production. Additionally, no statistically significant differences between smokers and non-smokers were observed, indicating both groups respond to treatment. However, IL-2 levels significantly increased in smokers (p<0.005). Multiple measures of immune function show improvements after short-term supplementation with fruit and vegetable extracts. Interestingly, improved immune function, as indicated by IL-2 levels, is evident in smokers and non-smokers. Supplementation with fruit and vegetable extracts offers a novel way to improve compliance with current nutritional recommendations in hopes to ultimately lower disease risk in the elderly.

**Paper 2:** The hormones DHEA and MLT decline with advancing age and are associated with immune dysfunction. The purpose of this study was to determine whether supplementation with DHEA and MLT have synergistic effects on improving immune function in old C57BL/6 mice. Mice were given DHEA, MLT, DHEA + MLT, or control diet and/or drinking water for a total of 12 weeks. MLT and DHEA + MLT significantly (p<0.05) increased B cell
proliferation in old mice. DHEA, MLT, and DHEA + MLT significantly (p<0.05) increased the Th1 cytokines, IL-2 and IFN-γ and decreased the Th2 cytokines, IL-6 and IL-10. DHEA and MLT each effectively modulate suppressed Th1 cytokine secretion and elevated Th2 cytokine secretion, however combined hormonal supplementation failed to produce significant synergistic effects.

**Overall Conclusions**

In general, Th1 cell cytokines function to stimulate cellular immune function, while Th2 cell cytokines stimulate the humoral immune response. During aging, cellular immune function is usually quite impaired and renders older individuals more susceptible to viral infections and cancer. Additionally, a somewhat enhanced humoral immune response can lead to the development of autoantibodies and exacerbate, or cause, development of autoimmune diseases. It is therefore essential to find ways to not only improve immune function, but more importantly, to balance both the humoral and cellular immune response.

This work demonstrates how DHEA, MLT or their combined supplementation can improve measures of immune function in aged C57BL/6 mice. Particularly, these supplemental hormones were able to correct the cytokine imbalance associated with age. We found major improvements in both Th1 and Th2 cell cytokines i.e. an increased Th1 response and a decreased Th2 response. Additionally, when old mice were compared to young mice, the cytokine profile of supplemented aged mice was similar to that of the young mice. This finding
further demonstrates the benefit of antioxidant hormone supplementation in aged mice, as the cytokine profile found in young mice leads to an effective and appropriate immune response. Further studies in this area should look at the effects of DHEA, MLT and DHEA+MLT supplementation in aged humans.

In humans, fruit and vegetable consumption is associated with a decreased risk of both cancer and cardiovascular disease. Increasing fruit and vegetable consumption however is difficult to achieve, as individuals are generally poorly compliant to such diets. Finding alternative ways to achieve the benefits offered by fruits and vegetables is consequently a challenge. In this study, the immunological effects of fruit and vegetable extracts were determined. Fruit and vegetable extracts were very effective at improving various measures of immune function in a non-smoking elderly population. The \textit{in vitro} proliferation of lymphocytes, NK cell activity, and IL-2 levels all significantly increased after only a short supplementation period. Furthermore, smokers also had significant increases in IL-2 levels. Increased production of IL-2, a Th1 cell cytokine, should result in a more effective cellular immune response, as IL-2 is an important regulator of cellular immune function. Increases in lymphocyte proliferation and NK cell activity are also indicators of an enhanced immune response. Fruit and vegetable extracts are consequently a potentially beneficial supplement for the elderly. Additional studies should, however, be conducted in order to test their long-term benefits on immune function, as well as their effects on lowering disease risk.

Although these studies show clear improvements in immune function by the
action of antioxidant supplementation, carefully designed studies still need to be conducted. These studies should investigate general health outcomes associated with improvements in immune function and antioxidant levels. Since it is clear that in general, biochemical imbalances lead to poor function, (e.g. free radicals damage immune cells), substantial improvements in immune function or antioxidant levels may also be harmful. Ultimately, studies should look at the health effects of an improved functioning immune system and higher serum levels of antioxidants. Perhaps we not only need to focus on which compounds improve immune function, but also look at their effect on other aspects of health. In the end, optimal doses of supplements that maximize health without producing any deleterious effects should be ascertained. One thing we know for sure is that fruit and vegetable consumption is beneficial and until the above issues are fully examined, recommendations should only focus on their promotion.
APPENDIX A
ANTIOXIDANTS AND IMMUNE FUNCTION

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FREE RADICALS, ANTIOXIDANTS AND IMMUNE FUNCTION

INTRODUCTION

Free radicals, reactive oxygen species (ROS), are produced during normal cellular metabolism by oxidation. It is the primary means by which humans and other animals derive energy. Oxidant catalysts provide the stable electrons that are necessary for oxidation. The most common biological oxidant catalysts are copper and iron, with iron being most abundant and most readily available. As electrons are transferred from oxidant catalysts to oxygen, a variety of new oxygen species are formed, each characterized by an unpaired set of electrons in their outer orbital. Therefore, one free radical can begin a destructive process of removing electrons from stable compounds and forming many ROS, transforming stable compounds into a variety of free radicals (Table 1-1). Other sources of free radicals are also common: inflammation, strenuous exercise, detoxification, exposure to certain chemicals, radiation, ultraviolet light, alcohol, cigarette smoke, air pollutants, excess free calcium, excess stored or unbound iron, and high fat diets.

ROS are toxic via their effects on cellular components such as, denaturing proteins, membrane lipids and DNA. The latter is a major initiator of cancer. Damage caused by ROS tend to accumulate over time and is a major reason facilitating cancer development in the elderly.

Free radicals can be detrimental by reacting with, and sometimes destroying, critical cellular components including the polyunsaturated fatty acids (PUFA) that
comprise lipoprotein particles and plasma membranes. ROS attack the unsaturated bonds of fatty acids in lipid membranes, altering membrane structure and function\textsuperscript{1}. The products of lipid peroxidation are diffusible and since lipoproteins travel throughout the body, the ensuing damage can spread far beyond the site of original attack. Therefore, in order to reach the point of oxidative stress a significant amount of oxidant exposure must occur. The basic prerequisite for lipid peroxidation, as well as other types of oxidative damage, is inadequate free radical scavengers. Free radicals that react with polyunsaturated long carbon chain lipids results in the formation of chemotactic products, aldehydric nonanoic acids, and various other aldehydes\textsuperscript{2}. Aldehydes can bind with biological amines such as proteins, nucleic acids and amino lipids and alter their structure and function\textsuperscript{2}. Free radical damage results in a loss of membrane fluidity, receptor alignment, potential cell lysis, damage to sulfur containing enzymes and other proteins resulting in inactivation, cross-linking and denaturation. Damage to carbohydrates can alter cellular receptor functions including those associated with cytokine activities and prostaglandin formation. Free radicals can also induce brain disorders\textsuperscript{3} atherosclerosis\textsuperscript{4} and colon cancer\textsuperscript{4}.

Since ROS are produced abundantly by a variety pathways, humans, and other animals have evolved defense mechanisms against these free radicals. Antioxidants are small molecules that act as scavengers of reactive oxygen species and prevent them from causing further cellular damage. In addition to antioxidants, free radicals can also be inactivated by enzymes. Aging results in a decrease in the amount of antioxidant enzymes. Such decreases in antioxidant enzymes contribute to the increased risk for
developing cancer and the increased incidence of immune dysfunction with aging. In addition to the decline in antioxidant enzymes the accumulation of free radical damage also contributes to these increased risk factors.

However, when free radicals are not excessive, they can play a positive role in human health and development. For example, the fetus uses oxidants to stimulate cellular differentiation. Free radicals can contribute to, and alter, gene expression. Free radicals also play a pivotal role in the activation of natural detoxification systems such as cytochrome P450. They also produced by neutrophils and macrophages in an effort to kill invading microorganisms. With chronic or high microorganism infection the antigen burden in white blood cells can produce excessive amounts of free radicals increasing the antioxidant requirements.

ANTIOXIDANTS MECHANISM OF ACTION

Antioxidant enzymes

Antioxidant enzymes have the capacity to lower the free radical burden. Free radical reactions can be broken down into three stages: initiation, propagation, and termination. Antioxidants enzymes can affect the generation of free radicals during all of these stages. The initiation phase of free radical reactions can be inhibited by two metalloenzymes; superoxide dismutase and catalase. They work by inactivating precursor molecules of free radicals, preventing the formation of ROS. Superoxide dismutase is a Mn-containing metalloenzyme in mitochondria and a Cu/Zn-containing
metalloenzyme in the cytoplasm. Catalase is an Fe-containing metalloenzyme in peroxisomes. It catalyzes the decomposition of hydrogen peroxide which is produced as a result of superoxide dismutase. Both enzymes however, catalyze the reaction seen in Table 1-2. In addition, glutathione peroxidase which contains selenium also works as an antioxidant. Glutathione peroxidase is important for the decomposition of hydrogen peroxides and lipid peroxides and thereby works by interfering with the propagation phase of free radical generation. Although Mn, Cu, Zn, and Se are necessary components, they are only considered antioxidant when incorporated into their respective enzyme.

Antioxidant Compounds

The following compounds act only by directly interfering with propagation of free radical generation: vitamin E, vitamin C and β-carotene. In addition to the direct action of these nutrients, riboflavin, a B vitamin, is a constituent of the enzyme glutathione reductase. Glutathione reductase is important for the re-generation of antioxidant defenses.

IMMUNE FUNCTIONS AND FREE RADICAL REACTIONS

Effect of free radicals on immune cells

Free radicals can be detrimental to lymphocytes. High levels of dietary polyunsaturated fatty acid (PUFA) are immunosuppressants. The unsaturated double
bonds found in PUFAs are prime targets for free radical damage and initiation of chain reactions resulting in lipid peroxide formation. Lipid peroxides and aldehydes can alter cellular, including immunocellular, functions and even result in lysis of oxidized cell membranes. Lipoproteins in the plasma can also be oxidized and become lymphotoxic\(^{10}\). Lipid peroxidation also causes a decrease in membrane fluidity. Loss of membrane fluidity in lymphocytes has been directly related to a decreased ability of lymphocytes to respond to immunological challenges\(^{11-13}\).

Antigen presenting cells (APCs) generate an oxidative burst in response to many stimuli which targets intracellular proteins. Intracellular proteins could either be normal cellular proteins which are not needed or they could be of viral origin. APCs contain proteolytic complexes, proteasomes, that selectively recognize and degrade oxidatively modified proteins. Oxidative modification exposes hydrophobic core residues which are acted on by proteasomes\(^{14,15}\) to generate peptides of nine amino acids in length. Normal rates of protein oxidation within cells "mark" proteins for proteolysis by proteasomes. Peptides of nine amino acids in length travel to the endoplasmic reticulum where they associate with Major Histocompatibility Complex (MHC Class I) molecules and B\(_2\)-macroglobulin. Such complexes traverse the golgi apparatus and get presented on MHC class I cell surface molecules. Once these proteins are properly presented on class I MHC, CD\(_8^+\) T cells can distinguish between self and non-self antigens\(^{16}\) and selectively destroy only those cells which are infected. In this scenario, oxidation and generation of free radicals is an essential component for the proper functioning of cell mediated immunity.
Free Radicals and T cell activation

Free radicals are necessary compounds for maintaining optimal immune function. The proliferation of T lymphocytes is a pivotal event in cell-mediated immunity and it too, requires the action of free radicals. Foreign antigens are partially degraded by antigen-presenting cells and presented on their surface in association with MHC Class II to CD4+ T-cells. This initiates a complex series of events with production of cytokines, particularly, interleukin-1 (IL-1) by accessory cells and IL-2 by CD4+ T cells. Cytokines are small, locally acting molecules which stimulate various events including proliferation of CD4+ T lymphocytes. These T cells also express cell surface receptors for IL-2 and the iron transport molecule, transferrin.

Hydroxyl radical scavengers such as dimethyl sulfoxide, thiouren, dimethyl urea and mannitol inhibit mitogenic responses of human peripheral blood lymphocytes to phorbol myristate acetate (PMA), ConA and phytohaemagglutinin (PHA) mitogens. These findings suggest that hydroxyl radicals might be involved in mediating the signal(s), perhaps those from cytokines, that trigger T cell activation and proliferation. Further evidence to support this theory is that antioxidants such as, butylated hydroxy anisole (BHA), desferrioxamine (DES) and desferrithiocin (DFT) inhibit the antigen-driven proliferation in a dose-dependent manner. These compounds however, do not inhibit the production of IL-1 by accessory cells or that of IL-2 by T cells, but they do inhibit cell surface expression of IL-2 receptors. An exception to this rule is the amino thiol cystamine can inhibit IL-2 production by human peripheral blood lymphocytes.
stimulated with mitogen. DES, DFT, BHA and ferriganide all inhibit DNA synthesis induced by PHA or PMA/ionomycine\textsuperscript{17}. It has therefore been proposed that free radicals are involved in the activation of T lymphocytes\textsuperscript{18}. Iron is also necessary for T cell activation as iron in the $^2$ state can convert O$_2$ to a free radical which then activates T cells. Iron chelators exert anti-proliferative effects through interactions with intracellular iron pools. These chelators might influence cellular activities by binding iron and preventing its involvement as a catalyst for hydroperoxides or by inhibiting ribonucleotide synthesis. Furthermore, Terada showed that small traces of iron are necessary for the production of the cell-cycle regulatory protein kinase, P34\textsuperscript{cdc219}.

B lymphocytes possess a functional NADPH oxidase, which also works by producing free radicals. The proliferation of B cells is also inhibited by antioxidants\textsuperscript{17} while T lymphocytes do not have NADPH oxidase\textsuperscript{20} other leukocytes provide the "help" necessary for T lymphocytes to produce ROS\textsuperscript{21}.

Cystamine amino thiol compounds block the binding of AP-1 and NF-KB to DNA. These two proto-oncogenes are necessary for T cell proliferation. Also, cysteamine inhibits intracellular DCFH oxidation, IL-2 mRNA, IL-2, and DNA synthesis. A speculative interpretation of these results is that mitogens induce intracellular formation of ROS in T cells leading to oxidation of AP-1 and/or NF-KB. They might need free radicals for their transport into the nucleus. Binding of transcription factors to DNA only occurs under reducing conditions. The redox factor, Ref-1, in the nucleus, is capable of reducing oxidized transcription factors. Among the
genes regulated by AP-1 is the gene encoding for IL-2 production. IL-2 production is essential for T cell passage through G1 into DNA synthesis.

Lipoxygenase (LO) inhibitor blockade increases intracellular Ca\(^{2+}\) in response to binding of the T cell receptor (TCR) to MHC and CD3 accessory cell surface molecules. This results in the inhibition of inositol-triphosphate synthesis which impedes signal transduction and ultimately cell activation. However, LO inhibitors do not effect the activation of Na\(^+\) and H\(^+\) antiport by PMA. These compounds can increase pH in stimulated cells and activate protein kinase C (PKC) which will amplify signal transduction pathways\(^{22}\). LO inhibitors decrease IL-2 production in Jurkat cells and do not inhibit IL-2 production in PMA-treated Jurkat cells\(^{22}\).

**Free radicals and cytokine production**

Since free radicals are extremely reactive they can modify various biochemical substances. Hydrogen peroxide elevates cytoplasmic free Ca\(^{2+}\) levels and activates PKC, facilitating signal transduction\(^{23}\). Additionally, hydrogen peroxide can cause reversible inhibition of DNA synthesis in murine osteoblastic cells when added during the late G1 phase. This function of hydrogen peroxide is also seen with TGF-β1. TGF-β1 and hydrogen peroxide both increase expression of the HIC-5 gene which encodes a novel Zn-finger protein (molecular weight 5 KDa). It also increases phosphorylation of 30 KDa heat shock protein. Thus, hydrogen peroxide can be a second messenger when activated by TGFβ1\(^{24}\). Low concentrations (10nm-1mM) of superoxide anions and hydrogen peroxide can stimulate growth or growth responses. Intracellular pH
increases within 10 seconds activating PKC. Production of superoxide anions and hydrogen peroxide involves the activity of a plasma membrane NADPH-oxidase. Cytokines are required for the generation of free radicals. TNF-a specifically induces extensive mitochondrial superoxide generation. In contrast to TNF-a, a variety of human tumor cells, neuroblastoma, melanoma, and colon, pancreatic, ovarian and breast carcinoma release large amounts of hydrogen peroxide without any specific growth stimulus. The growth responses that involves the release of superoxide or hydrogen peroxide may be mediated through the oxidative inactivation of serum proteinase inhibitors. This would allow serum proteinase to remodel the cell surface, or glycocalyx, thereby facilitating or modulating the action of normal growth factors.

Oncogenes or transformed cells which respond significantly better to growth promoting effects in the presence of low levels of superoxide or hydrogen peroxide. This may be due to the fact that reduced levels of antioxidant enzymes contribute to a cellular redox state. These free radicals facilitate the growth of neoplastic cells as part of a constitutively active autocrine system, or from adjacent inflammatory cells.

At low concentrations active oxygen is an important mediator of cellular response and growth. Exogenous addition of active oxygen to resting cells stimulates DNA synthesis and the induction of proto-oncogenes, c-fos and c-myc. The presence of oxygen radicals increases the production and reception of IL-1. Oxygen radicals can increase IL-1 production by monocytes and the proliferation of lymphocytes in PHA-induced blast transformation reactions stimulated by recombinant IL-2. There are two major mechanisms of lymphocyte stimulation by oxygen radicals, activation of
PKC and lipoxygenase. The effect of lipoxygenase activation is supported by the strong inhibitory effect of NDGA (a lipoxygenase inhibitor and antioxidant)\textsuperscript{31}.

Antioxidants, diamide and ascorbic acid, have inhibitory effects on protein tyrosine phosphatase in murine fibroblast cells transfected with human EGF-receptor. In view of its effects on cellular growth, oxidative stress plays a role in growth factor-mediated signal transduction\textsuperscript{32}. IL-1β stimulates the IL-6 secretion in a dose and time-dependent manner. The antioxidants pyrrolidine dithiocarbamate, N-acetyl-cysteine, two thiol-reacting molecules, trolox, and hydrosoluble analogue of vitamin E, completely inhibit IL-6 secretion in a dose-dependent manner. However, a mixture of verapamil (a calcium channel blocker) neomycin sulfate (a phospholipase C cascade inhibitor) and 2'5'-dideoxyadenosine (an adenylate cyclase inhibitor) did not affect IL-6 induction by IL-1\textsuperscript{33}.

The nuclear transcription factor, NF-KB is constitutively present in the cytoplasm as an inactive complex. NF-KB is involved in the transmission of signals from the cytoplasm to the nucleus by binding to the 5'-GGGACTTTCC-3' sequence in the K enhancer. NF-KB can activate genes involved in immune, inflammatory or acute phase responses. IL-1, TNF, PMA and other activating factors can activate NF-KB. Activated NF-KB translocates the nucleus where it recognizes a specific DNA sequence\textsuperscript{34} the gene coding for IL-6\textsuperscript{35}. N-acetylcysteine (NAC), an antioxidant, inhibits and diamid, an oxidant, stimulates NF-KB activation\textsuperscript{36}. The mechanism of action of NAC is that it increases intracellular glutathione and decrease reduced glutathione. NAC also blocks TNF-a induced NF-KB activation\textsuperscript{37} while hydrogen peroxide leads to
NF-KB activation. These changes should produce an immunological environment that contains higher levels of cytokines produced by T-helper 2 cells, TNF and IL-6. In murine and human AIDS, leukemia, and some cancers, high levels of IL-6 are associated with suppressed cellular immune defenses.

The role of cytokines in ROS production is still unclear. It has been shown that isolated rat islet cells exposed to a combination of cytokines had diminished insulin release, increased ROS production, and islet necrosis. The *in vitro* toxic effect of cytokines on islet cells is mediated by ROS release. However, *in vivo* effects of cytokines in the production of ROS and induction of oxidative damage are not well understood. In rodents, macrophages produce ROS following stimulation with other compounds. This process does not occur spontaneously. *In vivo* production of cytokines significantly enhanced macrophage response to infectious agents.

Biologically, cytokines such as TNF-α and IL-1 are highly conserved and are capable of causing extreme toxicity and even death at certain doses. Blockade of IL-1 by the use of its specific receptor antagonists and blockade of TNF by the use of TNF antibodies, preserved the effects of endotoxin i.e. bacterial lipoprotein polysaccharide (LPS) cell surface molecule. This implies that each cytokine is partially responsible for lethal endotoxin effects. Thus, both administration of cytokines and strategies to block their effects can be beneficial to the host.

Organisms need to evolve mechanisms to control or regulate cytokine responses. One mechanism of cytokine protection against cellular toxicity is generation of protective enzymes that limit the effects of ROS.
Summary

Although free radicals at high levels can decrease immune function, at physiologic concentrations they are vital for antigen presentation and cell proliferation. Cytokines like TNF-a are necessary for the generation of free radicals and at low concentration, free radicals act as second messengers to mediate cellular growth. Free radicals can also increase TNF-a, IL-6 and IL-1 production. IL-6 production stimulated by free radicals can occur via two mechanisms, either by increasing IL-1 or by activating NF-KB. IL-1 and TNF-a can be extremely toxic and even induce death at certain doses. IL-6 inhibits T-helper 1 cells and secretion of IL-2 is needed for normal cellular immunity. The body has therefore evolved defense mechanisms to detoxify free radicals. Instead of investigation ways to minimize the production of free radicals perhaps studies should focus on how to regulate their production more effectively.

ANTIOXIDANT NUTRIENTS AND IMMUNE FUNCTION

Effect of antioxidants on non specific immune responses

Antioxidants can increase immune responses by controlling the amount of free radicals generated in a cell. Neutrophils kill extracellular pathogens by generation of an oxidative bursts which are toxic to the invading organism. Neutrophils are not destroyed because they take up vitamin E\(^{44}\) before the oxidative burst. Following activation neutrophil vitamin C concentration is lower\(^ {45,46}\). This suggests that vitamin C works with vitamin E to decrease the free radical burden within the neutrophil\(^ {47}\).
Vitamin C and Vitamin E supplementation has also been found to normalize the reduced chemotactic and bactericidal activities of neutrophils in individuals with inherited phagocytosis disorders\textsuperscript{48}, as well as in newborns\textsuperscript{49}. Vitamin E deficient rats have impaired neutrophil and macrophage chemotaxis, reduced ingestion of complement coated beads, and decreased protection from auto-oxidative damage\textsuperscript{50}. Chronic immune-mediated inflammation such as that found in experimentally induced granulomas has been decreased in animals given superoxide dismutase, catalase, or vitamin E\textsuperscript{51}. The synovial fluid in the joints of rheumatoid arthritis patients contains high levels of reactive oxygen species with infiltrating neutrophils and T-lymphocytes in the infected joints. Local lipid peroxidation has been correlated with the degree of inflammation in animal models of arthritis. Administration of antioxidants such as superoxide dismutase and catalase directly into arthritic joints decreases inflammation\textsuperscript{52}. Ascorbic acid levels are low in patients with rheumatoid arthritis, despite normal ascorbic acid absorption\textsuperscript{53}. In this sense both a balanced generation of free radicals as well as an adequate level of antioxidant nutrients are essential to destroy invading organisms while preserving immune cells.

**Effect of antioxidants on specific immune responses**

In laboratory animals T and B-cell proliferation is correlated with dietary and serum vitamin E levels\textsuperscript{54}. Vitamin E deficiency affects T-lymphocytes to a greater degree than B-lymphocytes. T-lymphocyte function is reduced to a greater degree than that of B-lymphocytes, macrophages, and stem cells with age\textsuperscript{55,56}. T-lymphocyte cell
membranes in young mice are more fluid than B-cell membranes. However, as mice age, T-cells lose their fluidity whereas B-cells retain theirs this occurs because T-cell lipids are more susceptible to peroxidation than B-cell lipids are \(^5^7\). The ability of T-lymphocytes to form rosettes is significantly inhibited following exposure to oxygen radicals whereas B-lymphocyte rosette formation is not affected \(^5^8\). As aging progresses the level and activity of antioxidant enzymes decreases which results in impaired T-lymphocytes. Dietary β-carotene and carotenoids of similar chemical structure (but lacking pro vitamin A activity) enhance cytotoxic T-cell activity and lower tumor levels in animal models \(^5^5\).

Summary

Inflammation results in the production of excessive amounts of free radicals. Vitamin E, C and glutathione are necessary for increasing the immune response, controlling inflammation and reducing tissue damage.

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Table 1-1. Free Radical Produced by the Reduction of Dioxygen, by Ionizing Radiation, Reactive Metals, Enzymes and Other Endogenous and Environmental Initiators.

<table>
<thead>
<tr>
<th>Radical</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>.O$_2^-$</td>
<td>Superoxide</td>
</tr>
<tr>
<td>HO$_2^-$</td>
<td>Superoxide Conjugate Acid</td>
</tr>
<tr>
<td>1O$_2$</td>
<td>Singlet Oxygen</td>
</tr>
<tr>
<td>.OH</td>
<td>Hydroxyl Radical</td>
</tr>
<tr>
<td>R</td>
<td>Organic Free Radical</td>
</tr>
<tr>
<td>ROO</td>
<td>Peroxy Free Radical</td>
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</tbody>
</table>
Table 1-2. Antioxidant Enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mineral</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase (SOD)</td>
<td>CuZn</td>
<td>(2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2)</td>
</tr>
<tr>
<td>Glutathione peroxidase (GSHPx)</td>
<td>Se(4)</td>
<td>(H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O)</td>
</tr>
<tr>
<td>Catalase (CT)</td>
<td>Fe</td>
<td>(H_2O_2 \rightarrow H_2O + O_2)</td>
</tr>
<tr>
<td>Glutathione-S-transferase (GS-T)</td>
<td>None</td>
<td>(ROOH + 2GSH \rightarrow GSSG + ROH + H_2O)</td>
</tr>
</tbody>
</table>
ANTIOXIDANTS, VITAMINS AND HORMONES IN AIDS

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INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is a result of infection with the human immunodeficiency virus (HIV-1 or HIV-2) which eventually destroys a 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 Oxidative stress in HIV infection and AIDS is exemplified by an excess production of reactive oxygen species (ROS) and a general loss of antioxidant defenses.3 Therefore, the reduction of oxidative stress by antioxidants treatment may be a desirable therapy during 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 ROS producing systems and the antioxidant defense systems which normally function synergistically to prevent or destroy ROS.5 An increased production of ROS is caused by infecting agents in neutrophils and macrophages6 and as well as from abnormal production of TNF-α.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 viral replication machinery. TNF may play an important role in causing a further increase in the levels of oxidants by providing an 'amplification loop' that feedback to excite further production of ROS from macrophages and neutrophils. It may also react with T cells to enhance expression of autocrine cell activators, such as IL-2, and receptors, thereby promoting activation of T cells and generation of additional intracellular ROS. 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.

Apoptosis, programmed cell death of CD4+ lymphocytes is of fundamental importance in the progression of AIDS. The cascade of events that results from oxidative stress can initiate apoptosis, a possible pathway of immune cell loss in patients with HIV infection. It includes oxidation of cellular membranes, alteration in metabolic pathways, disruption of electron transport systems, depletion of cellular ATP, loss of Ca2+ homeostasis, endonuclease activation and DNA/chromatin fragmentation. DNA damage caused by oxidative stress may be related to HIV-associated malignancies and disease progression. Downstream events, secondary to these effects may also play a role in activation of the latent virus and subsequent viral replication. 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.\textsuperscript{13} TNF-α is an important activator of HIV by generating ROS which activates NF-κB.\textsuperscript{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.\textsuperscript{3} Deficiency is a result of malabsorption of nutrients, hypermetabolism, and drug-nutrient interactions.\textsuperscript{15} The antioxidant status of lymphocytes is important for their functioning, which is closely linked to their redox potential and particularly to cysteine and glutathione levels.\textsuperscript{5} In a weakened antioxidant system, DNA repair capacity of the cells may be altered and lymphocytes may be destroyed or impaired.\textsuperscript{16-18} Since ROS are involved in the signal transduction mechanisms for HIV activation, a possible therapeutic use of antioxidants in preventing HIV activation has been suggested.\textsuperscript{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.\textsuperscript{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. Depletion of GSH inhibits proliferation of T lymphocytes, particularly those from HIV-infected patients. 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.

HIV-infected patients have greatly decreased levels of GSH in their plasma and peripheral blood lymphocytes. The decreased levels of GSH are highly correlated with suppression of CD4+ cells. 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. 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.

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. 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. NAC has been reported to increase antibody-dependent cell mediated cytotoxicity of neutrophils. Early clinical trials have shown that NAC prevents the decline in CD4+ cells in GSH-deficient individuals. Unfortunately, oral and intravenous
GSH are not effective at enhancing cellular GSH stores. 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. Therefore, treating patients with NAC may be a useful strategy in slowing the progression of disease.

L-2-oxothiazolidine 4-carboxylate (OTC) is another pro-GSH drug that has been proposed for AIDS therapy. Although NAC and OTC blocked cytokine induction of HIV in vitro, NAC was far more effective than OTC. In isolated peripheral blood mononuclear cells, NAC fully replenishes depleted intracellular GSH whereas OTC only minimally replenishes GSH. Although NAC is markedly more effective at blocking HIV expression than OTC in vitro, both drugs could prove equally effective in the clinical setting. A report studying rats noted that procysteine, also a pro-drug for glutathione, effectively reduced ischemic heart damage by increasing levels of cellular GSH. Whether 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 ultimately preventing membranes lipid peroxidation. 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.\textsuperscript{15}

Plasma vitamin E levels are lower in HIV-infected patients than in controls.\textsuperscript{41} Vitamin E derivatives such as vitamin E acetate, $\alpha$-tocopheryl succinate and 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC) exhibited a concentration dependent inhibition of NF-$\kappa$B activation by TNF-$\alpha$.\textsuperscript{1} Thus, vitamin E acetate which is a natural, safe compound, and PMC which was demonstrated to be very effective in blocking NF-$\kappa$B activation, should be considered for possible inclusion in combination therapies for AIDS.\textsuperscript{1} The intake of supplementary vitamin E is significantly associated with slower progression to AIDS in HIV seropositive men.\textsuperscript{42} Vitamin E has also been shown to increase the CD4$^+$/CD8$^+$ lymphocyte ratio in AIDS patients by enhancing CD4$^+$ cell counts.\textsuperscript{43} In murine AIDS, a 15-fold increase in dietary vitamin E significantly restored mitogen-induced splenic T and B cell proliferation, stimulated NK cell activity, and alleviated hypergammaglobulinaemia by reducing immunoglobulin production.\textsuperscript{44,45}

\textbf{\textit{$\alpha$-LIPOIC ACID}}

Recently, $\alpha$-lipoic acid was found to exert antioxidant action in vivo and in vitro.\textsuperscript{46} In addition, $\alpha$-lipoic acid has been shown to inhibit HIV-1 replication in infected cells.\textsuperscript{47,48} This may be due to the inhibition of NF-$\kappa$B activation imposed by the antioxidant properties of dihydrolipoic acid (DHLA) generated from $\alpha$-lipoate.\textsuperscript{14} DHLA can causes a complete inhibition of NF-$\kappa$B activation induced by
TNF-α by scavenging free radicals and recycling vitamin E. The inhibitory action of α-lipoic acid was found to be very potent as only 4 mM was needed for a complete inhibition, whereas 20 mM was required for NAC, this suggests 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. Unfortunately, most of these studies were done in mice, and mice can synthesize vitamin C internally. In humans, vitamin C is properties seem to make it a potential anticancer treatment. 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 that are not toxic. Vitamin C is apparently more efficient than GSH or NAC in reducing of HIV-1 replication in chronically infected T lymphocytes. The effects of vitamin C on HIV can also be increased by the addition of NAC.

Large amounts of vitamin C are consummated by HIV-infected patients, however, no clinical benefit is associated with ingestion of vitamin C, in spite of the reports that vitamin C can improve CD4+ cell counts at high doses. A survey of the nutritional status of HIV seropositive patients showed a non-significant decrease in serum vitamin C, and no significant difference in the prevalence of a
low status even with an increase in vitamin C intakes (10 times the RDA) due to supplementation.

CAROTENOIDs

There is a severe deficit in plasma carotenoids including β-carotene levels in HIV-infected patients. 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.

OTHER VITAMINS

In a study of micronutrients in HIV-infected patients, there was a decrease in vitamin A and vitamin B2 (riboflavin) levels. Vitamin B2 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 B1 (thiamin), vitamin B6 (pyridoxal), folate and vitamin B12 were unchanged by HIV infection, whereas the prevalence of deficiencies in vitamin A and B6, vitamin B12 and E were significantly increased.

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 IL-2 gene expression.\(^5\) The addition of zinc to a serum-free culture medium increases the proliferation of T lymphocytes and the synthesis of IL-2.\(^6\) In the presence of zinc, thymulin assumes an active cyclic form enabling zinc to be recognized by high affinity receptors on T lymphocytes.\(^5\) This results in differentiation of T lymphocytes by induction of antigen B in response to concanavalin.\(^5\) It is very important to note that Zn\(^{2+}\) inhibits the endogenous endonuclase activated by Ca\(^{2+}\) which is responsible for apoptosis of CD4\(^+\) cells induced by TNF.\(^5\) Additionally zinc acts as an antiviral agent by inhibiting reverse transcriptase.\(^5\)

HIV-infected patients whose status remained stable for two years had normal plasma zinc levels,\(^5\) whereas zinc levels of those who progressed towards AIDS were lower. Thymulin, a good marker of zinc status, was found to be extremely low in the blood of patients with AIDS.\(^5\)

Faced with this decreased zinc status,\(^5\) the effect of zinc supplementation was investigated. The most worrisome risk was of an upsurge of viral activity due to the existence of several zinc-finger proteins in the structure of HIV-1.\(^6\) It has been reported that supplementation of zinc in AIDS patients can increase the CD4\(^+\)/CD8\(^+\) lymphocyte ratio,\(^5\) 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 for all immunological functions, administration of selenium is suggested as a supportive therapy in early, as well as in advanced stages of HIV infection. A characteristic of the protective effects of selenium against viral pathogens is that it is only beneficial at supplemental levels above physiological requirements. This suggests that it may have a role not associated with GPx. 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.

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

Selenium supplementation in HIV-infected patients causes symptomatic improvements, especially in appetite and intestinal functions, and possibly slows disease progression. During the period of supplementation, CD4+ cells tended to decline, however, this decline was often only slight, or not observed at all; CD8+
cells tended to decrease resulting in an increased ratio of CD4⁺:CD8⁺ cells.⁶⁸

COPPER

It is extremely difficult to study 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 proteins”, even in copper-deficient subjects.⁶⁹

Serum copper increased in AIDS patients after 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.⁵³, ⁶⁰, ⁷⁰

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 disease state.⁶⁹

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,⁷¹ while red cell SOD remains normal.⁶⁹

Serum catalase activity increased progressively with advancing HIV infection (i.e., AIDS > symptomatic infection >asymptomatic infection >controls).⁷¹ This correlates with increases in serum hydrogen peroxide (H₂O₂) scavenging
ability, and may reflect or compensate for systemic GSH and other antioxidant deficiencies in HIV-infected individuals.\textsuperscript{71}

Manganese-containing superoxide dismutase (MnSOD) is the key enzyme in cellular protection from TNF induced apoptosis.\textsuperscript{72} Expression of the gene for Mn-SOD and for metallothioneins.\textsuperscript{5} A decreased production of Mn-SOD was seen despite overproduction of its mRNA.\textsuperscript{73} This results from inhibition of translation of SOD mRNA caused by HIV tat protein binding to the RNA hairpin.\textsuperscript{5} The sequence on which tat binds presents a sequence homology to the viral RNA. This sequence is the biological target of tat and permits regulation of viral expression. The anomaly of Mn-SOD production is accompanied by signs of oxidizing stress in cells.\textsuperscript{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.\textsuperscript{74, 75} In animals, DDTC increased GSH levels in a variety of tissues.\textsuperscript{13} A significant reduction in the rates of new opportunistic infections were reported in AIDS patients receiving DDTC as compared to placebo.\textsuperscript{75} However, a subsequent cohort study of HIV-infected asymptomatic patients failed to demonstrate a benefit.\textsuperscript{13}
Desferrioxamine (DFX)

DFX is an iron chelator with strong antioxidant properties. DFX can inhibit in vitro HIV-1 replication in H-9 T lymphocytes. The rational for this work was to explain the low rate of symptomatic HIV-1 infection in multiply transfused thalassaemic patients intensively chelated with DFX.\textsuperscript{76}

PLANT-DERIVED METABOLITES WITH SYNERGISTIC ANTIOXIDANT ACTIVITY

Plants experience death due to oxidative stress which closely parallels the process of apoptosis in human, 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.\textsuperscript{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 are proposed as a mechanism by which viral replication and cell killing can be inhibited.\textsuperscript{3}

Phenolic compounds (hydroxyl derivatives of aromatic hydrocarbons)

\textit{Ubiquinone}
Although ubiquinone (coenzyme Q₁₀, CoQ₁₀) 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, sparing vitamin E in the process.⁷⁷

Patients with AIDS had significantly lower blood CoQ₁₀ levels than healthy controls, while patients with ARC and asymptomatic HIV infection had decreased blood levels of CoQ₁₀, but not to the extent of the of AIDS patients.⁷⁸

Supplementation with CoQ₁₀ retarded progression from ARC to AIDS and had positive effects on the T4/T8 lymphocyte ratio.⁶⁸ However CoQ₁₀ may actually increase the level of free radicals thereby increasing oxidative stress.⁶⁸

**Flavonoids**

Flavonoids (plant phenolic pigment products, particularly the catechins and quercitin), scavenge peroxyl and hydroxyl free radicals.⁷⁹ They are protective against lipid peroxidation probably by donating H⁺ atoms to peroxyl radicals and terminating chain reactions.⁸⁰ They can also control release of reactive oxygen products from macrophages and neutrophils by regulating enzymes such as NADPH oxidase.⁸¹ Quercitin, in particular, can inhibit the PKC-induced phosphorylation of I-κB that can liberate NF-κB and lead to viral replication.⁴¹,⁸²
**Coumarins (benzopyrones)**

Coumarins are effective against oxidative stress by acting in a similar manner to flavonoids.\(^8_3\)

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

Nitrogen containing compounds effectively inhibit lipid peroxidation and impede release of superoxide radicals from membranes. They exert their stabilizing affects by binding with negative charges on both nucleic acids and phospholipids.\(^8_4\) They inhibit protease and RNAase activity which is observed as a consequence of oxidative stress in plants.\(^8_4\) 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 as 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.\(^8_5\) 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.\textsuperscript{86}

\textit{Vitamins}

Vitamin C and E and the various carotenoids are ubiquitous in plants. In humans, however, they do not offer sufficient 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.\textsuperscript{87, 88}

HORMONES

Dehydroepiandrosterone (DHEA)

DHEA and its sulfate are major secretory adrenal products in humans and function primarily as precursors for peripheral conversion to active androgenic hormones.\textsuperscript{89} DHEA protects against certain viral infections in animal models and inhibits HIV-1 infection \textit{in vitro}. DHEA has a modest ability to downregulate HIV-1 expression in HIV-1-infected human cells.\textsuperscript{90} Clinical relevance of serum DHEA levels and progression of HIV remains to be established. The decline in serum DHEA levels before AIDS develops could be a manifestation of impaired adrenocortical function (adrenal glands), shown by diminished responses to adrenocorticotropic hormone stimulation in AIDS patients.\textsuperscript{91} However, glucocorticoid levels in the HIV
infection were elevated. DHEA has a direct antiglucocorticoid activity.\textsuperscript{92} Hence, a decreased level of DHEA may allow cortisol to act more effectively and lead to enhance immunosuppression.

**MELATONIN (MLT)**

MLT, the main pineal hormone secreted to induce sleep, influences many biological functions. MLT is two times more effective than vitamin E in scavenging peroxyl radicals.\textsuperscript{93} MLT protects against lipid peroxidation and chronic immune dysfunction in murine AIDS by reducing excessive free radical production.\textsuperscript{94} In addition, MLT can stimulate the action of the related antioxidant glutathione peroxidase, and inhibit HIV replication by reducting the binding activity of NFK-B.\textsuperscript{95} In addition, MLT can increase immunity in animals as MLT treatment prevented excessive Th2 cell cytokine production and immune dysfunction during murine AIDS.\textsuperscript{96} MLT, therefore, has the potential to be an effective antioxidant in the treatment of AIDS.

**CONCLUSION**

Oxidative stress is not merely an epiphenomenon, but is at the heart of the
pathology of HIV. This has incited researchers to test the effects antioxidants have in cell models which demonstrates the effectiveness of certain micronutrients and other antioxidants.

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. 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, but future studies will likely resolve which substances can be efficacious in treating HIV infection.
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MELATONIN, IMMUNE MODULATION AND AGING

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Running Title: Melatonin, Immune Modulation and Aging

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ABSTRACT

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 against 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.

Key Words: 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 (Fig. 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 its 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 MLT. Both immature (thymocytes) and mature (spleen lymphocytes) cells seem sensitive to MLT treatment. The epithelial component of the thymus, which is responsible for synthesizing and releasing 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 receptors, as well as receptors for other hormones/neuropeptides are present on these cell lineage’s, 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 lineage's 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-1[^12,13] MLT, TRH and IL-1 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 immuno-derived opioid agonists bind specific receptors on thymus and spleen cells and counteract the depression induced by stress-associated corticosteroids\cite{16,17}.

These agonists seem to mediate the immunoenhancing and anti-stress effects of MLT\cite{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\cite{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\cite{19}. MLT receptors are coupled to guanine nucleotide binding proteins which modulate guanine nucleotides in human lymphocytes\cite{19}. MLT increases cGMP production in human lymphocytes by binding specific receptors stimulating vasoactive intestinal peptides to activate cAMP\cite{20}. These peptides are not only potent activators of cAMP\cite{21,22}, but also exhibit inhibitory effects on mitogen-stimulated proliferation\cite{23}, decrease IL-2\cite{24} and modulate IgA and IgM production\cite{25}. MLT may therefore regulate immune function by its action on vasoactive intestinal peptides.

Human circulating T lymphocytes contain high-affinity binding sites for MLT, however these sites are not present on B lymphocytes\cite{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 had 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 and RZR\(\beta\)) which are part of the nuclear hormone receptor superfamily\(^{[32]}\). Very recently, MLT has been found to be a natural ligand of RZR and RZR\(\beta\). A response element on the promoter of 5-lipoxygenase (a key enzyme in the biosynthesis of leukotrienes/inflammatory mediators) binds specifically to RZR\(\beta\). 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\(\beta\)\(^{[32]}\).
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\[^{25}\] and restores zinc levels in old mice\[^{30}\]. The precise 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-\[^{24}\]. 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-\[^{24,39,40}\]. 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- production\[^{43}\]. Th2 cells are sensitive to IFN-, which selectively inhibits their proliferation and cytokine synthesis\[^{24}\]. IL-4 is able to inhibit IFN-production\[^{44}\] while IFN- 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\textsuperscript{[24]}. The existence of a pineal-immune axis which maintains a Th1/Th2 balance is of considerable relevance\textsuperscript{[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\textsuperscript{[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\textsuperscript{[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 immunosuppressed mice increased splenocyte response to LPS\textsuperscript{[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\cite{38}. Inhibiting MLT in mice results in depressed primary antibody response to sheep red blood cells and autologous mixed lymphocyte reactions\cite{47}. MLT induced increases in primary antibody (IgG and IgM) production which is attributed to opioid peptides released by MLT-induced Th cells \cite{5,38} (mediated by increases in IL-2).

**MELATONIN AND MECHANISMS 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\cite{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\cite{49}. Tumors that secrete cytokines like IL-6, which suppresses IFN-γ, could decrease serum IFN-γ levels enough to suppress MLT secretion\cite{45}. Enhanced peripheral metabolism of MLT in breast cancer patients\cite{50} may also account for its decline. Although this is not always seen, a similar decline in serotonin-N-acetyl transferase\cite{50}, the rate-limiting enzyme in MLT synthesis, was observed. These results were also replicated in patients with prostate cancer\cite{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\textsuperscript{[53]}. Studies in humans\textsuperscript{[50]} as well as animals\textsuperscript{[54,55]} illustrate an inverse relationship between tumor growth and MLT production. Thus, tumor growth itself, possibly by releasing neurotoxic agents\textsuperscript{[56]} or cytokines\textsuperscript{[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\textsuperscript{[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\textsuperscript{[57]}.

\textit{IN VITRO MODELS OF MELATONIN AND CANCER GROWTH}

Many studies have been performed using \textit{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 \textit{in vitro} against human breast cancers, but only against estrogen sensitive or estrogen receptor positive ones\textsuperscript{[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\textsuperscript{[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. When tamoxifen was given along with MLT it failed to slow the growth of the tumor. 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. This produced a 100 fold increase in the effectiveness of Tamoxifen. While the mechanism of action is still unclear, MLT seems to sensitize cells to respond to Tamoxifen treatment more effectively. MLT may therefore be useful at priming cancer cells treatment with Tamoxifen. Furthermore, MLT treatment resulted in a lower effective dose of Tamoxifen. On the other hand, 5-fluorouracil diminished the beneficial effects of MLT 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. 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-\(\gamma\), and TNF-a 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-\(\gamma\); 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-\(\gamma\) 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-\(\gamma\) 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\textsuperscript{[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\textsuperscript{[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\textsuperscript{[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\textsuperscript{[71]}, gastric cancer\textsuperscript{[72]}, colorectal cancer\textsuperscript{[72]} and other cancers of the gastrointestinal tract\textsuperscript{[72]}. MLT effectiveness in this combinational therapy limited the necessary dose, and subsequently, side effects of IL-2. Advanced solid neoplasms are usually resistant to treatment with IL-2 alone.
However, when IL-2 was given along with MLT, not only was tumor regression observed\textsuperscript{[72, 73]}, but there was also an increase in lymphocytes and eosinophils\textsuperscript{[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\textsuperscript{[74]}. Treatment with low-dose IL-2 plus MLT has been shown to normalize platelet counts in cancer patients with thrombocytopenia\textsuperscript{[74]}. This combinational therapy has been shown to be an effective treatment for renal cell carcinoma and may warrant further clinical studies\textsuperscript{[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 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\textsuperscript{[76, 77]}. Viral infection can increase glucocorticoids levels\textsuperscript{[78]} resulting in thymus and spleen involution and subsequently, immunosuppression\textsuperscript{[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)\cite{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\cite{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\cite{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\cite{79}. MLT stimulated production of immune components deficient in HIV patients i.e., T helper cells, NK cells, null cells, macrophages, IL-4, IFN-α, GM-CSF, IL-10, eosinophils, and red blood cells\cite{80}. Furthermore, MLT may act by repressing 5-
lipoxygenase therefore resulting in fewer leukotrienes; which contribute to the inflammatory response in AIDS\textsuperscript{[81]}. 

MLT may also help defeat chronic immune dysfunction in AIDS by reducing excessive free radical production\textsuperscript{[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\textsuperscript{[83]}. A decline in the ratio of CD4+ to CD8+ cells is one sign of progression from HIV infection to AIDS\textsuperscript{[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 (NF B); a element involved in promoting HIV replication, and further increases TNF production\textsuperscript{[85]}. MLT can stimulate the action of a related antioxidant, glutathione peroxidase, which prevents activation and subsequent binding of NF B. This suggests that MLT has the potential to be an effective antioxidant in the treatment of AIDS\textsuperscript{[86]}. 

**HORMONAL MODULATION BY MELATONIN: A MECHANISM OF IMMUNOREGREULATION**

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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Aging and the Pineal Gland

Dysfunctions

↓ Sleep
Immune suppression
↑ Cancer growth

Peak Melatonin (pg/ml blood)

Age (Years)
THE ROLE OF CYTOKINES AND CHEMOKINES ON TUMOR PROGRESSION

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Introduction

The term cytokine, introduced by Cohen in 1974, is now widely used to indicate a number of soluble peptide factors that include interleukins, cytotoxic factors such as tumor necrosis factors alpha and beta (TNF-α and TNF-β), growth factors, and differentiation factors such as colony stimulating factors (CSF).

Cytokines are crucial for regulation of cell growth and/or differentiation (1). These molecules display pleiotropic effects, both cellular and biochemical, thus stimulating antiviral activity. They direct cytocidal actions, as well as promote lymphocyte and myeloid progenitor cell growth. Furthermore they activate macrophage function in nonspecific host defense mechanisms (2).

Since cytokines are factors that regulate cell growth, and more importantly, cell proliferation, both tumor cells and immune cells are capable of producing them. The key difference between these two cell types is that cytokine production by tumor cells is often an unregulated process. Additionally, tumor cells typically, produce and use cytokines in an autocrine fashion.

The development of normal cells to cancerous cells is thought to be a multistep process of clonal evolution. This process is driven by a series of somatic mutations that initially convert normal cells to a pre-cancerous state. The progression involves inactivation of tumor suppresser genes; adenomatous polyposis coli gene (APC), deleted-in-colorectal cancer gene (DCC), and p53, as well as activation
of cellular oncogenes; K-ras. In addition to the inactivation of tumor suppressor
genes and the activation of oncogenes, up-regulation of growth factors
accompanies these changes which further enhances the tumor cells' ability to
proliferate unabated.

The immune system is capable of responding and eliminating mutated cells as well
as cancer cells however, so many individuals die each year from cancer. This
suggests that the immune response to tumors is often ineffective. Perhaps the
immune system is effective at removing tumor cells early in their development, but
once key mutations occur that eliminate tumor specific antigens, the immune
system becomes non-functional. The current approach to cancer immunotherapy
can help us to understand the role of cytokines. Cancer immunotherapy includes
both passive and active augmentation of host responses. Specific active
augmentation of immune effector cell functions involves immunization with tumor
cells, or better, with tumor rejection antigens. These approaches may elicit novel
or an enhanced immune response against tumor antigens. Non-specific stimulation
of immune effector cells is a result of exposure to selected cytokines such as
interferon’s and interleukins(3). Once exposed, the effector cells become activated
and capable of phagocytosing invading cells. The use of cytokines to
nonspecifically activate immune cells localized at the site of the tumor is a very
interesting approach to cancer therapy.
Growth Factors in Cancer

Cell growth is a subtly regulated process that responds to the specific needs of the organism (4-6). Autocrine and paracrine growth regulation has been observed in a number of tumor systems, thus suggesting that cells can respond to the same growth factors they produce. This is in agreement with the observation that tumor cells produce significant amounts of growth stimulating peptides, thus reducing the need for growth factors by malignant cells in vitro. Also, the desmoplastic response of normal stroma to many tumors is a clear indication of local growth factor synthesis. Growth factors induce cells to enter and proceed through the cell cycle. Initial experiments show that two types of restriction points exist in the cell cycle. One is at the G₀/G₁ transition point where cells come out of quiescence, and the second is within G₁, at which point the cell is irreversibly committed to division (4). Factors allowing transit through the first restriction point are termed competence factors and those permitting transition through the second are called progression factors. Many cytokines display properties of both types of factors.

Members of the family of epidermal growth factors (EGF), in addition to transforming growth factor alpha (TGF-α) has biological activity mediated through the EGF tyrosine kinase receptor. In contrast to EGF, TGF-α is often expressed in transformed cells and many carcinomas. TGF-β is a homodimeric peptide that
plays a crucial role in wound healing and angiogenesis. Depending on the cell type it can have either inhibitory or stimulatory effects. It has been shown that TGF-β has been unable to inhibit growth of some malignant cell lines (5).

Platelet derived growth factor (PDGF), secreted by a variety of tissues exists as both a homodimer and a heterodimer, and also exhibits tyrosine kinase activity. Another growth factor, which has been implicated in the pathogenesis of tumors, is basic fibroblast growth factor (BFGF), which not only stimulates angiogenesis, but also the proliferation of normal and malignant cells (6). The expression of insulin-like growth factor (IGF) is developmentally regulated. IGF-II messenger ribonucleic acid (mRNA) expression is highly expressed in fetal tissues and embryonic tumors such as nephroblastoma and hepatoblastoma (7). Angiogenic factors are also necessary for tumor growth since rapidly growing tumors are highly dependent on oxygen and nutrients (5).

Such angiogenic factors can be released by stromal cells in the course of an aberrant wound healing process following tumor cell proliferation or by the tumor itself.

Cytokines such as TGF-α, TGF-β, TNF-α, and interferon gamma (IFN-γ), can antagonize proliferation by other growth factors. In the case of TGF-α this is manifested as a G₂ phase block often followed by apoptosis (8). Programmed cell death is a frequent response by cells that are deprived of specific growth factors.
Recent evidence suggests protein expressed by the *c-myc* oncogene drives cells through the cell cycle and induces apoptosis in the absence of the autocrine or extrinsic growth factors necessary for survival (8). Several different mechanisms are known by which malignant cells undergo disruptions in growth factor regulation and signal transduction pathways, including alterations of growth factor peptides, decreased responsiveness to inhibitory factors, or by excess or inappropriate synthesis of the growth factors themselves. Several growth related genes have been cloned as a result of their oncogenic activity, including the *v-sis* transforming gene of simian sarcoma virus (SSV), the fibroblast growth factor (FGF) oncogene *hst*, as well as *erbA* and *erbB* which cooperate in the transforming action of avian erythroblastosis virus (AEV). Helyn has shown that a number of growth factors or promoters are required for in vitro growth of normal melanocytes (9). These include BFGF, IGF-1, insulin, 12-O-tetradecanoylphorbol-13-acetate (TPA), alpha-melanocyte stimulating hormone (α-MSH), and calcium.

**Inhibition of Tumor Growth**

The rate of tumor cell growth is limited by growth factor interactions. In theory, potential therapeutic interventions can be aimed at modulating different points within the stimulator mechanisms. If the factors are secreted it may be possible to sequester them using either anti-growth factor antibodies or soluble...
receptors. The high affinity binding that results in sequestering would thus prevent the growth factor from binding its normal signal transducing receptor. Certain growth factors signal cells to grow via phosphorylation of tyrosine residues present on protein molecules. Receptors for EGF, PDGF, and IGF-1, all exhibit a tyrosine specific protein kinase activity that is stimulated by ligand binding (10). It is therefore feasible to block tumor growth by inhibiting receptor tyrosine kinase activity. Erbstatin, an agent isolated from actinomycetes, is a tyrosine analog. Tyrphostins are related molecules which block phosphorylation of tyrosine residues and in culture have been shown to inhibit cell proliferation while displaying only minimal toxicity (11). Thus it may be possible to design tyrphostins that selectively inhibit tyrosine kinases. Furthermore the somertostatin analog octreotide (sms 201-95) has been used on patients with carcinoid and other neuroendocrine tumors since it reduces serum IGF-1 levels by effecting the growth hormone (GH)/IGF axis (12).

Inhibition of Malignant Cell Growth by Cytokines

Soluble interleukin-1 (IL-1) receptors have been shown to suppress growth of acute myelogenous leukemia blast cell progenitors and impairs deoxyribonucleic acid (DNA) synthesis in human astrocytes in vitro. In addition, synthetic analogues attained by replacements of constituent amino acids in growth factors have
effectively bound receptors without inducing biological response (13). Cancers such as hairy cell leukemia, carcinoid tumor, as well as head and neck cancer have all shown favorable responses to trials with interferon alpha (IFN-α). In vitro experiments have shown that IFN-γ can inhibit angiogenesis induced by FGF and PDGF by upregulation of TNF-α(14). IFN-γ, however, is also a potent stimulant of tumor associated macrophage (TAM) activity and may therefore boost production of angiogenic factors in situ. Clinical IL-2 cancer treatment exemplifies the enthusiasm associated with the immunotherapeutic approach. IL-2 acts as an immunostimulating agent by interaction with specific receptors not only on natural killer (NK) cells, but on T and B lymphocytes as well (15). Some tumors produce cytokines themselves (Table 1) but their effect on tumor progression is unclear.
Table 1. Cytokines Produced by Tumor Cells

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>IL-10</th>
<th>TGF-β</th>
<th>IL-6</th>
<th>IL-8</th>
<th>See</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>46, 57, 120, 126</td>
</tr>
<tr>
<td>Ovarian</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
<td>34, 51, 52, 57, 58</td>
</tr>
<tr>
<td>Colorectal</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>75</td>
</tr>
<tr>
<td>Endometrial</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>47, 58</td>
</tr>
<tr>
<td>Thyroid</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostate</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>101</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Renal Cell</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td>92, 97</td>
</tr>
</tbody>
</table>
Human recombinant interleukin-6 (IL-6) is a member of the multifunctional hematopoietic growth factor family of cytokines. When it is added to early stage melanoma cell lines, cell proliferation is inhibited. However, when IL-6 was added to advanced stage cell lines no effect was noted(16). Previous studies have shown that four out of eight advanced stage human melanoma cell lines produce IL-6 mRNA(17). When antisense oligonucleotide to the human IL-6 gene was added to melanoma cells in culture, there was significant inhibition of growth in those lines producing IL-6. No such inhibition was noted in cell lines not producing IL-6(17). By contrast, neutralizing antibodies to IL-6 were ineffective. This indicates that intracellular IL-6 may have evolved to function as an internal autocrine growth factor for melanoma cells, but only at the later stages of tumor progression(18). In
addition to IL-6, other cytokines such as IL-1α, IL-1β, TNF-α, and TGF-β, have also been shown to inhibit growth in early rather than late stage melanoma cell lines(19-20). The above mentioned cytokines have actually been shown to stimulate growth in some advanced stage cancers and *in vitro* production of IL-6 has been detected in several lung cancer cell lines(21).

High levels of IL-2 can stimulate cells that express a form of the receptor possessing 100-fold lower affinity. The selectivity and strength of the induced response is IL-2 dose dependent(22). Toxicity associated with high IL-2 results from the release by monocytes and macrophages of several mediators such as TNF, IFN-γ, and CSF(23). Both granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) shortened the duration of leukopenia after chemotherapy. It is suspected that pronounced side effects associated with GM-CSF treatment are due to monocyte/macrophage activation, which may account for tumor progression in tumors producing GM-CSF.

**Angiogenesis Factors in Cancer**

Malignant cells in solid tumors and their metastases communicate with neighboring stromal cells and with one another via a complex network of extracellular signals. Evidence suggests that these signals include not only a large
number of cytokines and soluble receptors but also hormones, antibodies, and components of the extracellular matrix (ECM). As seen in Figure 1 cytokines are mainly released by, and act on, cells within the tumor (11,12). Cytokines regulate the proliferation and metastatic activity of malignant cells and suppress the activity of infiltrating immune cells. Cytokines also enhance the establishment of stromal compartments by stimulating both angiogenesis and the deposition of matrix proteins (24). Angiogenesis is essential for early tumor growth. Angiogenesis is distinct from vascularization or vasculogenesis in that new blood vessels are not created from primitive stem cells, but instead bud off from existing vasculature present around the tumor. Cytokines that are involved in angiogenesis include vascular endothelial growth factor (VEGF), acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), TGF-α, TGF-β, EGF, TNF-α, platelet derived endothelial cell growth factor (PDECGF), pleiotrophin, hepatocyte growth factor/scatter factor (HGF/SF), IFN-α, IFN-γ, angiotrophin, thrombospondin, platelet factor IV, IL-1, IL-6, and IL-8. These factors may elicit several effects on endothelial cells either directly or indirectly, by stimulating angiogenesis-modulating activity of various cells at the tumor site. The direct-acting cytokines often alter endothelial cell proliferation, migration, and/or tube formation both in vitro and in vivo, whereas the indirect-acting agents may have little or no effect on these cells in vitro.

EGF and TGF-α stimulate endothelial cell proliferation both in vitro and in
vivo by binding to the same receptor(25). Both are capable of inducing
tetradecanoylphorbol acetate (TPA) production in HOME cells (human
microvascular endothelial cells with subsequent stimulation of tube formation(26).
Tumor cells and macrophages manufacture TGF-α in solid tumors(24,25). TGF-β
1 has a dose-dependent effect on angiogenesis induced in vitro by VEGF and FGF.
At concentrations <100ng/ml TGF-β1 has been shown to effect the activity of both
VEGF and FGF. At concentrations 5-10ng/ml TGF-β1 inhibited both VEGF and
FGF induced endothelial cell invasion and lumen formation(27,28).

Depending on dose, TNF-α(both in vitro and in vivo) can either stimulate or
inhibit angiogenesis. Low doses(0.01-1ng)produce stimulatory effects, while high
doses(1-5μg)cause inhibition(29). Since local tissue concentrations of TNF-α
rarely reach the μg range, these findings suggest that TNF-α may be a stimulating
factor in tumor angiogenesis. Macrophages are a major source of TNF-α in the
microenvironment of both ovarian and breast carcinomas(30,31). In certain cancer
cell lines, TNF-α induces prostaglandin(PG)production in vivo(32). Macrophage
production of TNF-α is inhibited by exposure to prostaglandin E2 (PGE2) (33). It is
therefore suspected that a potential feedback loop exists between tumor cells
making PGE2 and tumor macrophages secreting TNF-α. Some primary malignant
epithelial cells as well as certain tumor-associated macrophages(TAM) are capable
of making soluble TNF-α receptors(30,31). Please see reference 67 for an
interesting discussion of TAM. This may represent a mechanism whereby tumor
cells protect themselves from the immunomodulating (tumoricidal) activity of TNF-α. Tumor cells may simultaneously reduce local TNF-α concentrations and thereby inhibit the anti-angiogenesis effects of high levels of TNF-α.

The Role of TAM in the production of Factors Stimulating Cancer Progression: Solid tumors consist of malignant cells and stroma. Components of tumor stroma include new blood vessels, fibrin-gel matrix components, and cells responsible for the production of inflammatory leukocytes(34). The frequency of lymphoreticular infiltrate in human neoplasm’s reflects the origin of cancers at sites of previous chronic inflammation. TAM affects diverse aspects of the immunobiology of neoplastic tissues, including vascularization, growth rate, metastasis, stroma formation, and dissolution. There is evidence that in some neoplasm’s, including common human cancers, the pro-tumor functions of TAM prevail. The “macrophage balance” hypothesis emphasizes the dual potential of TAM(35). TAM can influence both neoplastic growth as well as regression, with pro-tumor activity prevailing in the absence of therapeutic intervention. Macrophage infiltration is not dependent on specific immunity. Factors derived from the tumor itself play a pivotal role in the regulation of macrophage levels in metastatic tumors that are poorly immunogenic(35). Murine and human tumors were found to release tumor-derived chemostatic factors(TDCF)capable of inducing chemostatic migration of monocytes(36-42). The chemokines that play an
important role in regulating TAM are shown in Table 2. MCP-1 is a chemo-attractant active on monocytes and inactive on neutrophils and lymphocytes. It belongs to the C-C branch of chemokines, which are a conserved region of four cysteine residues(43). MCP-1 interacts with a receptor coupled with a G protein that is sensitive to pertussis toxin. This interaction induces a rapid increase in intercellular calcium(44). MCP-1 affects several functions of those mononuclear phagocytes relegated to recruitment or effector activity(Table 2). C-C chemokines arm monocytes with the molecular tools needed to facilitate localized and polarized digestion of extracellular matrix components during recruitment. In tumor tissues, the release of lytic enzymes by MCP-1 stimulates TAM, and may provide a pathway for the invasion of tumor cells, thus contributing to augmented metastasis associated with inflammation(45). In addition, natural MCP-1 has been reported to induce IL-1 and IL-6, but not TNF-α(46).

Various human tissues express chemokines of the C-X-C family. Some neoplasm’s of the melanocyte lineage express GRO-α, and the related molecule IL-8, which induces proliferation and migration of melanoma cells(47). The pro-inflammatory cytokine IL-8 is chemotactic to neutrophils, and activates peptides isolated from the culture fluid of stimulated human monocytes(48). IL-8 is a member of a supergene family encoding a set of related cytokines possessing both inflammatory and growth stimulating potential(49). Melanoma growth-stimulating activity (MGSA) is also a member of the same supergene family. MGSA/gro is expressed in a tissue specific fashion and is induced by IL-1, TNF-α, as well as lipopolysaccharide(LPS) or thrombin. MGSA/gro regulates both normal and
malignant cells. IL-8 and MGSA/gro compete for the same receptor binding on
neutrophils. \textit{In vitro}, IL-8 is produced by various human carcinomas and brain
tumors, either spontaneously or after exposure to IL-1 and TNF-\(\alpha\)(50-60).
Table 2. Role of Tumor-Derived Chemokines in the Biology of TAM

<table>
<thead>
<tr>
<th>Family</th>
<th>Cytokine</th>
<th>Target Cells</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-C</td>
<td>MCP-1</td>
<td>Monocytes</td>
<td>Chemotaxis: Induction of uPA, uPAR, and gelatinase; Inhibition of NO synthase; Oxidative burst; Systemic anti-inflammation</td>
</tr>
<tr>
<td></td>
<td>MCP-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MCP-3</td>
<td>Basophils</td>
<td>Chemotaxis and Activation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PMN, Lymphocytes</td>
<td>Chemotaxis</td>
</tr>
<tr>
<td>IL-8</td>
<td></td>
<td>Melanoma Cells</td>
<td>Growth and Migration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Endothelial Cells</td>
<td>Angiogenesis</td>
</tr>
<tr>
<td>Gro-α</td>
<td>PMN</td>
<td>Chemotaxis</td>
<td></td>
</tr>
</tbody>
</table>

IL-8 has also been shown to have angiogenic activity(52). Furthermore, there is evidence that polypeptides other than chemokines are involved in regulating the recruitment of TAM. Ovarian carcinoma tumors produce M-CSF and TNF-α, as well as an unrelated polypeptide chemoattractant(35,53,54). The production of M-CSF by tumor cells, and the expression of c-fms (which codes for the M-CSF receptor) by TAM, facilitates the proliferation and survival of TAM in certain murine and human tumors. Tumor derived cytokines can thus play a role in causing the extravasation of monocytes in tumors (Figure 1) and advanced neoplasia. This has long been associated with defective capacity to mount inflammatory responses(56).

Inhibitors of chemotaxis antigenically related to a retroviral
immunosuppressive protein known as P15E coexist with TDCF in certain tumors(56). Thus a balance between chemotactic and inhibitory cytokines may regulate extravasation in tissues, including neoplasm's. IL-8 causes defective neutrophil recruitment when given in systemic circulation(57). This observation raises the possibility that cytokines such as MCP-1 and/or related chemokines (leaking from advanced tumors) contributes to the systemic defects in immunity and inflammation associated with neoplasia.

Tumor cells can produce TGF-β and an inhibitor polypeptide known as macrophage deactivating factor(58). These molecules inhibit various functions of mononuclear phagocytes. Various carcinoma lines produce IL-10(59). The in situ expression of this anti-inflammatory cytokine was demonstrated in skin tumors, and high levels of this cytokine are present in ascitic fluids from ovarian carcinomas(60,61). IL-10 and functionally similar mediators such as IL-13 may thus inhibit certain inflammatory responses observed in neoplasia. It should be noted, however that Richter, et al. found that IL-10 prevented tumor growth and macrophage infiltration when transfected into the ovary cells of immunodeficient Chinese Hamsters(62). These findings are consistent with the pro-tumor role of TAM. TAM can suppress T cells and natural killer cell-mediated reactions. It also suppresses its own function, in part by producing prostaglandin and tumor derived GM-CSF, which down regulates macrophage cytotoxicity(63). In the absence of deliberate stimulation, TAM produces substantial amounts of IL-6, but not IL-1 or
TNF-α(64,65). Macrophages are potent producers of nitric oxide, but tumor cells produce inhibitors nitric such as TGF-β, IL-8, and MCP-1(66).

Mononuclear phagocytes are capable of producing large amounts of growth factors, including PDGF, EGF, and TGF-β. Thus, in vitro, these cells can promote the growth of tumors. Usually, the growth promoting activity of TAM is best observed at low effector-to-target cell ratios, or with tumor cells in sub-optimal culture conditions(35,67). Ovarian carcinoma cells produce M-CSF and MCP-1, which promotes their survival and attracts monocytes. TAM in turn produces cytokines that stimulate cancer cell growth in a paracrine loop. In a series of pancreatic cell lines, TNF-α was shown to actually augment proliferation by inducing expression of TGF-α and its receptor(68).

Factors That Inhibit Immune Response and Induce Cancer Progression

Early research on immunosuppression in cancer was focused mainly on defective cellular responses, depressed polymorphonuclear(PMN) and macrophage cell function(particularly phagocytosis, migration, and bactericidal activity), depressed Ig production by B cells, reduced NK cytotoxicity, and most often the inability of lymphocytes to respond to mitogenic stimuli( such as lecithin, antigens, or allogens). Recent attention has shifted to defects in cytokine release(IL-2) and cytokine receptors. The exact mechanism of immunosuppression is uncertain, but a
multiplicity of factors has been postulated to contribute to the occurrence.

The peripheral blood lymphocytes (PBL) of patients and animals with cancer were unable to develop into effective lymphocyte activated killer cells (LAK) or cytotoxic T lymphocytes (CTL) (69, 70). Lymphocytes isolated from tumors are known as tumor infiltrating lymphocytes (TIL). TIL are found to proliferate poorly and to have only limited cytotoxicity (71). Interest now appears to have switched to the impaired production of cytokines or receptors by PBL in cancer patients (72, 73).

Culture supernatant from many types of tumor cells (isolated from different living species) has been found to contain strong immunosuppressive factors. Evidence of this is indicated by the capacity of this material to inhibit a variety of immune reactions such as delayed type hypersensitivity (DTH) macrophage accumulation at inflammation sites, macrophage chemotaxis, as well as phagocytosis and cytotoxicity. Other immune reactions inhibited by the supernatant of these cultured tumor cells includes skin graft rejection, lymphocyte proliferation in response to mitogenic stimuli, antibody synthesis, lymphokine production, and generation of LAK, TIL and CTL.

TGF-β was identified as a tumor-associated immunosuppressive molecule following a series of studies of immunosuppression in glioblastoma. TGF-β mRNA was found in all tumors tested and was made by many tumor cell lines including prostate adenocarcinoma, as well as colorectal, breast, endometrial, and thyroid
carcinomas(74). IL-2 is a potent inducer of LAK cells that release TGF-β(75). Unlike most cells, which secrete latent TGF-β, the LAK cells secrete a biologically active form of the molecule(75).

Lymphocyte blastogenesis inhibitor factor (LBIF) is produced by U037 macrophage cell lines and inhibits IL-1, IL-2, as well as antigen and lecithin-induced proliferation(76). It has been shown to inhibit expression of the p75 IL-2 receptor. It acts on both murine and human lymphocytes and arrests lecithin-stimulated T cells at early G1 phases(77).

Although CSF is not immunosuppressive by itself, they stimulate proliferation and differentiation of hemopoietic cells and induce the appearance of immunosuppressive macrophages(77). Daily injections of GM-CSF into mice causes immunologic alterations similar to those noted in tumor-bearing animals. The macrophage-associated suppression was attributed to cell-to-cell contact as well as to release of PGE₂, since the effect could be partially reversed by indomethacin(78). Acute-phase proteins such as haptoglobin, α1-acid glycoprotein, ceruloplasmin, and c-reactive protein are produced by the liver in response to inflammation, and are found in increased amounts in sera of patients with cancer(79). Some of these factors were found to depress lymphocyte proliferation. Synthesis of acute phase proteins is mediated by a number of cytokines, which may be produced directly by the tumor cells or indirectly by tumor products acting on macrophages(80,81).
IL-6 is an autocrine growth factor for various carcinomas, including renal cell, bladder, and ovarian. It also serves as a growth factor for glioblastoma and certain T and B cell lymphomas(82-87). Moreover, elevation of serum IL-6 levels is an adverse prognostic factor in patients with metastatic renal cell carcinoma(89). IL-6 is also increased in lung cancer(90). Information about the role of IL-6 in patients with various malignancies is not extensive. Furthermore, the mechanism responsible for elevated levels of serum IL-6 in these patients remains unclear. It may be the result of abnormal production by tumor cells, or it may arise from an immunological response to the tumor.

It is interesting to note that dehydroepiandosterone(DHEA) and melatonin, whose levels in humans decline with increasing age, have both been shown to suppress IL-6 production in immunosenescent old mice. Also, Yanagawa found that lung cancer patients with detectable serum IL-6 levels have lower serum albumin levels than those without detectable IL-6(90). Renal cell carcinoma patients who experienced weight loss had higher blood IL-6 levels than those who did not(91). IL-6 directly and indirectly contributes to tumor progression through inhibition of the anti-tumor response by host cells. It is thus somewhat of a paradox that detectable serum levels of IL-6 are not frequently observed in the progression of B-cell chronic lymphocytic leukemia(92).

High levels of IL-6 secretion are found in normal human ejaculate, in prostate epithelial primary culture, and in anaplastic androgen-independent human prostate
cancer cell lines. Unlike most solid tumors, prostate cancer (PCA) can cause death without metastasis to vital organs (93). IL-6 was tested as a molecular mediator of human PCA morbidity. TNF-α, which stimulates the production of IL-6 and mediates cachexia, is not an exocrine human PCA cell protein. Additionally, IL-6 is a well documented cytokine mediator of inflammation and wasting syndromes in other diseases (94). In experimental models chronic, systemic exposure to IL-6 causes a cachexia syndrome that is independent of tumor burden (95,96). Other studies have shown the presence of human IL-6 receptor and quantitative production of bioactive human IL-6 in three human PCA cell lines (97). IL-6 causes a dose-dependent decrease in human hepatocyte secretion of cholesterol and is part of the physiology of cancer cachexia (98). In vitro, IL-6 causes elevated lactate dehydrogenase (LDH levels which decreased serum cholesterol (99). IL-6 has also been shown to block programmed cell death induced by the following cytotoxic agents; TGF-β1, and wild type p53 (100,101).

Normal melanocytes require a number of exogenous growth factors in contrast to most malignant melanomas. Melanoma cells endogenously produce human IL-8 as a growth factor (102). When recombinant human IL-8 was exogenously added to human melanoma cells, weak cellular proliferation was observed. In Vitro exposure of sk-MEL13 and sk-MEL23 to antisense oligonucleotide inhibited cell proliferation. These oligonucleotides were targeted against two different regions of human IL-8 mRNA. They were also targeted against a monospecific immune
serum, and two IL-8 specific Monoclonal antibodies (mAb). It may thus be postulated that endogenous IL-8 is involved in immunomodulation and melanoma metastases.

IL-8 is also a chemotactic factor and immunomodulator for lymphocytes. It works by upregulating CD11b/CD8 and macrophage-1 antigen (Mac1) (103) which play important roles in metastases and lymphocytic tumor infiltration. Also, it has been reported that IL-8 causes leukocytes to lose their ability to bind to IL-1 activated endothelial cells (104).

IL-10 has also been shown to have inhibitory effects, in particular its ability to inhibit cytokine production by Th-1 cells. Epstein-Barr virus (EBV) and human immunodeficiency virus (HIV) associated lymphomas provide a model to investigate the possible roles of IL-10 in the neoplastic process. High levels of hIL-10 but not eIL-10 were produced by 8 out of 15 B cell lymphomas, although 7 of the 8 tumors with high levels of hIL-10 contained the EBV genome (105). Just 1 out of 11 lymphomas from HIV-seronegative patients produced IL-10, and only one of the tumors (IL-10 negative) contained the EBV genome. The observation that HIV and EBV increased tumor IL-10 expression lends credence to the notion that IL-10 plays a role in lymphoma development and B cell hyperactivation common in AIDS patients. Additionally, patients suffering from non-Hodgkin’s lymphoma exhibited significantly higher serum levels of IL-10 (7.98 pg/ml) than healthy volunteers (< 5 pg/ml) (88). In patients with basal cell carcinomas (BCC), reverse
transcriptase polymerase chain reaction (RT-PCR) have elevated levels of IL-4 and IL-10(106). Furthermore, in comparison to benign growths, higher levels of IL-10 were associated with squamous cell carcinomas (SCC). Treatment of BCC with intralesional IFN-α induced tumor regression with a concomitant upregulation of IL-2 (on TILs), and a down regulation of IL-10 mRNA expression in lesions (on tumor cells). Tumor IL-10 production may thus provide a mechanism for evading the local T cell-mediated immune response.

Patients with certain types of cancer have profound immunosuppression(107,108). In vitro, TIL isolated from autologous tumor cells exhibited marked functional defects(109). As previously described in this review, it is suspected that the tumors themselves produce immunosuppressive factors that contribute to immune dysregulation.

In conclusion, tumor cells can directly or indirectly induce tumor progression by producing a range of molecules that include cytokines, growth factors, tumor derived chemotactic factors and immunosuppressive factors. Examples of direct induction of tumor progression would include activation of the κ-ras oncogene, inactivation of anti-oncogenes, and increased angiogenesis. Indirect induction of tumor progression occurs when tumor cells suppress immune cells, and indirectly facilitate tumor progression.
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IMMUNE FUNCTION IN ELDERLY SMOKERS AND NON-SMOKERS
IMPROVES DURING SUPPLEMENTATION WITH FRUIT AND
VEGETABLE EXTRACTS

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Abstract  **Background:** Epidemiological evidence suggests fruits and vegetables reduce the risk of cardiovascular disease (CVD) and cancer. Immune function declines with age as CVD and cancer incidence rises and may be related to poor antioxidant status.  

**Objective:** To investigate how fruit and vegetable extracts (Juice Plus™) containing multiple antioxidants and phytonutrients, effects immune function in the elderly.  

**Design:** Subjects (n=53; ages 60-86; mean=68 yrs.) consumed extracts for eighty days and 2 blood samples were taken at baseline and then one at days 40 and 80.  

**Results:** Significant increases were found in the serum antioxidants when baseline values were compared to day 80; Lutein/zeaxanthin (p<0.005), α-carotene (p<0.0001), β-carotene (p<0.0001), lycopene (p<0.05) and α-tocopherol (p<0.005). Peripheral blood mononuclear cells (PBM) stimulated with LPS (20ng/ml) significantly increased (p<0.0001). Spontaneous proliferation of PBM cells, significantly increased (p<0.0001). NK cell cytotoxicity significantly increased at effector to target cell ratios of 100:1 (p<0.0001), 50:1 (p<0.0005), and 25:1 (p<0.005). Supernatant from PBM cells stimulated with PHA (10μg/ml) resulted in significant 2-fold increases in IL-2 (p<0.0001). Additionally statistically significant increases in IL-2 production were seen in smokers (p<0.005).  

**Conclusions:** Fruit and vegetable extract supplementation significantly enhanced multiple measures of immune function in elderly subjects, and improved IL-2 levels in smokers. Fruit and vegetable extract supplementation offers a novel way to improve compliance with current nutritional recommendations and may potentially lower disease risk.  

**Key Words** carotenoids, α-tocopherol, fruits and vegetables, immune function, aging,
smoking.

Introduction

The incidence of cardiovascular disease (CVD) and cancer rises dramatically with age as immune function declines. Recently, development of atherosclerosis has been attributed to the oxidation of LDL (1). The imbalance of antioxidants and free radicals may be a contributing factor which further impairs immune function in the aged and leads to an inappropriate induction of the inflammatory response. Preventing immune damage with antioxidants may be a way to decrease CVD risk. Additionally, initiation of cancer cells, may also be a result of free radical damage and poor antioxidant status. The lymphocytes, natural killer (NK) cells are capable of eliminating cancer cells. Impaired NK cell cytotoxicity in the elderly may be another event which increases cancer risk.

Epidemiological evidence suggests a beneficial role of dietary fruits and vegetables in reducing risk for cancer and CVD. The observed inverse relationships between fruit and/or vegetable consumption and lung cancer (2,3), colorectal cancer (4), total cancer mortality (5), and CVD (6) have been attributed to dietary antioxidants like β-carotene and vitamin E. Interestingly, no decrease in the incidence of disease has been observed with supplemental β-carotene (7) and, in fact, increased risk of lung cancer was observed in smokers (8,9). Whether these studies failed to supplement with the optimal dose, or whether other protective factors (10) are present in fruit and vegetables that work alone or in combination with antioxidants, like β-carotene, remain to be answered. Vitamin E has consistently been shown to improve cellular immunity in rats and mice (11-13). Similarly,
\(\beta\)-carotene increased the \textit{in vitro} stimulation of mouse splenocytes (14), however, the evidence is not as convincing for humans (15,16). Therefore, current public health messages encourage increasing consumption of fruit and vegetables (17) to at least five servings per day instead of promoting supplement use. Unfortunately, consumption has not changed (18), despite such strong recommendations, and is particularly low in adolescents (19) and patients with increased risk of colorectal cancer (20). Furthermore, large discrepancies have been seen with self-reported and actual fruit and vegetable intake, as individuals tend to overestimate their actual intake (21). Thus, nutritional recommendations tend to be ineffective (21). We have therefore used fruit and vegetable extracts to supplement dietary intake. Since increasing consumption of fruits and vegetables tends to be difficult, perhaps supplemental extracts could be a way to provide some, or all, of the benefits supplied by fruits and vegetables themselves.

The aim of the current study is to determine the effect supplementation with fruit and vegetable extracts has on various measures of immune function among smokers and non-smokers.
Subjects and Methods

Subjects

55 subjects over the age of sixty were recruited for the 3-month study. Subjects were recruited from a database of participants in previous studies. This database consists of approximately 200 healthy males and females over the age of 60 years. Additional subjects were recruited from an advertisement in a newspaper from a local retirement community. Subjects were screened for past medical history, alcohol consumption and smoking status. Subjects over the age of 60 years were eligible for participation. Subjects with known active cancers or uncontrolled diabetes were not eligible for participation. A total of 46 subjects completed the study. Nine individuals dropped out during the course of the study period; 4 subjects moved away from the area, 2 subjects developed a hive-like rash, and 3 subject decided not to continue. (Table 1). Data obtained from subjects who dropped out after day 40 of treatment are included in the results. Since only 2 individuals dropped out before day 40, data from a total of 53 subjects were available for use in the final analysis. Blood samples were taken two separate days over one week prior to supplementation (visits 1 and 2) and two samples were taken during the supplementation period (once after 40 days of supplementation and one after 80 days; visits 3 and 4). The study was approved by the Human Subjects Committee of the University of Arizona and all subjects provided written informed consent prior to their participation. During visits 2 and 3 subjects were given a supply of supplements to last until their next visit. Subjects were instructed to consume 2 fruit capsules in the morning with an 8 oz glass of water and
a meal and 2 vegetable capsules in the evening with an 8 oz glass of water and a meal. This information was emphasized verbally and in writing at visit 2 and 3. Written information was provided as a label on the supplements. Subjects were otherwise instructed to eat as they normally would. Subjects were also instructed to bring any remaining pills to the clinic at day 40 and day 80, at which time pill counts were conducted.

Supplements

The fruit and vegetable extracts were obtained from NSA International (Memphis, Tennessee) and consisted of dried fruit and vegetable powders prepared as previously described (22). Briefly, fruit juice supplements contained 850 mg fruit powder per capsule and contained extracts from apples, oranges, pineapples, papaya, cranberries and peaches. Vegetable supplements contained 750 mg vegetable powder per capsule and contained extracts from carrots, parsley, beets, broccoli, kale, cabbage, spinach and tomatoes.

Blood Handling

Blood samples were collected using sodium heparinized Vacutainer® tubes (Becton Dickinson, Franklin Lakes, New Jersey). Samples were centrifuged within 2 hours of collection for 10 minutes at 1200g. Immediately after centrifugation serum was collected and three 1.5 ml aliquots were taken and stored at -70°C.

ELISA for Cytokines

Peripheral blood mononuclear cells were isolated from whole blood using a density
gradient centrifugation on Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden) at 1900g for 20 minutes. Cells were then collected and washed twice using sterile phosphate buffered saline (PBS). Cell concentration was counted and adjusted 1x10^6. Mononuclear cells were cultured in triplicate on a 96-well flat-bottomed culture plate (Falcon 3072, Lincoln Park, New Jersey) in RPMI-1640 culture medium with added 10% fetal calf serum. Cells were then stimulated with LPS at 20ng/ml or 20μg/ml for 24 hours for induction of IL-6 or TNF-α, respectively, or with PHA at 10μg/ml for 24 hours for induction of IL-2 or 72 hours for induction of IFN-γ. Cells were incubated at 37°C in a 5% CO₂ incubator. After incubation, the plates were centrifuged for 10 minutes at 800g. Supernatant fluids were collected and stored at −70°C until analysis. The samples were then determined by sandwich ELISA (23) as we have described previously (24).

Mitogenesis
LPS-stimulated proliferation was determined by [³H]thymidine incorporation as described previously (25). Briefly, cells in 0.1ml of CM (1x10^6 cells/ml) were cultured in 96-well flat bottomed culture plates (Falcon) with LPS (20ng/ml). They were incubated at 37°C in a 5% CO₂ incubator for 44 hours for LPS and spontaneous proliferation, and then pulsed with [³H]thymidine (0.5 μCi/well, New England Nuclear, Boston, MA). After 6 hours cells were harvested by a cell sample harvester (Cambridge Technology, Cambridge, MA). Radioactivity was determined by a liquid scintillation counter (Tri-Carb, 2200 CA Packard, Laguna Hills, CA). Data were presented as counts per minute (cpm).
NK Cell Activity
NK cell activity was measured using a fluorescence release assay modified from the murine method of Poet et al. (26). In brief, K562 cells-target cells were labeled with a fluorescent dye and incubated at different effector (PBMCs):target cell ratios (100:1, 50:1, and 25:1) in a u-bottom tissue culture plates at 37°C in a humidified atmosphere of 5% CO₂ for 3 hours. Epifluorescence of each well was determined and specific percent toxicity was calculated from fluorescence contained in target cells.

Determination of Carotenoids and Vitamin E in Serum
250μl of ethanol (containing 0.1% BHT anti-oxidant) were added to a 250μl aliquot of serum in order to precipitate proteins. After vortexing, samples underwent 2 hexane extractions, evaporated under nitrogen, and then re-dissolved in mobile phase (solvent A). 50μl of extractant were injected directly into the high-performance liquid chromatography (HPLC) system. Carotenoids and α-tocopherol were separated by a 5μm ultrasphere ODS column (4.6x250mm; Beckman Instruments, San Ramon, CA) and detected at the wavelengths of 452 nm and 300 nm by use of the method of Xu et al. (27). The solvent system consisted of 95% acetonitrile (CAN)-tetrahydrofuran (THF) (85:15, v/v) with 250ppm butylated hydroxytoluene (BHT) and 0.05% triethylamine (TEA) and 5% 50mM ammonium acetate in methanol with 0.05% TEA and was delivered at a flow rate of 2.5 ml/minute. The retention times for lutein/zeaxanthin, β-cryptoxanthin, α-tocopherol, lycopene, α-carotene, and β-carotene were 1.94, 4.24, 4.83, 5.50, 9.45, and 10.13 minutes, respectively. The total run time for a single analysis of sample was 13
minutes. Analytic quantification was performed by the external standard method. Extinction coefficients were used to spectrophotometrically validate the final solution concentrations. Standard reference material 968b (fat-soluble vitamins and cholesterol in human serum) supplied by the National Institute of Standards and Technology (NIST, Gaithersberg, MD) was used for assigning values to in-house control materials.

**Lymphocyte Subpopulation Measurement**

Mononuclear cells were isolated as described above and adjusted to $2 \times 10^6$ cells/ml, 0.5 ml/tube for subsequent lymphocyte surface marker determination as previously described (28). Anti-human CD3 FITC/CD4 PE, CD3 FITCCD4 PE or CD3 FITC/CD16 PE + CD 56 PE were obtained from Becton Dickinson (San Jose, CA). Samples were analyzed using a FacStar flow cytometer (Becton Dickinson, San Jose, CA) with the consort 40 program.

**Statistics**

Statistical significance was determined by using paired t tests for the nutrient data (Microsoft Excel software, Copyright © Microsoft Corporation). Immune function data were analyzed separately for non-smokers and smokers using one way analysis of variance (ANOVA) (STATA). Bonferroni analysis was done to determine differences between means at baseline and day 40, baseline and day 80, and day 40 and day 80 (STATA). A p value of < 0.05 was considered statistically significant.
Results

Subjects and Compliance

Subject characteristics are reported in (Table 1). Subjects reported regular consumption of fruit and vegetable supplements. The supplements were well tolerated and improvements in bowel habits and overall feelings of well being were noted. Pill counts were conducted each month revealing that 99.99% of the fruit extract was consumed and a 99.96% of the vegetable extract was consumed. No differences in immune function or serum antioxidants were observed for males and females (data not shown).

Serum Antioxidants

The average serum carotenoid and tocopherol concentrations are presented in Table 2. Lutein/zeaxanthin (p<0.05), α-carotene (p<0.0001), β-carotene (p<0.0001) lycopene (p<0.05), and α-tocopherol (p<0.005) levels significantly increased after supplementation. No significant increase was seen for β-cryptoxanthin.

Mitogenesis & Lymphocyte Subpopulations

Proliferation of PBM cells in response to LPS (20 ng/ml) significantly (p<0.0001) increased after treatment in non-smokers (Figure 1). The increase in LPS-induced mitogenesis occurred between baseline and day 40 (p<0.0001) and between baseline and day 80 (p<0.0005). No significant increase was observed between days 40 and 80. Spontaneous proliferation of PBM cells significantly (p<0.0001) increased in non-smokers
The increase in spontaneous cell proliferation is seen when baseline is compared to day 40 (p<0.0001) and day 80 (p<0.05), and a slight decrease is seen when day 40 is compared to day 80 (p<0.01). No significant differences were observed for smokers (data not shown).

No significant changes were noted in the amount of T helper cells (CD3/CD4+), cytotoxic T cells (CD3/CD8+), or of NK cells (CD3/CD16/CD56+) (data not shown).

Natural Killer Cell Cytotoxicity

NK cell cytotoxicity at an effector:target cell ratio of 100:1 (p<0.0001), 50:1 (p<0.0001), and 25:1 (p<0.005) significantly increased in non-smokers (Figure 2). The increase in NK cell cytotoxicity is seen when baseline is compared to day 80 and when day 40 is compared to day 80 at effector:target cell ratios 100:1 and 50:1 (p<0.005). At the effector:target cell ratio of 25:1 a significant increase is only seen when day 40 is compared to day 80 (p<0.005). No significant differences were observed for smokers (data not shown).

Cytokines

IL-2 production in supernatant from PBM cells stimulated with PHA (10μg/ml) significantly increased in smokers (p<0.05) and non-smokers (p<0.0001) (Figure 3). In smokers, significant increases in IL-2 were observed between baseline and day 80 and day 40 and day 80 (p<0.001). In non-smokers, significant increases were observed between
baseline and day 40, baseline and day 80, and between day 40 and day 80 (p<0.0001). In non-smokers, IL-6 production in supernatant from PBM cells stimulated with LPS (20ng/ml), significantly decreased (p<0.005) between baseline and day 40 however, between day 40 and day 80 IL-6 level significantly increased (p<0.05). No statistically significant changes were observed when baseline values were compared to day 80 (data not shown). No significant changes were observed for either TNF-α or IFN-γ.
Discussion

The present study was the first to describe how fruit and vegetable extracts can be used to significantly improve immune function in the elderly. The supplements were processed by a unique method which extracts the constituents of fruits and vegetables so their bioavailability is enhanced.

Aging results in a dysregulation of immune function. There is a tendency for cell mediated immunity to become suppressed while humoral immunity is elevated. The decline in cell mediated immunity, IL-2, and T cell function is thought to be a result of thymus involution. B cells, on the other hand, are generated throughout life in humans (29). The decline in T cell function in combination with the continued production of B cells leads to a dysregulated immune response. Increased humoral immunity results in aberrant antibody production which can be quite devastating, as indicated by the prevalence of autoimmune diseases in the elderly. Impaired cell mediated immunity is associated with an inability to destroy viruses and cancer cells. Interestingly, as immune function declines the incidence of CVD and cancer rises. Finding ways to reduce risk for these diseases, as well as improve immune function would be extremely useful. Increasing consumption of fruits and vegetables may be one way to lower risk. Since few individuals consume the optimal amounts of fruits and vegetables the need for a dietary supplement becomes evident. At first, studies in animals (11-14) given supplemental antioxidants were encouraging, however the evidence in humans (15,16) is not as clear. T and B cell proliferation in old rat splenocytes, along with IL-2 levels have been shown to increase in
response to dietary vitamin E (11). In old mice fed high vitamin E diets, IL-2 was also shown to increase (12,13). However, in a study where elderly humans were supplemented with 100 mg of vitamin E, no changes in PBM cell proliferation was observed (15). Similarly, β-carotene increased the \textit{in vitro} stimulation of mouse splenocytes (14), but failed to increase PBM cell proliferation in humans (16). Additionally, IL-2 production was also unchanged in elderly subjects supplemented with β-carotene (16). Antioxidants like β-carotene and vitamin E may need to work in tandem with other nutritive and/or non-nutritive compounds to exert \textit{in vivo} effects as when a mixture of vitamins and trace elements were supplemented in a group of healthy elderly subjects it prevented further suppression of cell mediated immunity (30). This therefore suggests that nutrients are working together to exert their functions. Since not all of the beneficial agents present in fruits and vegetables have been identified, at best we can only get a prevention of further suppression when nutrients are given in combination (30). We propose that the improvements we observed in immune function are due to the action of nutritive and non-nutritive agents present in the extracts, as our findings show improvements in IL-2 levels, NK cytotoxicity, and LPS and spontaneous PBM cell proliferation.

Decreases in IL-2 production in the elderly are attributed to decreases in cell-mediated immunity. Our data demonstrate that fruit and vegetable extracts can dramatically improve IL-2 levels, in both smokers and non-smokers, as we observed 2-fold increases after only 40 days of supplement use. A benzene derivative found in cigarette tar, p-benzoquinone (p-BQ), and nicotine, were both found to inhibit IL-2
production in PBM cells and locally suppress lung cell-mediated immunity (31,32). The increase in IL-2 we observed may therefore have added benefits for elderly smokers. Since smokers are at such high risk for developing lung cancer, it is essential to find ways to reduce their risk. However, recent evidence (8,9) suggests that antioxidant supplementation increases the risk of developing lung cancer in smokers. The importance of other antioxidants or substances in fruits and vegetables, and their synergistic effects came to be appreciated. We are now able to demonstrate that fruit and vegetable extracts, containing both nutritive and non-nutritive compounds, can be of use to the smoking and nonsmoking elderly populations, as indicated by enhanced production of IL-2.

Another important aspect of our findings is that 2-fold increases in IL-2 and spontaneous cell proliferation occur after only 40 days of supplementation. The age-associated decline in spontaneous mitogenesis has been attributed to a decline in IL-2 (33). Additionally, IL-2 antagonists increase with age thereby decreasing IL-2's effectiveness (34). Since we too observed increases in LPS and spontaneous cell proliferation and IL-2 levels, perhaps some components in the fruit and vegetable extracts work to up-regulate IL-2 production. Increased IL-2 could then modulate other aspects of immune function and improve cell-mediated immunity.

IL-6, an inflammatory cytokine, also involved in activation, growth, and differentiation of T cells (35) is known to be higher in the elderly (36). Our data show significant decreases in IL-6 levels between baseline and day 40, however, these levels significantly increase from day 40 to day 80. Overall, when baseline values were
compared to day 80 no significant changes occurred (data not shown). It is therefore unclear whether fruit and vegetable extracts have effects on IL-6 levels.

Our finding of improved NK cell cytotoxicity, after 80 days of supplement use, at each of the effector:target cell ratio is also of extreme importance. Since the incidence of cancer increases with age, finding ways to reduce risk is an effective approach to reducing cancer mortality rates. NK cells are capable of destroying cancer cells and have been shown to have lower function in older mice (37), older humans (38) and patients with oral cavity cancer (39). We observed that the number of NK cells did not change, as indicated by our flow cytometry data (data not shown), but that the cells became more cytotoxic. This finding is in agreement with previous data we observed in mice supplemented with vitamin E (12). Others have observed increased NK activity in humans supplemented with β-carotene (40) which was not due to increases in the number of NK cells (16,41,42). The mechanism by which NK activity is increased may be mediated by IL-2 levels. IL-2 is reduced in the elderly, and also capable of enhancing NK cytotoxicity (43). Since we have also shown that IL-2 production by PBM cells increased after supplementation, the increase in NK cytotoxicity may be occurring secondary to the enhanced IL-2 production.

We observed a highly significant increase in serum carotenoids and α-tocopherol levels indicating that the supplements were well tolerated and compliance was high. The pre-treatment values for these nutrients are similar to those found by others (21,44,45) which illustrates that our sample was representative of most elderly populations. In other
subjects given diets high in fruits and vegetables, a similar increases in these nutrients after two 15-day periods on the diet were observed (45). This demonstrates that fruit and vegetable extracts contain many of the same components that fruits and vegetables themselves have, and that the antioxidants we measured are digested and absorbed in a similar fashion. This finding is extremely relevant in view of the fact that few individuals consume the recommended number of servings of fruits and vegetables (18), and that until now, an acceptable alternative has not been available.

In conclusion, fruit and vegetable extracts are a safe and well-tolerated supplement to dietary fruits and vegetables. They are also beneficial at improving immune function in the elderly who have increased risk for developing infections, cancer, and heart disease. The supplements were also useful in smokers, as indicated by the increase in IL-2. Ultimately, fruit and vegetable extracts could be a useful tool to improve immune function in elderly.
References


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Table 1: Subject’s Characteristics

<table>
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<tr>
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<th>Men (N=21)</th>
<th>Women (N=32)</th>
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<td></td>
</tr>
<tr>
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<td>Yes</td>
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<td>10</td>
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<tr>
<td><strong>Age Distribution</strong></td>
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<tr>
<td>76-80</td>
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<td>3</td>
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<tr>
<td>&gt;80</td>
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<td>2</td>
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<td>9</td>
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<tr>
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<td>6</td>
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<tr>
<td>Hypothyroid</td>
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<tr>
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<td>3</td>
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<tr>
<td>Arthritis</td>
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<td>3</td>
</tr>
<tr>
<td>Other (ulcer, pulmonary disease)</td>
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<td>2</td>
</tr>
<tr>
<td><strong>Hormone Replacement</strong></td>
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<td>13</td>
</tr>
<tr>
<td><strong>Vitamin/Mineral Supplementation</strong></td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td><strong>Average BMI</strong></td>
<td>23.97</td>
<td>23.89</td>
</tr>
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</table>

1 Available data from 53 subjects were used in the analysis. A total of 9 subjects dropped out of during the course of the study. 2 subjects dropped out before day 40 of treatment and this data was excluded. The seven remaining subjects dropped out after day 40 and if available, this data was used in the final analysis. Of the 7 subjects 2 were male non-smokers, 1 was a male smoker, 3 were female non-smokers, and 1 was a female smoker.
Table 2: Effect of Fruit and Vegetable Extracts on Serum Carotenoids and \(\alpha\)-Tocopherol Levels (N=46)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>80 days</th>
<th>% Change*</th>
<th>(p)†</th>
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<tr>
<td>Lutein/Zeaxanthin ((\mu)g/ml)</td>
<td>0.21 ± 0.084</td>
<td>0.28 ± 0.118</td>
<td>29.26</td>
<td>0.0024</td>
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<tr>
<td>(\beta)-Cryptoxanthin ((\mu)g/ml)</td>
<td>0.08 ± 0.048</td>
<td>0.09 ± 0.047</td>
<td>12.20</td>
<td>NS</td>
</tr>
<tr>
<td>Lycopene ((\mu)g/ml)</td>
<td>0.30 ± 0.141</td>
<td>0.35 ± 0.141</td>
<td>15.53</td>
<td>0.0424</td>
</tr>
<tr>
<td>(\alpha)-Carotene ((\mu)g/ml)</td>
<td>0.07 ± 0.040</td>
<td>0.28 ± 0.130</td>
<td>308.08</td>
<td>0.0001</td>
</tr>
<tr>
<td>(\beta)-Carotene ((\mu)g/ml)</td>
<td>0.33 ± 0.311</td>
<td>0.88 ± 0.554</td>
<td>165.24</td>
<td>0.0001</td>
</tr>
<tr>
<td>(\alpha)-Tocopherol ((\mu)g/ml)</td>
<td>22.56 ± 8.459</td>
<td>28.75 ± 9.815</td>
<td>24.43</td>
<td>0.003</td>
</tr>
</tbody>
</table>
Figure 1: Effect of fruit and vegetable extracts on LPS and spontaneous proliferation of PBM cells in elderly non-smoking subjects. Each sample was done in triplicate and averaged. N=41, 35, and 34 for baseline, day 40, and day 80, respectively. The values are mean ±SEM. *p<0.0001.

Figure 2: Effect of fruit and vegetable extracts on NK cell cytotoxicity of PBM cells in elderly non-smoking subjects. Each sample was done in triplicate and averaged. N=25, 31, and 35 for baseline, day 40, and day 80, respectively. The values are mean ±SEM. *p <0.0001, **p <0.005.

Figure 3: Effect of fruit and vegetable extracts on IL-2 production by PBM cell stimulated with PHA in non-smoking and smoking elderly subjects. Each sample was done in triplicate and averaged. For non-smokers N=39, 23, and 12 for baseline, day 40 and day 80, respectively. For smokers N=8, 6, and 4 for baseline, day 40 and day 80, respectively. The values are mean ±SEM. *p<0.0001, **p<0.05.
Figure 1

Days of Supplementation

cpm

LPS*  Spontaneous*

0 40 80
Figure 2

Days of Supplementation

% NK Cytotoxicity

- 100:1*
- 50:1*
- 25:1**
Figure 3

- Non-Smokers *
- Smokers **

Days of Supplementation

Days of Supplementation

pg/ml IL-2
MODULATION OF CYTOKINE PRODUCTION BY DEHYDROEPIANDOSTERONE (DHEA) PLUS MELATONIN (MLT) SUPPLEMENTATION OF OLD MICE

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Host parasite Interaction/Immunology/Microbiology/Virology

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Running Title: DHEA, Melatonin, and cytokines

Funding: Wallace Genetic Foundation, Inc., Phi Beta Psi Sorority and NIH L59794.
ABSTRACT

Tissue levels of the antioxidants melatonin (MLT) and dehydroepiandrosterone (DHEA) decline with age which 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.

Key words: Cytokines, Age, Dehydroepiandrosterone, Melatonin, Oxidation, and Immunomodulation.
INTRODUCTION

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. Age-related changes in hormone levels, therefore, 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, being lipid soluble, it can readily pass membranes without the aid of carrier proteins. These 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 NADPH₂(10). NADPH₂ is necessary for the generating of 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 serotonin, 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- 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 which 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 (3-4 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 h 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 (8 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 Fg/mouse/day) and then 0.06% DHEA diet for next 9 weeks (18.66 Fg/mouse/day) with 0.05% ethanol in the drinking water, (C) Unsupplemented diet with 10 Fg/ml melatonin (MLT) dissolved in 0.05% ethanol drinking water (49.8 Fg/mice/day) for 12 weeks and (D) 0.02% DHEA supplemented diet for the first 3 weeks and then 0.06% DHEA for the other next weeks with 10 Fg/ml MLT in 0.05% ethanol drinking water (for 12 weeks). Four groups of old mice (4 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, Pennsylvania) using the same AIN 93A diet, pelleted and color coded. MLT was purchased from Sigma and dissolved in 95% ethanol. It was then diluted in distilled water. The final concentration of MLT in the drinking tap water was 10 Fg/ml with 0.05% ethanol. The treatment period was 12 weeks for all groups.
Standard Cytokines and Their Antibodies

Rat anti-murine IFN-gamma, IL-2, IL-4, IL-6, IL-10 purified antibodies, rat anti-murine IFN-gamma, IL-2, IL-4, IL-6, IL-10 biotinylated antibodies, and recombinant murine IFN-gamma, IL-2, IL-4, IL-6, IL-10 were obtained from Farmington (San Diego, CA).

ELISA for Cytokines

IFN-gamma, 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 mM 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 1x10^7 cells/ml. Splenocyte viability was more than 95% as determined by trypan blue exclusion. 0.1 ml/well of splenocytes (1x10^7 cell/ml) from 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 Fg/ml, 0.1 ml/well, Sigma) for induction of IL-2, IL-4 and IL-10 with 24 h
incubation, IFN-gamma with 72 hours incubation at 37°C, 5% CO₂ incubator.

Splenocytes were also stimulated by lipopolysaccharide (LPS, 10 Fg/ml, Gebco, Grand Island, NY) for 24 h induction for IL-6 and TNF-α production. After incubation, the plates were centrifuged for 10 min at 800 x g. Supernatant fluids were collected and stored at -70°C until analysis. They were determined by sandwich ELISA (17), and as we have described previously (16).

**Mitogenesis of Splenocytes**

Splenic T and B cell proliferation was determined by ³H-thymidine incorporation as described previously (18). Briefly, splenocytes in 0.1 ml of CM (1x10⁷ cell/ml) were cultured in 96-well flat-bottom cultured plates (Falcon) with Con A and LPS (10 Fg/ml). They were incubated at 37°C, 5% CO₂ incubator for 44 h for Con A and LPS-induced T and B cell proliferation respectively, and then pulsed with ³H-thymidine (0.5 FCi/well, New England Nuclear, Boston, MA). After 6 h, 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 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, Maine). 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 $4 \times 10^4$ target cells/100 FI. The plate was centrifuged (90 x g) for 3 min to facilitate cell to cell interaction. The cells were then incubated at $37^\circ C$ in a humidified atmosphere of 5% $CO_2$ for 3 h. After incubation, 20 FI of 1% inert fluoricon polystyrene assay particles was added to each well of plate (Pandex Harvesting Plate, IDEX, Portland, Maine), and 70 FI aliquot from each well of irradiation plate was 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:

$$\frac{\text{Spontaneous Release} - \text{Experimental Fluorescence}}{\text{Spontaneous Release} - \text{Maximum Release}} \times 100$$

**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 $1-2 \times 10^5 / 0.1 \text{ ml/tube}$ 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 California). Tissues from each mouse were counted and assessed separately, with 4 mice/group. Samples were analyzed using a FacStar flow cytometer (Becton Dickinson) with the consort 40 program.

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

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 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 CD₃⁺/CD₈⁺ cells from thymic glands was not found to be significantly different and was not affected by treatments (data not shown). The percentage of CD₃⁺ 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. 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). MLT supplementation, however, significantly (p<0.05)
increased T cell proliferation in young mice as compared to MLT treated old mice (Fig. 2B). Natural Killer (NK) 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 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 who 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-. 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- 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-) 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. A lack of cellular immunity, additionally, 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, suppressing
immune function. DHEA, MLT, and DHEA + MLT significantly decreased IL-6
production in young and old mice. These results demonstrate that
supplementation 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:cortisol 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, demonstrates 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.
Fig 1. Effect of DHEA, MLT and DHEA + MLT on splenocyte numbers in old and young mice. The values are mean ± SE. The data represent 8 young mice per group and 4 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.

Fig 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 8 young mice per group and 4 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.

Fig 3. Effect of DHEA, MLT and DHEA + MLT on (A) IL-10 and (B) IL-4 production by Con A stimulated splenocytes from old and young mice. Each sample was done in triplicate and averaged. The values are mean ± SE. The data represent 8 young mice per group and 4 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.

Fig 4. Effect of DHEA, MLT and DHEA + MLT on IL-6 production by Con A stimulated splenocytes from old and young mice. Each sample was done in triplicate and averaged. The values are mean ± SE. The data represent 8 young mice per group and 4 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.

Fig 5. Effect of DHEA, MLT and DHEA + MLT (A) IFN-γ and (B) IL-2 production by Con A stimulated splenocytes from old and young mice. Each sample was done in triplicate and averaged. The values are mean ± SE. The data represent 8 young mice per group and 4 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.
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Figure 1

Splenocyte $\times 10^7$/spleen

- Control
- DHEA
- MLT
- DHEA+MLT

- young
- old

Legend:
- a*
- a**
- b*
- c*
Figure 2B

T-cell proliferation (cpm)

- young
- old

Control  DHEA  MLT  DHEA+MLT
Figure 3A

IL-10 production (pg/ml)

Figure 3B

IL-4 production (pg/ml)
Figure 5A

- young
- old

IFN production (pg/ml)

- Control
- DHEA
- MLT
- DHEA+MLT

Significance levels:
- b***
- b**
- b*
- a**
- a***
Figure 5B

IL-2 production (pg/ml)

- young
- old

Control | DHEA | MLT | DHEA+MLT

a** | b*** | b* | b**

Significance levels: a, b, ** indicate statistical significance.