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EFFECT OF ANTIOXIDANTS IN THE ELDERLY

by

Sussan Kaboudanian Ardestani

A Dissertation Submitted to the Faculty of the
COMMITTEE ON NUTRITIONAL SCIENCES
In Partial Fulfillment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College
THE UNIVERSITY OF ARIZONA
1996
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Sussan Kaboudanian Ardestani entitled "Effect of antioxidants in the elderly" and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of ______.

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

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SIGNED: Sussan K. Ardestani
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The achievement of earning a Ph.D has been accomplished with the help and support of many people. I owe a debt of thanks to Dr. Ronald R. Watson for his assistance during my Ph.D program both as an academic advisor and as dissertation director. I also am very appreciative of his financial support during my studies at the University of Arizona. I express my appreciation to Dr. Cleamon Eskelson and Dr. Harris Bernstein for their valuable insight and teaching. I also want to express my appreciation to Dr. Marilyn Halonen and Dr. Ralph L. Price for serving on my committee and for all their assistance. My sincere thanks to Dr. Steven M. Wood, Mr. Bailin Liang, Ms. Paula Inserra, Ms. Carla Beckham and Ms. Anabell Castro for their informative discussions and helpful encouragements.
DEDICATION

I would like to dedicate this work to all the teachers of the world, and especially to the greatest teacher of all times our prophets: Adam, Ibrahim, Moses, Essa and Mohamad.

I would also like to dedicate this work to the most patient teacher, to my husband.

SIGNED

Sussan K. Ardeshir
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURE</td>
<td>11</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>14</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>15</td>
</tr>
<tr>
<td>CHAPTER I: BENEFICIAL IMMUNOLOGICAL EFFECT OF ANTIOXIDANT NUTRIENTS IN PREVENTION OF FREE RADICAL DAMAGE</td>
<td>17</td>
</tr>
<tr>
<td>I. Free radicals, Antioxidants and Immune function</td>
<td>19</td>
</tr>
<tr>
<td>A. INTRODUCTION</td>
<td>19</td>
</tr>
<tr>
<td>1. Free radicals. definition</td>
<td>19</td>
</tr>
<tr>
<td>2. Antioxidant’s mechanism of action</td>
<td>22</td>
</tr>
<tr>
<td>Enzymes</td>
<td>22</td>
</tr>
<tr>
<td>Compounds</td>
<td>25</td>
</tr>
<tr>
<td>B. IMMUNE FUNCTION AND FREE RADICAL REACTIONS</td>
<td>25</td>
</tr>
<tr>
<td>1. Effect of free radicals on immune cells</td>
<td>25</td>
</tr>
<tr>
<td>2. Free radicals and T cell activation</td>
<td>26</td>
</tr>
<tr>
<td>3. Free radicals and cytokine production</td>
<td>28</td>
</tr>
<tr>
<td>4. Summary</td>
<td>32</td>
</tr>
<tr>
<td>C. ANTIOXIDANTS AND IMMUNE FUNCTION</td>
<td>33</td>
</tr>
<tr>
<td>1. Effect of antioxidants on nonspecific immune response</td>
<td>33</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS—continued

2. Effect of antioxidants on specific immune response .......... 34

D. SUMMARY .................................................................. 35

II. ANTIOXIDANTS AND CANCER .................................... 35

A. INTRODUCTION .................................................. 35

B. β-CAROTENE .......................................................... 38

C. VITAMIN C ............................................................... 43

D. VITAMIN E ............................................................... 48

E. SELENIUM ................................................................. 57

F. GLUTATHIONE ........................................................... 62

G. SUMMARY ................................................................. 65

CHAPTER II. AGING AND IMMUNE DYSFUNCTION .......... 66

A. INTRODUCTION .................................................. 68

1. Theories of aging ..................................................... 68

2. Changes in antioxidant defence mechanism with aging ........ 69

B. AGING AND IMMUNE SYSTEM .................................. 71

1. Immune cell and organs ............................................ 71

   Lymphoid organs .................................................. 72

   Cell count and cellular marker ................................ 74

2. Immune function ..................................................... 76

   a. Cell mediated immunity ..................................... 76
TABLE OF CONTENTS—continued

Mitogenesis.................................76
Cytotoxicity.................................77
Cytokine production.........................78
b. Humoral immunity..........................88
c. Gut mucosal immunity......................91
d. Cancer........................................92

CHAPTER III. EFFECT OF LONG TERM ANTIOXIDANT NUTRIENT SUPPLEMENTATION IN THE ELDERLY..............................94

A. ABSTRACT..................................95

B. INTRODUCTION............................96

C. MATERIAL AND METHODS..................99

2. nutrient solution for in vitro application.....................99
3. Cell culture for antioxidant application for in vitro.........100
4. Natural killer cell cytotoxicity................................101
5. Cytokine stimulation for in vitro supplementation studies....102
6. ELISA assay for cytokines..................................102
7. In vivo study population: Blood collection and lymphocyte preparation.........................................................103
8. Dietary and nutrient analysis....................................104
TABLE OF CONTENTS—continued

9. Mitogenesis of lymphocytes ................................................. 104
10. Cell preparation for flow cytometry analysis .......................... 105
11. Phospholipid and lipid analysis ........................................... 106
12. Cholesterol analysis .......................................................... 106
13. Lipid peroxidation analysis .................................................. 107
   Fluorescence and conjugated diene analysis .............................. 107
   Malondialdehyde determination ............................................ 107
14. Statistical analysis .......................................................... 108

D. RESULTS ............................................................................. 110

Effect of antioxidant nutrients on lipid profile and lipid peroxidation 111
Modification of lymphocyte function in vivo by application of antioxidants
   Effect of antioxidant nutrients on T-cell population .................. 111
   Effect of antioxidant nutrient on T-cell function ...................... 112

E. DISCUSSION ........................................................................ 112

CHAPTER VI. MODULATION OF IMMUNE FUNCTION AND CYTOKINE
PRODUCTION BY DHEA AND MELATONIN IN OLD MICE .......... 133

A. ABSTRACT ........................................................................ 134
B. INTRODUCTION .................................................................. 135
C. MATERIAL AND METHODS .................................................. 137
**TABLE OF CONTENTS—continued**

1. Animal and diets.................................................................137
2. Standard cytokines and their antibodies.................................138
3. ELISA for cytokines..............................................................139
4. Mitogenesis of splenocytes....................................................140
5. Natural killer cell cytotoxicity..............................................140
6. Lymphocyte subpopulation measurement ..................................141
7. Statistical analysis ..................................................................142

D. RESULTS.................................................................................142
1. Spleen cell number.................................................................142
2. Spleen cell function...............................................................143

E. DISCUSSION...........................................................................144

G. REFERENCES........................................................................158
LIST OF FIGURES

Figure-1. Model for age associated shifts in T cell subset frequencies that lead to altered T cell lymphokine repertoires.................................................................87

Fig.3-1: The number of individuals in the placebo and supplemented group in each months. .................................................................118

Fig.3-2: Effect of antioxidant nutrient supplementation on plasma concentration of lipid before during and 2 month after supplementation. .........................119

3-3: Effect of antioxidant nutrient supplementation on plasma concentration of TBRs before during and 2 month after supplementation..........................120

Fig.3-4: Effect of antioxidant nutrient supplementation on plasma concentration of Conjugated diene and fluorescence before during and 2 month after supplementation.................................................................121

Fig.3-5: Effect of antioxidant supplementation on percentage of CD3^+ cell before and after 2 and 6 months of supplementation.........................................................122

Fig.3-6: Effect of antioxidant supplementation on percentage of CD4^+ cell before and after 2 and 6 months of supplementation.........................................................123

Fig.3-7: Effect of antioxidant supplementation on percentage of CD8^+ cell before and after 2 and 6 months of supplementation.........................................................124

Fig.3-8: Effect of antioxidant supplementation on percentage of CD3/CD56CD16^+ cell before and after 2 and 6 months of supplementation. .........................125
LIST OF FIGURES—continued

Fig. 3-9: Effect of antioxidant supplementation on PHA-induced T cell proliferation before, during and 2 month after supplementation. .......................... 126

Fig. 3-10: Effect of antioxidant supplementation on LPS-induced B cell proliferation before during and 2 month after supplementation. .......................... 127

Fig. 3-11: Effect of antioxidant supplementation on IL-2 production by PHA-activated T cell before during and 2 month after supplementation. ......................... 128

Fig. 3-12: Effect of antioxidant supplementation on IFN-γ production by PHA-activated T cell before during supplementation............................................. 129

Fig. 3-13: Effect of antioxidant supplementation on LPS-induced PMNC IL-6 production before during and 2 month after supplementation. ......................... 130

Fig. 3-14: Effect of antioxidant supplementation on Plasma concentration of IL-6 before during and 2 month after supplementation. .............................. 131

Fig. 3-15: Effect of antioxidant supplementation on NK cell cytotoxicity before during and 2 month after supplementation.............................................. 132

Fig 4-1. Effect of DHEA, MLT and DHEA plus MLT on splenocyte count in old and young mice................................................................. 150

Fig 4-2. Effect of DHEA, MLT and DHEA plus MLT on B cell proliferation in old and young mice. ................................................................. 151

Fig 4-3. Effect of DHEA, MLT and DHEA plus MLT on T cell proliferation in old and young mice................................................................. 152
LIST OF FIGURES--continued

Fig 4-4. Effect of DHEA, MLT and DHEA plus MLT on IL-10 production in old and young mice. ................................................................. 153

Fig 4-5. Effect of DHEA, MLT and DHEA plus MLT on IL-4 production in old and young mice. ................................................................. 154

Fig 4-6. Effect of DHEA, MLT and DHEA plus MLT on IFN-\( \tau \) production in old and young mice. ................................................................. 155

Fig 4-7. Effect of DHEA, MLT and DHEA plus MLT on IL-2 production in old and young mice. ................................................................. 156
LIST OF TABLES

Table 1-1. Free radical production by the reduction of dioxygen....................20
Table 1-2. Antioxidant enzymes........................................................................24
Table 1-3. Major sources of carotenes, vitamin C, and vitamin E in the american diet..........................................................36
Table 1-4. Selected case-control and cohort studies of the relationship between dietary carotenes, vitamin E, and breast cancer risk.................................37
Table 1-5. Vitamin C and nonspecific immunity..................................................44
Table 1-6. Vitamin C and specific immunity.........................................................45
Table 1-7. Effect of vitamin E supplementation on immune function..............56
Table 2-1. Summary of markers whose expression differs between naive and memory T cell..................................................................................75
Table 2-2. Functional differences between naive and memory T cells ..............76
Table 2-3. Presentation of T cell subsets during aging......................................80
Table 2-4. Cytokine production in naive and memory T cells............................84
Table 3-1. Characteristic of participants by group.............................................110
ABSTRACT

An increasing proportion of the population will be sixty-five years of age or more in the first decade of twenty-first century. Predictably, the spectrum of health and disease problems will also rise.

Aging is a progressive deterioration of biological functions causing increased susceptibility to disease. This increased risk of disease is associated with a concomitant decrease in immune "responsiveness". Antioxidants are a group of compounds that prevent oxidative damage and disease. Researchers have focused on anti-oxidizing factors in disease prevention, however, nutrients and hormones which prevent oxidative damage may also have immunomodulating properties. Aging is associated with a decrease in antioxidant defence mechanisms and a decrease in dehydroepiandrosterone (DHEA) and melatonin (MLT). Vitamin E, Vitamin C, β-carotene, glutathione, DHEA, and MLT have all been shown to decrease the incidence of cancer in the elderly. We measured lipid profile, lipid peroxidation, and the functional responsiveness of immune cells, in supplemented healthy elderly and aged mice.

Human subjects were randomly assigned to receive a daily placebo, 1.8 mg β-carotene or antioxidant nutrients, 800 IU vitamin E plus 500 mg vitamin C plus 30 mg β-carotene plus 70 mg glutathione for 6 months. Antioxidant nutrients decreased the amount of melandialdehyde in plasma of the supplemented group and also increased IL-2 production by activated T cells after 4 months. But we did not find a significant change in cell populations and other cytokine production in supplemented group compared with
placebo.

Also we investigated the effect of DHEA (19 μg/day), MLT (50 μg/day), or DHEA plus MLT in aged (16 months) C57BL/6 female mice. DHEA, MLT, or DHEA plus MLT increased IL-2, IFN production and decreased IL-6 and IL-10 production in aged mice.

In conclusion, antioxidant nutrients can decrease one parameter of lipid peroxidation and improve IL-2 production in healthy aged participants. MLT and DHEA can enhance immune function in aged mice and which explain their disease preventive properties.
CHAPTER I

Beneficial Immunological Effects of Antioxidant Nutrients in Prevention of Free Radical Damage
TABLE OF CONTENTS

I. Free radicals. Antioxidants and Immune function

A. Introduction

1. Free radicals, definition

2. Antioxidant’s mechanism of action
   Enzymes
   Compounds

B. Immune function and free radical reactions

1. Effect of free radicals on immune cells

2. Free radicals and T cell activation

3. Free radicals and cytokine production

4. Summary

C. Antioxidants and immune function

1. Effect of antioxidants on nonspecific immune response

2. Effect of antioxidants on specific immune response

D. Summary

II. Antioxidants and Cancer

A. Introduction

B. β-carotene
I. Free radicals, Antioxidants and Immune function

A. Introduction

What is a free radical? Where do free radicals come from? Free radical production begins with oxidation. Oxidation is the primary means by which humans and other animals derive energy. Oxidation requires stable electrons that are readily provided by oxidant catalysts from neighboring atoms or compounds. The most common 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. (See table 1-1)
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>( \cdot 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>( \cdot 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>
</tr>
</tbody>
</table>

In addition to the continuous production of reactive oxygen intermediates during the normal cellular metabolism, the most common sources of free radicals are inflammation, strenuous physical exercise, natural detoxification, and acute or chronic exposure to oxidant chemicals. Radiation, ultraviolet light, alcohol, cigarette smoke and marijuana, air pollutants, excess free calcium, excess iron storage, excess unbound iron, and high fat diets all can increase the amount of reactive oxygen.

Reactive oxygen can damage the body by denaturing proteins, damaging nucleic acids and unsaturated bonds of fatty acids in lipid membranes which thereby alter membrane structure and function (1). Since oxidative damage to nucleic acids leads to genetic damage, and the initiation of cancer, it is reasonable to hypothesize that antioxidants could protect a cell at the initiation stage. Because the presence of oxidative
compounds can be life threatening, the body has many overlapping defense mechanisms
to protect against oxidation such as small molecules that act as scavengers of reactive
oxygen species.

If not checked by free radical scavengers i.e. antioxidants, each free radical will
begin a destructive process. The free radical takes an electron from a stable compound,
which in turn, is transformed into a new free radical. These chain reactions can be
stopped by enzymes, antioxidants, or by combination of two free radicals.

Unfortunately nature's protective mechanisms sometimes fail. Excess free
radicals contribute to degenerative diseases and aging. They do this by reacting with and
sometimes destroying critical cellular components including the polyunsaturated fatty
acids (PUFA) that comprise lipoprotein particles and plasma membranes. Since the
products of lipid peroxidation are diffusible, and since lipoprotein 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 is inadequate free radical
scavengers. Once these chain reactions are set into motion, they can lead to a variety
of damaging effects.

Free radicals that react with polyunsaturated long carbon chain lipids lead to the
formation of chemotactic products, aldehydric nonanoic acids, and various other
aldehydes (2). The resulting aldehydes can bind with biological amines such as proteins,
nucleic acids, and amino lipids and alter their structure and functions (2).
Free radicals can also induce atherosclerosis (5), colon cancer (5), and nerve and brain disorders (4). 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 (6). Free radicals also play a pivotal role in the activation of natural detoxification systems such as cytochrome P450 (7). Free radicals are also produced by neutrophils and macrophages in an effort to kill invading microorganisms (7).

The primary target for free radical reactions are unsaturated bonds in lipids. Free radical damage results in a loss of membrane fluidity, receptor alignment, and potential cellular lysis. Free radical damage to sulfur containing enzymes and other proteins results in inactivation, cross-linking and denaturation. Additionally nucleic acids can be attacked subsequently causing damage to DNA which leads to mutations and possibly cancer. However oxidative damage is not limited to lipids. Damage to carbohydrates can alter cellular receptor functions including those associated with cytokine activities and prostaglandin formation.

2. Antioxidant 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 which can affect the generation of free radicals during any of these stages. There are two metalloenzymes which can interfere with the production of free radicals during the initiation phase by inactivating precursor molecules. Superoxide dismutase is one metalloenzyme. It is a Mn-containing enzyme in mitochondria and a Cu/Zn-containing enzyme in the cytoplasm. Both enzymes can catalyze the reaction seen in table 1-2. Catalase is another metalloenzyme. It is an Fe-containing enzyme which is found in peroxisomes and catalyzes the decomposition of the hydrogen peroxide produced as a result of superoxide dismutation (or by other reactions). In addition to metalloenzymes glutathione peroxidase which contains selenium, also works as an antioxidant. Glutathione peroxidase is important for the decomposition of hydrogen peroxides and lipid peroxides. Although Mn, Cu, Zn, and Se are necessary components they are only considered antioxidant when incorporated into their respective enzyme (3).
<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></td>
<td>Mn</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fe(bacterial)</td>
<td></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></td>
<td></td>
<td>$ROOH + 2GSH \rightarrow GSSG + ROH + H_2O$</td>
</tr>
<tr>
<td>Phospholipid hydroperoxide</td>
<td>Se(1)</td>
<td>$H_2O_2 + 2GSH \rightarrow GSSG + H_2O$</td>
</tr>
<tr>
<td>glutathione peroxidase (PLGSHPx)</td>
<td></td>
<td>$ROOH + 2GSH \rightarrow GSSG + ROH + H_2O$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$PLOOH + 2GSH \rightarrow GSSG + PLOH + H_2O$</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>

Table 1-2. Antioxidant Enzymes
Antioxidant Compounds

Three essential nutrients act only by directly interfering with propagation of free radical generation, there are vitamin E, vitamin C, and β-Carotene. Even though glutathione is not an essential nutrient it can also interfere with the propagation stage. In addition to the direct action of these nutrients, riboflavin, a B vitamin, is a constituent of the enzyme glutathione reductase which is important in the re-generation of antioxidant defenses (8).

B. Immune Functions and Free radical reactions

1. Effect of free radicals on immune cells

High levels of dietary poly unsaturated fatty acid (PUFA) has been shown to be an immunodepressant (9). The unsaturated double bonds found in PUFA are prime targets for free radical damage and initiation of chain reactions resulting in the formation of lipid peroxides. Lipid peroxides and aldehydes can alter cellular functions and even result in lysis of oxidized cell membranes. Lipoprotein in the plasma can also be oxidized and become lymphotoxic (10). Lipid peroxidation also causes a decrease in membrane fluidity. Loss of membrane fluidity has been directly related to a decreased ability of lymphocytes to respond to immunological challenges (11-13).

Antigen presenting cells (APCs) exhibit an oxidative burst in response to many stimuli and express a proteolytic complex (proteasome) that selectively recognizes and degrades
oxidatively modified proteins. Oxidative modification from intracellular protein oxidation, or from an enzymatic oxidative burst exposes hydrophobic core residues for 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. Nonnumeric peptides seem to travel to the endoplasmic reticulum where they associate with MHC class I molecules and B2-macroglobulin. Such complexes traverse the golgi apparatus and get inserted into the cell membrane. Properly presented class I self proteins are recognized by CD8+ T cells as self antigens (16).

2. Free Radicals and T cell activation

The proliferation of T lymphocytes is a pivotal event in the cell-mediated responses. Foreign antigens are partially degraded by antigen-presenting cells and presented on their surface in association with major histocompatibility complex (MHC II) to T-cells bearing the cell surface marker CD4. This initiates a complex series of events. Key events in this process are the production of interleukin-1 (IL-1) by accessory cells and IL-2 by CD4+ T cells. The 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 human peripheral blood lymphocytes mitogenic response to phorbol myristate acetate (PMA), ConA and phytohaemagglutinin (PHA) (17). These findings suggest that hydroxyl radicals might be involved in mediating the signal(s) that trigger
T cell activation.

Antioxidants such as butylated hydroxy anisole (BHA), desferrioxamine (DES) and desferrithiocin (DFT) inhibit the antigen-driven proliferation in a dose-dependent manner. These compounds do not inhibit the production of IL-1 by accessory cells or that of IL-2 by the T cells, but they inhibit the cell surface expression of IL-2 receptor. However, 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 (18). Therefore, Chaudhri et al. proposed that free radicals are involved in the activation of T lymphocytes (19).

Iron chelators exert their anti-proliferative effects through interaction with intracellular iron pools. These chelators might influence cellular activities by preventing the involvement of iron in the catalysis of hydroperoxides or inhibit ribonucleotide synthesis. Terada showed that small traces of iron are necessary for the production of the cell-cycle regulatory protein kinase, P34\textsuperscript{cdc2} (20). B lymphocytes possess a functional NADPH oxidase, the proliferation of which is also inhibited by antioxidants (18). T lymphocytes do not have NADPH oxidase (21). Other leukocytes provide the "help" necessary for T lymphocytes to produce reactive oxygen species (ROS) (22). Cystamine amino thiol compounds block the binding of AP-1 and NF-Kappa B to DNA. These two prooncogenes 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-Kappa B. Ap-1 and/or NF-Kappa B 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 increase of intracellular Ca^{2+} in response to TCR triggering, and due to this inhibit inositol-triphosphate synthesis and cell activation. However, LO inhibitor does not effect the activation of Na^+, H^+ antiport by PMA, that can increase PH in stimulated cell and activate PKC (23). As explained by this mechanism, LO inhibitor decreases IL-2 production in Jurkat cells. LO inhibitor does not inhibit IL-2 production in PMA-treated Jurkat cells cultured with vanadate (23).

3. Free radicals and cytokine production

Free radical is extremely reactive and can modify various biochemical substances. At low concentrations, active oxygen acts as a mediator of cellular response and growth. Hydrogen peroxide acts like insulin. It elevates cytoplasmic free Ca^{2+} levels and activates protein kinase C (24). Exogenous addition of active oxygen to resting cells stimulates the induction of the proto-oncogenes; c-fos and c-myc as well as DNA synthesis (25). On the other hand, like TGFβ1, hydrogen peroxide can cause reversible
inhibition of DNA synthesis in mouse osteoblastic cells when added during the late G1 phase. TGFβ1 and hydrogen peroxide, both increase expression of the HIC-5 gene which encodes a novel Zn-finger protein (molecular weight 55 KDa). It also increases phosphorylation of 30 KDa protein. 30 KDa protein is a heat shock protein. Thus, hydrogen peroxide can be a second messenger of TGFβ1 (26).

Diamide and ascorbic acid have inhibitory effects on protein tyrosine phosphatase in intact murine fibroblasts transfected with human EGF-receptor. In view of its effects on cellular growth oxidative stress plays a role in growth factor-mediated signal transduction (27).

Low concentrations (10nm-1mM) of superoxide anions and hydrogen peroxide can stimulate growth or growth responses. Intracellular pH increases within 10 seconds activating protein kinase C (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 (28). TNF-α specifically induces extensive mitochondrial superoxide generation (29). In contrast of TNF-α, a variety of human tumor cells such as those that cause neuroblastoma, melanoma, and colon, pancreatic, ovarian and breast carcinoma are all reported to release large amounts of hydrogen peroxide without any specific growth stimulus (30). The growth responses that involve 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 and/or modulating the action
of normal growth factors (31). Oncogeny transformed cells 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 either as a part of a constitutively active autocrine system or from adjacent inflammatory cells (31).

The effect of oxygen radicals on the production and reception of IL-1 are elevated. Oxygen radicals can increase IL-1 production by monocytes and the proliferation of lymphocytes in the PHA-induced blast transformation reaction stimulated by recombinant IL-2 (32). There are two major mechanisms of lymphocyte stimulation by oxygen radicals. The first one is the activation of protein kinase C. The second is the activation of lipoxygenase. The effect of lipoxygenase activation is supported by the strong inhibitory effect of NDGA (a lipoxygenase inhibitor, and antioxidant on lymphocyte proliferation) (33).

IL-1B stimulates the IL-6 secretion in a dose and time-dependant manner. Antioxidants like pyrrolidine dithiocarbamate, N-acetyl-cysteine, two thiol-reacting molecules, trolox, and hydrosoluble analogue of vitamin E are also able to completely inhibit IL-6 secretion in a dose-dependant manner. However, a mixture of verapamil; a calcium channel blocker: neomycin sulfate, a phospholipase C cascade inhibitor, and 2'5'-dideoxyadenosine; a adenylate cyclase inhibitor does not affect IL-6 induction by IL-1 (34).
The transcription factor NF-Kappa B 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-Kappa B can activate genes involved in immune inflammatory or acute phase response. IL-1, TNF, PMA and other activating factors can activate NF-Kappa B. The activated NF-Kappa B translocates to the nucleus where it recognizes a specific DNA sequence (35). For instance, NF-Kappa B can regulate the gene coding for IL-6 (36). N-acetylcysteine (NAC) inhibits, and diamid stimulates, NF-Kappa B activation (37). NAC is known to increase intracellular glutathione and decrease reduced glutathione. NAC also blocks TNF-α induced NF-KB activation (38). Cell exposure to H₂O₂ also leads to NF-KB activation (39).

The role of cytokines in ROS production is still unclear. Rabinovitch and associates (40) showed that isolated rat islet cells exposed to a combination of cytokines exhibited 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 is not well documented. In rodents, macrophages (peritoneal or alveolar) produce ROS following stimulation with other compounds. This process does not occur spontaneously. Wolf and Mossof showed that in vivo production of cytokines significantly prime macrophages to an enhanced response to infectious agents or particles (41).

The in vivo effect of rHG-CSF has been investigated in human. Seven adults
with lymphoma were given G-CSF. Neutrophils were harvested before, during, and after treatment. Super oxide anion release was enhanced in all patients following treatment as well as in vitro stimulation with PMPL. Additionally, in vitro incubation with G-CSF does not increase oxidative response in most patients (42). This suggests that there is an "unpriming" or tolerance induction with excessive or repetitive exposure to the priming 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 physiological doses. Blockade of IL-1 by the use of its specific receptor antagonists and blockade of TNF by the use of TNF antibodies, reserved the effects of endotoxin. This implies that each cytokine is partially responsible for the lethal endotoxin effects (i.e. LPS). Thus, both administration of cytokines and strategies to block their effects can be beneficial to the host because cytokines are ubiquitous, pluripotent and potentially toxic. 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 (43).

4. Summary

Free radicals can decrease immune cell function, but in physiologic concentrations, they are necessary for antigen presenting and cell proliferation. Cytokines like TNF-α are necessary for the generation of free radicals and at low concentration, free radicals act as second messengers for cytokines and mediate cellular response and growth. Free
radicals can also increase TNF-α and IL-1 production. Also free radicals can increase IL-6 production by two mechanisms. They can act either by increasing IL-1 or by activating NF-KB. However IL-1 and TNF-α can be extremely toxic and even induce death at certain physiological doses. For these reasons the body has defence mechanisms to detoxify free radicals.

C. Antioxidant Nutrients and Immune Function

1. Effect of antioxidants on non specific immune responses

Antioxidants can increase immune responses by controlling the amount of free radical. During the oxidative bursts, neutrophils take up vitamin E (44) and following activation, the vitamin C concentration is reduced (45,46). Vitamin C can decrease the damaging products of the oxidative burst without decreasing the intracellular concentration of reactive bactericidal molecules (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 (48) as well as in newborns (49).

Vitamin E deficient rats have impaired neutrophil and macrophage chemotaxis, reduced ingestion of complement coated beads, and decreased protection from auto-oxidative damage (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 (51). The synovial fluid in the joints of rheumatoid
arthritis patients contains high levels of reactive oxygen species. Infiltrated neutrophils and T-lymphocytes are found 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 (52). Ascorbic acid levels are low in patients with rheumatoid arthritis, despite normal ascorbic acid absorption (53).

2. Effect of antioxidants on specific immune responses

In laboratory animals, T and B-cell proliferation is correlated with dietary and serum vitamin E levels (54). Vitamin E deficiency affects T-lymphocytes to a greater degree than B-lymphocytes. As laboratory animals age, T-lymphocyte functions are reduced to a greater degree than that of B-lymphocytes, macrophages, and stem cell activities (55,56). T-lymphocyte membranes in young mice are more fluid than B-cell membranes. However, as mice age, T-cells lose their fluidity whereas B-cells retain the same level. T-cell lipids are more susceptible to peroxidation than are B-cell lipids (57). The ability of T-lymphocytes to form rosettes is significantly inhibited following exposure to oxygen radicals whereas B-lymphocyte rosette formation is not significantly affected (58). As aging progresses, the level and activity of antioxidant enzymes decrease.

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 (55).
3. Summary

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

II. Antioxidant and Cancer

A. Introduction

Case-control and cohort studies show an increase risk of cancer with concentration of antioxidants nutrient in the body. Vitamin E, vitamin C, and β-carotene are essential nutrients which cannot be synthesized by humans. The major source of carotenes, vitamin C and vitamin E in the American diet are listed in table 1-3.

Although epidemiologic studies are useful for generating hypotheses, they are susceptible to ecologic changes. These fallacies includes the appearance of spurious associations between diet and cancer that may be only casually related factors. Therefore, we will investigate the immune function, as a controlling mechanism of cancer with antioxidant supplementation.
Table 1-3. Major sources of carotenes, vitamin C, and vitamin E in the American diet

<table>
<thead>
<tr>
<th>Food</th>
<th>Carotenes %</th>
<th>Food</th>
<th>Vitamin C %</th>
<th>Food</th>
<th>Vitamin E %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Carrots</td>
<td>37.8</td>
<td>1. Orange Juice</td>
<td>26.5</td>
<td>1. Mayonnaise</td>
<td>14.6</td>
</tr>
<tr>
<td>2. Tomatoes</td>
<td>51.0</td>
<td>2. Grapefruit (and juice)</td>
<td>33.7</td>
<td>2. Potato chips</td>
<td>18.8</td>
</tr>
<tr>
<td>3. Sweet potatoes</td>
<td>56.7</td>
<td>3. Tomatoes (and juice)</td>
<td>39.9</td>
<td>3. Apples</td>
<td>22.9</td>
</tr>
<tr>
<td>4. Yellow squash</td>
<td>62.3</td>
<td>4. Fortified fruit drinks</td>
<td>45.7</td>
<td>4. Nuts</td>
<td>27.0</td>
</tr>
<tr>
<td>5. Spinach (cooked)</td>
<td>67.9</td>
<td>5. Oranges</td>
<td>50.6</td>
<td>5. Peanut butter</td>
<td>30.9</td>
</tr>
<tr>
<td>6. Cantaloupe</td>
<td>71.7</td>
<td>6. Potatoes (not fried)</td>
<td>54.8</td>
<td>6. Oil and vinegar</td>
<td>34.2</td>
</tr>
<tr>
<td>7. Mixed vegetables</td>
<td>75.4</td>
<td>7. Potatoes (fried)</td>
<td>58.9</td>
<td>7. Tomatoes</td>
<td>37.4</td>
</tr>
<tr>
<td>10. Spinach (raw)</td>
<td>83.0</td>
<td>10. Broccoli</td>
<td>67.2</td>
<td>10. Tomato sauce</td>
<td>45.9</td>
</tr>
</tbody>
</table>


The relationship between breast cancer risk and dietary antioxidant intake is listed in Table 1-4.
Table 1-4. Selected case-control and cohort studies of the relationship between dietary carotenes, vitamin C, vitamin E and breast cancer risk.

<table>
<thead>
<tr>
<th>Dietary</th>
<th>Study Design (n:N)b</th>
<th>Extremes compared by relative risks</th>
<th>Relative risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>carotenes</td>
<td>cohort (123:10,473)</td>
<td>Tertiles</td>
<td>1.2</td>
</tr>
<tr>
<td>carotenes</td>
<td>c:c (12 studies)</td>
<td>Quartiles</td>
<td>1.2</td>
</tr>
<tr>
<td>carotenes</td>
<td>c:c (139:139)</td>
<td>Quartiles</td>
<td>4.8</td>
</tr>
<tr>
<td>carotenes</td>
<td>c:c (200:420)</td>
<td>Tertiles</td>
<td>3.4</td>
</tr>
<tr>
<td>carotenes</td>
<td>c:c (133:289)</td>
<td>Below vs above 2mg</td>
<td>1.1</td>
</tr>
<tr>
<td>carotenes</td>
<td>c:c (409:515)</td>
<td>Tertiles</td>
<td>1.0</td>
</tr>
<tr>
<td>vitamin C</td>
<td>c:c (439)</td>
<td>Quartiles</td>
<td>0.6</td>
</tr>
<tr>
<td>vitamin C</td>
<td>c:c (250)</td>
<td>Quartiles</td>
<td>1.4</td>
</tr>
<tr>
<td>vitamin C</td>
<td>c:c (118)</td>
<td>Decile</td>
<td>1.0</td>
</tr>
<tr>
<td>vitamin C</td>
<td>c:c (12 studies)</td>
<td>Quartiles</td>
<td>1.5</td>
</tr>
<tr>
<td>vitamin C</td>
<td>c:c (139:139)</td>
<td>Quartiles</td>
<td>3.1</td>
</tr>
<tr>
<td>β-carotene</td>
<td>cohort 8 year (30:59)</td>
<td>Quintile</td>
<td>0.9</td>
</tr>
<tr>
<td>vitamin E</td>
<td>cohort 8 year (30:59)</td>
<td>Quintile</td>
<td>0.6</td>
</tr>
<tr>
<td>vitamin E</td>
<td>c:c (250)</td>
<td>Quartiles</td>
<td>1.0</td>
</tr>
<tr>
<td>β-carotene</td>
<td>c:c (210)</td>
<td>Quintile</td>
<td>1.2</td>
</tr>
<tr>
<td>β-carotene</td>
<td>c:c (377)</td>
<td>Quintile</td>
<td>1.2</td>
</tr>
<tr>
<td>vitamin E</td>
<td>c:c (439)</td>
<td>Quintile</td>
<td>0.6</td>
</tr>
<tr>
<td>vitamin E</td>
<td>c:c (109)</td>
<td>Tertiles</td>
<td>0.6</td>
</tr>
</tbody>
</table>
General patterns are often observed which show increased risk with low levels of circulating antioxidants.

B. β-CAROTENE

β-carotene is one of a large group of carotenoids. β-carotene is a purported anti cancer agent, which is believed by some to have antioxidant action of a radical-trapping type. Recently they show that, it exhibits good radical-trapping antioxidant behavior only at partial pressure of oxygen significant less than 150 torr, such low pressures are found in most tissue under physiological condition. At high oxygen pressures, β-carotene loses its antioxidant activity and show an autocatalytic prooxidant effect, particularly at relatively high concentration (231). Blood level of healthy people of β-carotene generally reflect β-carotene intake (59,60). β-carotene consumption is most significantly associated with improved survival. Only one death occurred in the group with the highest consumption of β-carotene while there were eight and twelve deaths in the intermediate and lowest groups of consumption, respectively (61). However, it is particularly
important because it is the major provitamin A, and is converted in the gut to vitamin A and is degraded to retinoic acid. Retinoic acid affects the differentiation and growth of many tissues (62,63). The induction of differentiation has been shown in embryonal carcinoma cell lines, leukemia cells, and numerous epithelial tissues. In addition, anti proliferative or growth-inhibition effects have been observed in vitro in a great variety of cells, including lines derived from mammary carcinomas (64). Such differentiating anti proliferative properties appear central role to retinoid suppression malignant phenotype in vitro and in vivo. The mechanisms by which retinoic acid affects cell differentiation is poorly understood. Retinoic acid receptors probably participate in this process by signal transduction. A case-control study of 317 cases of breast cancer from Italy and France observed no significant differences between cases and controls in mean consumption of preformed vitamin E and β-carotene (65,66). Potischman (67) observed a marginally significant decline in blood β-carotene levels with increasing stages of breast cancer. The Finnish Social Insurance Institution Mobile Clinic collected blood sample from 2,300 women from 1968-1971. Subsequently, 67 women developed breast cancer and were each watched. The samples were then analyzed for retinol, β-carotene, α-tocopherol, and selenium. The relative risks of developing cancer was 1.0 for retinol and α-tocopherol was close to 1.0 but the risk for selenium was 1.7 and for α-carotene the risk was 0.4. The beneficial effects of β-carotene remain statistically significant even after adjusting for other variables (68). In their study, Potischman et al (67) showed that β-carotene blood levels were significantly lower in breast cancer cases than in controls.
Although there was no overall association between plasma retinol levels and breast cancer, there was a positive relationship between retinol and breast cancer in the subgroup with low β-carotene levels (67). Three major limitations of the studies conducted to date are 1) the possibility that disease or preclinical diseases can affect serum β-carotene levels 2) the small sample size of prospective studies, and 3) inadequate storage conditions. Studies of serum carotenoid and vitamin E levels and cancer should ideally be large prospective studies and should utilize storage conditions of -70°C or below and have comparable treatment of case and control specimens (69).

Chemoprevention trials using β-carotene have been initiated for many malignancies (70). A trial of a retinoic acid derivative is currently under way in Italy. The aim to evaluate any reduction in the frequency of contralateral breast cancer in patients with previously treated primary breast cancer (71). Because of β-carotene's ability to act as both a quenched of single oxygen and free radicals and its epidemiological association with lower cancer risk, there are now several groups studying the possible role of β-carotene as an immunomodulator (72).

Several complications arise in research with β-carotene. It is highly insoluble making in vitro experiments difficult. Studies using rodents are also difficult since they efficiently convert β-carotene to vitamin A, making it questionable whether any immune enhancement seen is due to the known immunostimulatory capability of vitamin A. Another carotenoid like canthaxathin can serve as an appropriate dietary control since it is a singlet oxygen quenched but it cannot be converted to vitamin A.
Neutrophils serve as the first line of cellular defense against bacterial infection. When human neutrophils are incubated with β-carotene and bacteria, the bacteria are killed efficiently and the neutrophils were not damaged by the free radicals. In contrast, neutrophils not incubated with β-carotene are damaged by their oxidative products (73). Colorless bacteria are effectively killed by neutrophils, whereas carotenoid-containing bacteria are protected from oxidative destruction. In fact, carotenoids may protect surrounding host cells, as well as neutrophils, from inadvertent indiscriminate damage (73).

In addition to neutrophils, which contribute to the nonspecific arm of the immune response, the immune system has the ability to mount specific defenses against a particular pathogens. The cells involved in the generation of specific immune responses are T and B-lymphocytes and macrophages. T and B-lymphocytes can be adversely affected by oxidative products as well as directly by free radicals. Under high oxygen tension, which would increase the potential for the generation of free radicals, there is a depression in T and B-lymphocyte proliferative response (74). If lipid peroxides are formed by free radical damage to cellular lipid membranes, they can depress the T and B-cell response (75). A high level of polyunsaturated fatty acids in the diet can also have a depressant effect on T and B-lymphocyte function (76). Thus, oxidative products generally have adverse affect on specific immune responses.

In a series of experiments, rats were fed nutritionally complete diets containing a high level of β-carotene or canthaxanthin. Both dietary β-carotene and canthaxanthin
enhanced T and B-lymphocyte proliferative. In this study, mitogen responses were assessed with both carotenoids and then compared with plasma and tissue vitamin A, β-carotene and canthaxanthin levels. β-carotene has an immunoenhancing capacity which is hypothesized to be linked to the antioxidant function shared by both carotenoids and is not related to its pro-vitamin A activity (77).

The three immune cells capable of killing tumor cells are macrophages, cytotoxic T-lymphocytes, and natural killer cells. Macrophages have been shown to have an increased ability to kill tumor cells if the study animals were fed β-carotene, canthaxanthin, or vitamin A. β-carotene protects macrophage receptors from oxidative damage (78). When mice are fed β-carotene, the thymus and the thymocytes are protected against radiation damage (79). There is also enhanced proliferation and an induction of cytotoxic T cells in mice fed diets containing β-carotene (80).

In a preliminary report (81), natural killer cells lysed more tumor cells when they were incubated in media containing either β or α-carotene as compared with incubation in media alone. β-carotene and canthaxanthin increased the production of tumor necrosis factor; which directly kill tumor cells (82).

Tumors can produce molecules that suppress certain macrophage functions. β-carotene suppresses molecules which are synthesized by tumors (83). β-carotene and canthaxanthin at concentrations of 70mM or 300mM were shown to inhibit the proliferation of cultured human squamous cells (SKMES lung carcinoma and SCC-25 oral carcinoma in 5h. cell density assay). Onset of the responses to β-carotene alone or
in a combination with α-tocopherol is signalled within 1-2 hours of treatment by the appearance of a unique 70 KD heat-shock protein (84). Willett (85) reported that during a period of 16 weeks, a daily α-tocopherol supplement of 800 IU caused a decrease in plasma carotenoid levels. They also showed β-carotene doses of 15-60 mg/d caused profound reductions in plasma α-tocopherol concentrations only after 6 consecutive months of treatment (166).

β-carotene increases T and B cell proliferation. It can increase the ability of macrophages and natural killer cells to kill tumor cells. Additionally it can increase cytotoxic T cells and TNF-α production by activated T cells. β-carotene influence on the immune system can result in a decrease risk of cancer.

C. Vitamin C

Vitamin C is an important component of the overall antioxidant defense system.

Plasma and leukocyte vitamin C levels reflect vitamin C intake. Vitamin C intake reflects a more time integrated measure of vitamin C status (86). Although the results from 3 case control studies, as well as results from a combined analysis of nine case control studies, consistently show a protective effect of vitamin C existing data in breast cancer is inconsistent. At high levels of vitamin C consumption, there were significantly fewer deaths from breast cancer (61). Vitamin C (ascorbic acid) has a number of biochemical functions which are linked to immune function. Table 1-5 and 1-6 shows the effect of vitamin C on the immune system (232).
Table 1-5. Vitamin C and Nonspecific Immunity

<table>
<thead>
<tr>
<th>Target</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>skin</td>
<td>required for collagen synthesis</td>
</tr>
<tr>
<td>linings of mouth, gut</td>
<td>maintains basement membrane</td>
</tr>
<tr>
<td>interferon</td>
<td>important in production and secretion</td>
</tr>
<tr>
<td>complement components</td>
<td>required for the synthesis of C1q</td>
</tr>
<tr>
<td>phagocytic cells</td>
<td>enhances chemotaxis, protects cells from free radical damage</td>
</tr>
</tbody>
</table>
Table 1-6. Vitamin C and Specific Immune Response

<table>
<thead>
<tr>
<th></th>
<th>Deficiency</th>
<th>Supplementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocyte Proliferation</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>Cytotoxicity</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>Antibody Titers</td>
<td>No effect</td>
<td>Increased</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No effect</td>
</tr>
<tr>
<td>Delayed Hypersensitivity</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>Response to Vaccines</td>
<td>Decreased</td>
<td>ND*</td>
</tr>
<tr>
<td>Inflammatory Responses</td>
<td>Decreased</td>
<td>ND</td>
</tr>
<tr>
<td>Autoimmune Responses</td>
<td>Decreased</td>
<td>ND</td>
</tr>
</tbody>
</table>

*ND = no data available

Vitamin C is involved in collagen synthesis. This function includes the acceleration of hydroxylation reaction required for formation of hydroxyproline from proline, enhancing collagen formation. Collagen is an essential component of basement membranes which serves to enhance attachment of epithelial cells lining the digestive, respiratory, urinary and reproductive tracts. In addition, collagen is an important component of skin and bone tissues. Vitamin C is therefore involved in the maintenance of natural barriers.

Singlet oxygen is not a free radical, it can participate in the formation of free radicals. Vitamin C can quench singlet oxygen (87) and block the formation of carcinogenic
nitrosamines in test tubes as well as in the digestive tract (88).

For rodent and most animals, vitamin C is not a vitamin since it is synthesized in their livers. Unlike those animals, humans do not have the ability to synthesize vitamin C. Therefore, studies using animals with a vitamin C requirement are emphasized. Vitamin C has been shown to be required for the secretion of hydroxyproline-rich complement component, C1 (89). Vitamin C concentration in neutrophils and macrophages is approximately 150 times the concentration of plasma vitamin C. During the oxidative burst, neutrophils take up vitamin C and following activation, the vitamin C concentration is reduced (90). Vitamin C supplementation enhances the chemotactic response to neutrophils in healthy individuals (91).

Anderson (92) have shown that vitamin C can decrease the damaging effects of oxidative burst products without decreasing the intracellular concentration of reactive bacterial molecules. In addition, vitamin C decreases lipoxygenase activity therefore, causing an increase in human phagocytic activity (93).

In scorbutic animals, there is a depressed response to vaccination and delayed hypersensitivity response; both indicators of immune memory function. Responses to foreign tissues are also significantly diminished, resulting in the increased survival of grafted skin and decreased autoimmune responses.

Ascorbic acid, the reduced form of vitamin C, can donate an electron to tocopherol free radicals and, regenerate the antioxidant form of vitamin E (71). Due to this effect, vitamin C supplementation may indirectly improve immune responses by
helping to maintain circulating and tissue levels of vitamin E (94). Vitamin C has been found to suppress growth in isolated leukemia cells. Vitamin C supplementation has also been shown to enhance survival and reduced tumor burden and growth in several animal models. However, there are no studies which have specifically examined the ability of vitamin C to enhance tumor immunity. However, the enhancement of interferon production and T-cell activities should encourage such experiments. Some animal studies have documented that vitamin C inhibits tumor development while others have shown either no effect or enhanced tumor growth and metastasis (95). Vitamin C supplementation (at 1 g/day) enhances human in vitro lymphocyte proliferative and antibody responses (13). Delayed hypersensitivity responses are also enhanced following oral supplementation of vitamin C (1g/day) in a study (13) of young adults following injections of 500 mg/day of vitamin C and in another study in the elderly population (96). Oral supplement (2 gr/day) in an elderly population enhances the in vitro lymphocyte proliferative responses but does not affect delayed hypersensitivity responses in another study (91).

Vitamin C can act directly or indirectly on the immune system. It increases the non specific and specific immune response, or indirectly, by trapping free radicals and regenerating tocopherol. The effect of vitamin C on the immune system reduce risk of developing cancer.
D. Vitamin E

Vitamin E is a fat-soluble vitamin. Traditionally its activity is expressed in international units (IU), where 1 IU is equivalent to 1 mg dL-α-tocopherol acetate. In these terms the 1980 Recommended Daily Allowance (RDAs) are 15 mg/d (15 IU/d) for adult men and 12 mg/d (12 IU/d) for adult women. A newer unit of measure, the Tocopherol Equivalent (TE), has also been used to express vitamin E. One TE is equal to 1 mg/d-α-tocopherol, or 1.49 IU. In these terms, the 1980 RDAs are 10 TE for men and 8 TE for women. Vitamin E is present in the blood as d-α-tocopherol and is well accepted as the major antioxidant in lipid body tissues. Vitamin E supplementation has resulted in inconsistent effects on serum lipids and lipoprotein levels. Lipoprotein effects of vitamin E given in dosages of 400 IU/d to 800 IU/d were examined in nine studies. No effect on high density lipoprotein (HDLs) was seen in five of the studies and increases were observed in four (97). Animal studies showed that vitamin E is not mutagenic, carcinogenic, or teratogenic (97). In human double-blind protocols with large population, oral vitamin E supplementation resulted in few side effects, even at doses as high as 3200 IU/d. However, in vitamin K-deficient individuals, the administration of high levels of vitamin E can exacerbate coagulation defects. The mechanism for vitamin E synergism with anticoagulant therapy may involve the blocking of the oxidation of vitamin K, thus altering the balance between the inactive and active forms by increasing the levels of the inactive form. Vitamin E intake by humans at dosage of 1600 IU/d decreases platelet thromboxane production which could consequently reduce the potential
for thrombosis formation (97).

Blood α-tocopherol levels reflect dietary intake of vitamin E (86) and thus can be used to assess vitamin E status. In a nested case-control study, Russel (98) observed a nonsignificant increase in blood α-tocopherol levels among 30 cases of breast cancer as compared to controls. Data compared on the relationship between dietary intake of vitamin E and breast cancer are inconsistent.

In a longitudinal study in six geographic areas in Finland men with higher blood vitamin E levels had lower risk of subsequent cancer. Adjusted relative risks in the two highest quintiles of blood vitamin E concentrations, compared to all other quintiles were 0.7 for all cancers combined, and 0.6 for cancers unrelated to smoking (99). In a study which was performed in England, 5 times greater breast cancer risk was observed among women with vitamin E levels in the lowest quintile than for those in the highest (100). In a study evaluating the risk of subsequent female cancers in Finland, subjects with low serum selenium and vitamin E levels had a 10 fold higher risk of breast cancer. According to the researchers, their results suggested that a low serum vitamin E concentration can predict cancer development in women (101). High vitamin E intake was also associated with a significantly lower risk of cervical cancer (102). Average plasma vitamin E and β-carotene levels were significantly reduced in women with cervical dysplasia or cancers compared with the control group.

Vitamin E blood levels were significantly lower for subjects who developed cancer. Subjects with vitamin E blood levels in the lowest quintile had a 4.4-fold higher
cancer risk as compared to subjects with levels in the highest quintile. Based on the results obtained by Kok et al (103), they suggested that low vitamin E blood level may be a risk factor for cancer.

Vitamin E is considered the first line of defense against cell-membrane damage due to peroxidation. It scavenges free radicals, terminates chain reactions, and confines damage. In addition to its role as a free radical scavenger, vitamin E at high levels enhances the immune response which may play a role in cancer defense. The initiation of the immune response occurs at the cell membrane. Vitamin E is an essential constituent of all membranes found in cells including plasma, mitochondrial, and nuclear membranes (104). If vitamin E is absent from the diet, mitochondrial membranes from lymphocytes, reticulocytes, and platelets show swollen and disrupted areas (105). All circulating cells which are derived from the common hemopoietic stem cell precursor demonstrate membrane changes when vitamin E is absent from the diet. Red blood cells lyse in the presence of hydrogen peroxide. Platelets will increase and be more adhesive and produce more thromboxane (106,104). The dietary α-tocopherol requirement for maintenance of optimal immune responsiveness may be higher than the levels necessary for normal growth and reproduction. Chavance (107) found that subjects with serum vitamin E levels of 1.35 mg/dL had significantly lower numbers of infections over a three year time span. Average serum vitamin E levels in U.S. are 0.9-1.0 mg/dL. ConA can increase the mitogenic response of lymphocytes and increase IL-2 production. In the study, IL-2 production of a vitamin E-treated group was significantly increased
with stimulation by ConA (400 IU dL-α-tocopherol acetate in soybean for 30 days)(108).
The vitamin E content of lymphocytes and mononuclear cells are approximately ten times
greater than that found in platelets and red blood cells. When macrophages are exposed
to oxidative stress, the vitamin E contents of these cells is significantly reduced (109).

Free radical mediated reactions can result in membrane lipid peroxidation. Peroxidation of lymphocyte membranes significantly depresses the in vitro responses to
mitogens as well as the primary antibody responses. Addition of vitamin E to a
lymphocyte culture overcomes the immunosuppression (110). α-tocopherol succinate or
vitamin E acetate exhibits a concentration dependent inhibition of NF-KB activation. In
contrast, α-tocopherol does not block NF-KB activation. PMC is the most potent
inhibitor among the vitamin E derivatives. Oct-1 DNA binding activity, which is
constitutively expressed in jurkat cells, is inhibited by α-tocopherol succinate at a
concentration that is also effective at inhibiting NF-KB. Only α-tocopherol succinate
inhibits the DNA binding activity of activated NF-KB. This shows a lipophilic
antioxidant to be effective in inhibiting TNF-α induced NF-KB activation (111).

Vitamin E acetate is known to become deesterified to a biologically active
antioxidant form, α-tocopherol (112). Addition of α-tocopherol is ineffective in
inhibiting signal transduction. Alpha-tocopherol is localized in the internal
compartments of cell architecture, such as mitochondria, where vitamin E acetate, but
not α-tocopherol, can be reached before deesterification. d-α-tocopherol inhibited cell
growth approximately 50%. However, d-β-tocopherol, an analogue of d-α-tocopherol
lacking a methyl group in position 7 of the chromanol ring, did not show any inhibition in cell proliferation (113). α-tocopherol strongly inhibited protein kinase C activity, whereas β-tocopherol was much less effective. This shows that the growth inhibitory effect of α-tocopherol was not related to its antioxidant properties (114). The molecular basis of d-α-tocopherol sensitivity is not clear. It can be based on different signalling pathways used for proliferation in various cell types. Depending on the cell type, it may be possible that d-α-tocopherol transport and metabolism is different. Finally, it may be conceivable that d-α-tocopherol binding proteins, related to d-α-tocopherol inhibition, are present in some cells and not in others (115). Addition of d-α-tocopherol at the G<sub>i</sub>/G<sub>i</sub> phase results in a 75% inhibition of kinase activity. If the addition is made later in the cycle, progressively less inhibition of protein kinase C activity is seen. The cell cycle phase when d-α-tocopherol is added appears to be critical in the onset of d-α-tocopherol inhibition.

The membrane by which α-tocopherol is transported to intracellular organelles has been identified. Rat liver cytoplasm contains a protein, with a molecular weight of 30-40 kDa, that binds to α-tocopherol with high affinity and specificity. The biological role of this protein is to transfer α-tocopherol through the membrane. Two isoforms of α-tocopherol transfer protein exist that transfer α-tocopherol from liposome to microsomes. The purified transfer proteins recognize a certain tocopherol structure as a substrate and the two isoforms are not distinguishable with respect to the substrate specificity (116). Topical application of α-tocopherol increases the level of α-tocopherol
in the skin but not in the plasma. The presence of 1% beta carotene in the diet resulted in a significant decrease in of \( \alpha \)-tocopherol in skin of the uv-irradiated mice (166).

Vitamin E protects vitamin A from destruction in the body and spares selenium. Vitamin E also inhibits the conversion of nitrites present in smoked, pickled, and cured foods to nitrosamines. Nitrosamines are strong tumor promoters. Vitamin E can protect the conjugated double bonds of \( \alpha \)-carotene from oxidation.

In cell-culture studies, vitamin E and selenium have been effective in inhibiting the transformation of normal mouse embryo cells to cancerous cells after exposure to radiation and chemicals. Studies of mouse cancer cells have shown that incubation of cells with vitamin E succinate also inhibited the growth of cancer. Vitamin E succinate also inhibits the growth of four representative cancerous murine cell lines in a dose-dependant manner (117).

One of the biological changes seen with aging is an increase in free radical damage. The mortality rate was lower in those who took a daily vitamin E supplements containing 100-999 IU/day as compared those who had not taken vitamin E supplements. The mortality rate of those using > 1000 IU of vitamin E/day was similar to the mortality rate of those who did not use supplements (118).

Plasma vitamin E levels were positively correlated with positive DTH responses to Diphtheria toxoid, and Candida and Trichophyton antigen. Subjects with \( \alpha \)-tocopherol levels greater than 135 mg/L were found to have higher helper-inducer/cytotoxic-suppressor ratios (119). Supplementation with 800 IU/day for approximately one month
significantly enhanced the delayed hypersensitivity responses, while the level of circulating peroxides were diminished (120).

When lymphocytes were cultured with a synthetic oxidant, such as 2-mercaptoethanol, the lymphocytes from vitamin E deficient rats and mice did not proliferate when stimulated with mitogens to the level found with lymphocytes from animals fed vitamin E (107). This implies that vitamin E must be present during the synthesis of all cellular components. Low levels of dietary vitamin E (7.5 and 15 mg/kg in the diet) were insufficient at enhancing T and B-lymphocyte mitogenic responses even though the purified diet contained all other nutrients at levels recommended by the American Institute of Nutrition (107).

A ten fold increase in dietary vitamin E may be required to achieve a twofold increase in serum vitamin levels with fat soluble vitamins. The level of vitamin E in the diet was correlated with plasma vitamin E levels. More importantly, plasma vitamin E levels were correlated with optimum responses to T and B-lymphocyte mitogen responses (107). The dietary requirement for optimum T and B-lymphocyte responses to mitogens was greater than 50 mg/day and was correlated with plasma vitamin E levels over a range of 0.04-19\(\mu g/ml\) (107). Meydani showed that high levels of dietary vitamin E decreased the level of splenocyte PGE\(_2\) and enhanced lymphocyte functions (120).

Vitamin E produces a greater response in young mice than in old mice maintained on either diet. This effect might be due to a greater utilization and/or incorporation of \(\alpha\)-tocopherol in cells of young mice (108). The ability of splenic natural killer cell to lyse
tumor cell was not significantly altered when either mice (121) or rats (122) were fed vitamin E deficient or supplemented diets. However, when tumor cells were injected into mice, tumors were more numerous and grew faster in vitamin E deficient mice (123). Since the mechanism of natural killer cell lysis does not seem to involve oxidative damage (124), we assume that vitamin E protects other immunoenhancing substances such as protecting vitamin C and A from oxidation. The concentration of tumor necrosis factor secreted by activated macrophages was significantly enhanced in the vitamin E-treated group (125). The dietary α-tocopherol requirement for maintenance of optimal immunoresponsiveness may be higher than the level necessary for normal growth and reproduction. Vitamin E deficiency can cause peroxide hemolysis of red blood cells. In addition, a decrease in α-tocopherol levels in the blood could lead to a higher risk of arteriosclerotic coronary artery disease, by causing an increase in proliferation of vascular smooth muscle cells (126).
Table 1-7. Effect vitamin E supplementation on immune function

<table>
<thead>
<tr>
<th>Number of Individuals</th>
<th>Dosage</th>
<th>Findings</th>
</tr>
</thead>
</table>
| 3                     | 2179 IU/day one week | a. neutrophil phagocytosis increased  
b. neutrophil bactericidal activity decreased  
c. auto-oxidative damage to neutrophils decreased |
| 3                     | 2179 IU/day two weeks | hydrogen peroxide level decreased in neutrophils                |
| 1                     | 544 IU/day/3months | normalization of neutrophil functions; no new bacterial infections |
| 9                     | 406 IU/day/3weeks  | a. no change in DOTH to PHA  
b. decrease in mitogen response to PHA  
c. decrease in bactericidal activity |
| 4                     | 1632 IU/day/3 weeks | no change in MLC with vitamin E supplemented  
responder lymphocytes; decrease with vitamin E supplemented stimulators |
| 14                    | 2040 IU/2-3 weeks | normalization of depressed monocyte chemotaxis in diabetic subjects |
| 34                    | 1088 IU/day/one month | a. significantly enhanced delayed hypersensitivity responses in vitamin E group  
b. increase in mitogen response to ConA |

α-tocopherol can inhibit NF-κB activation by TNF-α, thereby improving immune dysfunction and reducing the risk of developing cancer in elderly. In addition to its antioxidant activity in decreasing the risk of cancer, α-tocopherol also inhibits cell proliferation and protein kinase C and subsequently inhibits tumor growth.

E. SELENIUM

Selenium, in addition to its inclusion in glutathione peroxidase, enhances immune response and potentially increased resistance to cancer initiation (94).

In four of five case-controlled studies of breast cancer (127-131), cases were found to have significantly lower blood selenium levels than did controls. In another such study, authors reported no significant differences in nail selenium levels between cases and controls (132).

Selenium levels in Finland are among the lowest in the world. This observation is compatible with an increased risk of breast cancer among women. Subjects with blood selenium levels in the lowest third showed a 5.7 fold adjusted risk of developing fatal cancer compared to those with higher selenium levels. However, the existing data does not support a protective effect of high tissue selenium levels on breast cancer (133).

Vitamin E can protect against lipid peroxidation in cell membranes. The enzyme glutathione peroxidase can reduce lipid peroxidation within the cell. Selenium is required for the activation of metalloenzyme glutathione peroxidase. Glutathione peroxidase is the second line of defense that destroys peroxides before they damage cell membranes.
Both vitamin E and Selenium are required for optimal immune function. Bendich (54) have found that Selenium can not replace vitamin E by enhancing T and B-lymphocyte responses. Vitamin E and Selenium appear to function cooperatively and have additive effects in cancer studies. Results of a study on induced mammary (breast) tumors in rats showed that selenium supplementation alone leads to a modest decreases in tumors compared to controls. Although vitamin E by itself had no effect a combination of selenium and vitamin E significantly produce the fewest tumors (134).

A major function of selenium in immune cells, and especially in cells that elicit a phagocytic respiratory burst, (135,136) is to control excessive production of peroxidative substrates, such as H$_2$O$_2$.

Selenium and/or glutathione peroxidase is found in thrombocyte, eosinophils, neutrophils, monocytes, macrophages, natural killer cells, cytotoxic T-suppressor, T helper cells, and B cells. Normal levels of dietary selenium and doses exceeding that which is needed for glutathione peroxidase synthesis, is known to reduce the frequency, growth, and size of tumors in experimental animals and in vitro (137,138). Selenium given to rats in drinking water at 0.5 or 2.0 ppm, exhibited enhanced NK cell cytotoxicity against tumor cells in vitro. Five ppm selenium in drinking water was slightly suppressive to NK cells (139). Dimitrov (139+1) have shown that humans who ingest 400 $\mu$g/day of sodium selenite (160$\mu$g selenium) had increased plasma selenium and enhanced NK cell activity against $^{51}$Cr-labeled K562 target cells. Drug resistant tumor cells with high intracellular glutathione are highly sensitivity to selenite (140).
Since selenium becomes toxic (141,142) by producing $O_2$ and $H_2O_2$ upon oxidation of glutathione by selenite (143,144), these NK cell effects could be related to selenium toxicity. The question remains whether dietary selenium is cytotoxic in vivo for immune cells. The highest selenium excess decreased vitamin A storage and enzymatic antioxidant defense in rat liver (145). High dietary selenium can sometimes enhance carcinogenesis (145). Direct addition of selenium at 1.0 mg/ml to target and effector cells significantly suppressed NK activity of normal lymphocytes. When lymphocytes were preincubated with selenium at concentrations as low as 0.2 $\mu$g/ml for a period of 48 hours, a significant inhibitory effect on NK activity was observed. In the LAK cell assay, direct addition of selenium at concentrations of 0.2-1.0 mg/ml in the presence of selenium at 0.8 $\mu$g/ml, showed significant inhibition of function (146). Lymphocyte proliferation responses to T-cell mitogens such as PHA and ConA were also significantly suppressed by direct addition of selenium at 0.5-1.0 $\mu$g/ml (146).

Mulhern (147) reported that lymphocyte mitogen response of first generation selenium deficient, vitamin E adequate mice was the same as animals who were fed the selenium adequate diet. However specific antibody responses (IgG) were reduced following challenge with sheep red blood cells. When mice were kept selenium deficient through two generations, mitogenesis, specific and nonspecific antibody titers, as well as thymic size were depressed even though mice were fed adequate levels of vitamin E. Selenium decreased the activation of certain cancer-promoting agents in cell culture studies (148).
Selenium enters the food chain through plants. These plants incorporate selenium into sulfur compounds, resulting in the formation of selenomethionine, and to a lesser extent, selenocysteine and other analog of sulfur amino acids. The predominant form of selenium in animal tissues is selenocysteine. Selenium content, in soil over 500 mg/kg produces chronic selenium toxicity in domestic animals. Hemostasis of selenium in animals is achieved through regulation of excretion. Selenium is methylated for excretion and dimethyl selenite is exhaled by animal given a large amounts of the element. Trimethylselenonium is a urinary metabolite but the identity of most of the selenium excreted in the urine is not known.

The typical american diet provides an average daily selenium intake of 100μg for adult men and 70 μg for adult women (149). The daily requirement for selenium is 50-300 μg for adults, while the toxic dose is regarded as 5 mg daily (150). Due to its role in glutathione peroxidase, selenium could interact with any nutrient that affects the antioxidant/pro-oxidant balance in cells. For example, the selenium requirement of chicks is inversely proportional to their dietary vitamin E intake (151).

There are three biological functions of selenium 1) Glutathione peroxidase is used to reduce hydrogen peroxide and nonesterfied organic hydroperoxides. It is made up of four identical subunits, each of which contains one selenocysteine. Glutathione peroxidase serves primarily to metabolize hydrogen and therefore acts against injury to the cell. 2) Phospholipid hydroperoxide glutathione peroxidase inhibits lipid peroxidation and can reduce fatty acid hydroprooxide. This enzyme might provide some
antioxidant properties of selenium. 3) Selenoprotein P is a single polypeptide chain containing seven or more selenocysteine residues. It probably has redox functions and antioxidant functions. Selenium is considered to protect organisms in case of poisoning with lead, mercury or cadmium (150). Selenium deficiencies lead to marked changes in many biochemical systems. The cytochrome P450 system and glutathione metabolism are affected by selenium deficiency. Pure selenium deficiency does not usually cause clinical illnesses. Selenium deficient animals are more susceptible to injury by certain chemicals. Toxicities of the redox cyclers paraquat, diquat and nitrofurantoin, and of Mercury and other heavy metals are increased by selenium deficiency.

However, the epidemiologic evidence linking low selenium status and increased incidence of cancer is conflicting (152). It is often based on small differences in plasma selenium levels between controls and subjects who later develop cancer (153). Some animal experiments show that high levels of dietary selenium can protect against certain chemically or viral induced cancers. However selenium itself can stimulate tumorigenesis in some rodent models (154).

Selenium is a component of glutathione peroxidase. Selenium deficiency decreases the specific and nonspecific immune response. However high concentrations of selenium can be carcinogenesis and toxic, we will therefore not use this compound in our study.
**F. Glutathione**

Glutathione (GSH) is the most abundant low molecular weight thiol-containing compound in living cells. Glutathione is of the major significance as an antioxidant (155) because it participates directly in the destruction of reactive oxygen compounds. It also maintains reduced forms such compounds as ascorbate and tocopherol, which also exert an antioxidant effect. Glutathione is involved in the reduction of dehydro ascorbate. Due to these it increase immune cell function and reduce risk of cancer.

Buthionine sulfoximines, a selective transition state inhibitor of glutamylcysteine synthetase (the enzyme that catalyses the first and rate limiting step in the synthesis of glutathione). It produces markedly decreased levels of cellular glutathione and cause oxidative tissue damage. Glutathione deficiency induced in adult mice damages certain tissues such as skeletal muscles, lung, jejunum and colon. It is associated with mitochondria degeneration, whereas other organs such as liver, kidney, heart, and stomach are relatively unaffected. In contrast, glutathione deficiency in newborn rats produces sever damage to liver, kidney, brain, lung, cerebral cortex, and leads to formation of cataracts. Glutathione deficiency in newborn rats leads to death within a few days. Tissue damage and mortality are decreased by administration of glutathione monoesters or ascorbate. The level of mitochondrial glutathione increased 2.7 to 6.0 in various tissues after administration of ascorbate (156). Glutathione and ascorbate can function in the destruction of reactive oxygen compounds. These two compounds can replace each other. However, each of them is able to participate in certain reactions that
Glutathione has been shown to regulate the binding, internalization and degradation of IL-2 (157). Depletion of intracellular glutathione inhibits the lectin-induced activation response of human T-lymphocytes. In particular, lectin-stimulated human lymphocytes progress into an early stage of G₁ (termed G₁a) which is characterized by IL-2 receptor expression, IL-2 production, and some RNA synthesis. Neither IL-2, nor expression of the IL-2 receptor, appear to be critical for this transition. However, in order for lymphocytes to progress further into G₁ (termed G₁b), IL-2 is absolutely essential. At this point, the lymphocytes express new IL-2 receptors, respond to IL-2, and proceed into the S phase. DL-buthionine-SR-sulfoximide (BSO) and 2-CHX deplete intracellular GSH synthesis, thereby allowing a gradual depletion of intracellular GSH. In contrast, 2-CHX is a cyclic aromatic compound which is conjugated via glutathione-S-transfer to form a cysteine-substituted derivative called mercapturic acid (158).

GHS-depleted lymphocytes are found to incorporate significant quantities of IL-2 and express a substantial density of IL-2 receptors but they fail to incorporate ³H-uridine or ³H-thymidine. This means GHS-depleted lymphocytes undergo a partial activation in response to lectin, but fail to undergo blast transformation. Exogenous IL-2 cannot restore activation in GSH-depleted lymphocytes. Furthermore, lymphocytes remain highly susceptible to inhibition induced by GSH depletion even after 48 hours of lectin stimulation, this amount of lectin is usual sufficient to induce early activation events in the G₀→G₁ transition, such as IL-2 receptor expression and IL-2 production (159).
GSH alone has little effect on thymidine incorporation of CT.4R cells. It enhances the response of CT.4R to IL-4 and increases the levels of thymidine incorporation up to more than 60-fold in a concentration dependent manner. GSH affects the binding of IL-4 to cellular receptors. It increases receptor numbers from 1173 to 2112 molecules per cell. Internalization and degradation studies of IL-4 show that the amount of IL-4, internalized and degraded in the GSH-treated cells, is about two fold higher than that in cells without GSH treatment (160). In many aspects, the effect of IL-4 on T cells is similar to that of IL-2. Whether or not IL-2 and IL-4 exert their effects by a similar mechanism is unclear. Generation of CD3-AK cells, which are CD3⁺ and CD8⁺ T cells was shown to be regulated by IL-4 and protein kinase (161). The proliferation and cytolytic activity of IL-4-dependent CD3-AK cells is also found to be regulated by cellular GSH, and is very similar to that of IL-2-dependent CD3-AK cells. The inhibitory effect by BSO can be reversed by adding exogenous GSH. Further, this confirms that the proliferation and cytotoxic responses of both IL-2-driven and IL-4-driven CD3-AK cells is regulated by GSH (162). The cytolytic activity of CD3-AK and LAK cells is found to generally parallel the levels of BLT-esterase produced in these killer cells (161). IL-4 is found to up regulate this function (161,162).

The approximate minimal intracellular GSH concentration necessary to sustain a normal activation response is 2 nmol per 10⁷ lymphocytes. Decreased GSH levels as a function of age has been reported to occur in liver, kidney, heart, and blood of mice. It has been suggested to be responsible for progression of aging (163). Methionine, a
precursor of GSH biosynthesis, has been reported not to have an immunostimulatory effect \cite{164}. An immunostimulatory effect in experimental animals has been reported for another sulphydryl-containing compound, 2-mercaptoethanolamine \cite{165}. However, this antioxidant is not suitable for human use.

Glutathione has antioxidant activity and also increases IL-2 and IL-4 binding and internalization. This function is necessary for T cell activation, proliferation and LAK cell formation.

**G. Summary**

The excessive production of free radicals can induce severe problems which increase risks of cancer and facilitate aging. Animal studies, prove the effect of antioxidants. Antioxidants can inactivate free radicals and improve immune responses. However, since human studies have large variations between individuals, we did not find any significant effects improving of the immune system. As animal studies show some effects of each antioxidant. We suggest that experiments be done in large populations using multiple antioxidants and examine the incidence of cancer.
CHAPTER II

AGING AND IMMUNE DYSFUNCTION
TABLE OF CONTENTS

I. Introduction
   A. Theories of aging
   B. Changes in antioxidant defence mechanism with aging

II. Aging and the immune system
   A. Immune cell and organs
      1. Lymphoid organs
      2. Cell count and cellular marker
   B. Immune function
      1. Cell mediated immunity
         - Mitogenesis
         - Cytotoxicity
         - Cytokine production
      2. Humoral immunity
      3. Gut mucosal immunity
      4. Cancer
I. Introduction

After reaching maturity, humans show a universal and progressive decline in almost all physiological processes to the point that such decrements limit their survival (167). Although the aging phenotype is well known, the basic nature of the aging processes is still unclear.

A. Theories of aging

There has been a number of theories of the aging process that have been proposed. Some of these theories are described here. In 1928, Peart (168) proposed that the duration of life is inversely related to energy expenditure. In 1959, Szilard (169) proposed that genetic mutations accumulate with time resulting in functional failure. In 1968, Bjorksten (170) proposed "The Cross-Linkage Theory". According to this theory, the functional alterations seen in aging are due to the cross-linking of proteins and nucleic acids. Several gerontologists, during the past two decades, suggest that aging is not intrinsic to most cells of complex multicellular organisms, but rather secondary to attractions in neuroendocrine pacemaker cell (171). In 1956, Harman (172) proposed "The free radical theory". In recent years, this concept has been related to fuel use, specifically, to the generation of oxygen free radicals of reactive oxygen compounds during oxidative metabolism of fuel (173). This subject will be more extensively covered later in this section. The glycation theory of aging also relates to fuel use. It is hypothesized that nonenzymatic reactions of glucose and other reducing sugars with
amino groups of proteins and nucleic acid result in a series of events which alter protein and nucleic-acid structure and function. DNA is especially important in aging. DNA ordinarily occurs in two copies per cell, as compared with the numerous copies of most other macromolecule. Therefore, each deoxyribonucleotide is a potential target for damaging agents. DNA damage is a DNA alteration that causes an abnormal structure, such as a single-strand break, a double-strand break, or an interstrand cross-link. The accumulation of DNA damage is associated with a decline in gene expression, as well as a decline in cellular, tissue, and organ function. An increase in the rate of accumulation of DNA damage appears to cause an increase in the rate of aging. DNA damage can be caused either by intrinsic intercellular processes, such as oxidation hydrolysis and alkylation, or by extrinsic chemical and radiation that enters the organism (174). Chromosomes shorten as cells divide and become senescent (175,176). Also, deletions in mitochondria DNA increase markedly with advancing age which is presumably secondary to damage from oxygen radicals (177). Ames (178,179) presumed that, excretion rates of oxidative DNA fragments into urine are higher in short-lived animals than in long-lived animals and are proportional to the oxygen consumption rate.

B. Changes in antioxidant defence mechanism with aging

Normal function and survival is dependent on the ability of cells to sustain homeostasis via their ability to adapt or resist stress, and to repair or replace damaged molecules and organelles. The body contains numerous compounds, such as antioxidants, that can
attenuate these harmful reactions. Also Tolmasoff (180) showed that the ratio of superoxide dismutase activity (unit per milligram of protein) in the liver, brain, or heart homogenate metabolic rate (calorie per gram weight per day) increases with age. Since host defenses against environmental factors also become attenuated, the molecular damage associated with aging may increasingly perturb normal homeostasis. Sohal (184) found that maximum life-span is positively correlated with the SOD activity in liver, and catalase activity in the heart, and glutathione peroxidase activity in the brain. A negative correlation was also found with glutathione concentration in the liver and brain. Except for the above case, however, correlations between maximum life-span, level of antioxidant enzyme activity and biological antioxidant concentrations are significant (184). Thiol concentration (cysteine and reduced glutathione) decreases with advance age (185). Vitamin E concentrations in the liver and lung increase with advancing age, although that of the cerebrum does not change (186). In senescent cells, growth factors induce the transcription of jun and ras protoncogenes and ornithine decarboxylase genes. This is similar to nonsenscent cells which are normal and a necessary part of proliferation (187). Senescent cells can not proliferate properly. It is known that fos and jun proteins will dimerize to form a transcriptional complex known as AP-1, which in turn can activate other genes required for growth. Thus, this suggests that the senescent cells’ theme is to block expression of one or more genes.

Also, genetic systems have evolved to detect specific forms of damages and activate genes, such as heat shock protein genes (HSP), whose products increase cellular
resistance to DNA damage.

HSPs facilitate the removal of damaged structures as well as their replacement. The expression of a major HSP, HSP70 strikingly reduces in magnitude with age and senescence (188). Also, exposure to oxidative stress induces the transcription of a number of genes. Enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and heme oxygenase, protect cells and tissues from oxidative damage (189). However, Semsei (190) found a gradual decrease in Cu/Zn superoxide desmutase and catalase as animals aged.

II. Aging and the immune response

The following section will address changes in different parts of immune systems of elderly subjects.

Decline in immune function is one of the most important consequences of aging. This age-associated decline of immune functions increases the elderly's susceptibility to infectious agents and cancer (114). The elderly are susceptible to unusual cancers and infections. Also, their body responds to vaccination very differently. In the following pages we will address this immune dysfunction in the elderly. We need to find a way for preventing or decreasing the diseases that accompany aging.

1. Immune cells and organs

Studies on the aging of the immune system indicate that not all immune-cell functions are
degraded uniformly (192-194). Actually, immune senescence is not an immune deficiency state, but an immune dysregulated state (195). Among functions of the cells involved in natural immunity, natural killer (NK) cells are most sensitive to aging and macrophage function is more resistant to the effects of aging. Among T cells, CD8⁺ cells decline more rapidly than CD4⁺ cells do, and the CD4⁺ :CD8⁺ ratio becomes very high in some individuals over the age of 70. Some CD4⁺ cells, especially the Th2-type become hyperfunctional and production of interleukins such as IL-4 and IL-5 is even enhanced, while the production of IL-2 is slightly decreased.

**Lymphoid organs**

The primary lymphoid organs are the thymus and bone marrow. A lymphocyte is immunologically incompetent until it becomes competent within the primary lymphoid organs. The thymus, the organ responsible for maturation of T lymphocytes, develops until puberty and then it begins to rapidly involute becoming rudimentary thereafter (196). Rose (197) showed age-related changes in immunoregulation caused by an asymmetrical involution of the thymus. Age-related thymic involution causes a relative decrease in the number of antigen-specific suppressor T-cells. Immunoregulatory T cells undergo a distinct maturational pathway. Since antigen-specific CD8 immunoregulatory cells do not under go MHC class II selection, therefore they undergo modest positive selection in the cortex. Negative selection occurs when thymocytes encounter class I MHC expressing stromal cells in either the corticomedullary junction or the medulla.
In the natural process of aging, the thymic cortex atrophies more rapidly than the medulla. Auto-antigen-specific immunoregulatory T cells decrease because they undergo less proliferation in the cortex and more negative selection in the medulla. As these autoantigen-specific CD8^+ cells fall with age, non-specific cells (such as NK cells) tend to increase. Non-specific immunoregulatory cells, which lack the T cell receptor (TCR), fail to undergo either positive or negative selection in the cortex and multiply in the medulla. This occurs in response to homeostatic signals designed to maintain total peripheral lymphocyte numbers (198). The decrease in thymic function contributes to the onset of T-lymphocyte immaturity seen during aging. Hormone injection and thymus transplantation restore cell-mediated immunity in elderly animals and humans (199-202), while thymectomy can aggravate age-related immune dysregulation and accelerated aging in young mice (203-205). Thymic epithelial cells (TEC) produce cathepsin L (PCL), a type of cysteine protease. PCL lacks a protease activity, but can promote proliferation of immature thymocytes in the presence of IL-1 and IL-7 (206). These PCL positive thymic epithelial cells are most pronounced in newborn and decline after young adulthood (206).

Extrinsic factors also regulating thymic functions. The suppressive effect of glucocorticoid and the regulator effects of sex hormones is already well known. The thymus involutes after puberty, in addition to thymic hyperplasia seen with gonadectomy (207) occurs not only in young adult animal but also in aged ones. Moreover, this effect is not observed in animals who were previously hypophysectomized (208). These
findings suggest that pituitary hormones are concerned with thymic hyperplasia after gonadectomy. Also, destruction of the anterior portion of hypothalamus brings about a striking thymic hyperplasia (2-or-3-fold in weight as compared with control) (209). The number of splenic T cells and their function decline slightly in relation to the age of the thymus donors (less than a 2-fold difference)(210).

Conventionally, immune responses have been divided into two types: humoral immunity and cellular immunity. Most immune responses involve the participation of both T and B lymphocytes. The effector limb of immune response is mediated either by direct intercellular contact (cytotoxicity) or by soluble molecules, such as antibodies secreted by B lymphocytes and a variety of cytokines secreted by T lymphocytes. B lymphocyte activation and antibody specificity will only occur in the context of T lymphocytes. Therefore the following section will look at changes in T cell number, phenotype and function in the elderly.

**Cell Count and cellular marker**

The number of peripheral lymphocytes in humans decreases slightly (15-20%) or not at all with age (211). In rodents, it was shown that the number of T lymphocyte decreased with age in most of the compartments studied (thymus, peripheral blood, spleen, digestive mucosa) (212,213).

The number of mature T cells (CD3+) lessens with age (214). This is due mainly to a decrease in CD8+ lymphocytes, as CD4+ lymphocytes show little change. In
parallel, a sharp raise in the number of NK cells was reported in the course of aging (215). This augmentation of NK cells appeared to be due to an increase in immature CD2⁺ CD3⁺ T cell population (216). Also, aging is accompanied by an increase in CD45RBlow or PG P1⁺ memory population to the detriment of naive CD45RBhi cells (217,218). Table 2-1 and 2-2 indicate the differences between markers and functions of memory T-cells from naive T cell.

Table 2-1. Summary of markers whose expression differs between naive and memory T cell

<table>
<thead>
<tr>
<th>Molecule</th>
<th>naive CD45R-high</th>
<th>Memory CDw29-high</th>
<th>comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFA-3</td>
<td>1</td>
<td>&gt; 8</td>
<td>Ligand for CD2, involved in adhesion and signaling</td>
</tr>
<tr>
<td>CD2</td>
<td>1</td>
<td>3</td>
<td>Mediates leukocyte adhesion and activation</td>
</tr>
<tr>
<td>LFA-1</td>
<td>1</td>
<td>3</td>
<td>Mediates leukocyte adhesion and signaling</td>
</tr>
<tr>
<td>CDw29</td>
<td>1</td>
<td>4</td>
<td>a part of VLA family of adhesion molecules</td>
</tr>
<tr>
<td>CD35</td>
<td>1</td>
<td>30</td>
<td>Lowest molecular weight form of CD45</td>
</tr>
<tr>
<td>Pgp-1</td>
<td>1</td>
<td>2</td>
<td>No defined function</td>
</tr>
<tr>
<td>CD45R</td>
<td>10</td>
<td>1</td>
<td>Highest molecular weight forms of CD45; Cd45 is implicated in natural killer function and cell mediated lympholysis</td>
</tr>
<tr>
<td>CD3</td>
<td>1</td>
<td>1</td>
<td>Part of antigen-specific receptor complex</td>
</tr>
</tbody>
</table>
Table 2-2. Functional differences between naive and memory T cells

<table>
<thead>
<tr>
<th>Function</th>
<th>naive</th>
<th>memory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation/recall antigen</td>
<td>-</td>
<td>+++++</td>
</tr>
<tr>
<td>Proliferation/CD3 and CD2 mAbs</td>
<td>++</td>
<td>+++++</td>
</tr>
<tr>
<td>Helper function/Ig production(CD4^+ )</td>
<td>-</td>
<td>+++++</td>
</tr>
<tr>
<td>Cytotoxic precursor cells(antigen-specific)</td>
<td>+</td>
<td>+++++</td>
</tr>
<tr>
<td>Cytotoxic precursor cells(alloantigen-specific)</td>
<td>++++</td>
<td>+++++</td>
</tr>
<tr>
<td>Cytotoxic effector cells(alloantigen-specific)</td>
<td>-</td>
<td>+++++</td>
</tr>
<tr>
<td>Adherence to endothelium/augments vascular permeability</td>
<td>+</td>
<td>+++++</td>
</tr>
<tr>
<td>Response to chemotactic stimuli</td>
<td>+</td>
<td>+++++</td>
</tr>
<tr>
<td>Inhibition of proliferation by CD25 mAb</td>
<td>++</td>
<td>+++++</td>
</tr>
<tr>
<td>Proliferation to allogenic cells</td>
<td>++++</td>
<td>+++++</td>
</tr>
<tr>
<td>Proliferation to mitogens</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>Proliferation to autologous cells</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>Induction of suppressor function</td>
<td>++++</td>
<td>+/-</td>
</tr>
<tr>
<td>Inhibition of proliferation by CD7 antibodies</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

2. Immune function

a. Cell mediated immunity

Mitogenesis

Decreases in the proliferation potential is a gradual process that occurs throughout adulthood (219), but it is less severe in the very oldest population (220,233,234). Decline in the proliferation response is partly due to increases in immature T-cell subsets. Decreases in intra cellular calcium is severe in the lymphocytes of elderly subjects (221). Also, aging is accompanied by changes in membrane lipid composition resulting in
lymphocyte proliferation in hypertriglyceridemic subjects. Moreover, macrophage secretions play a positive (IL-1) or a negative (PGE$_2$) role in the regulation of mitogenesis. Lymphocytes of elderly subjects are more sensitive to exogenous PGE$_2$ than those of younger people (218). PGE$_2$ interferes with the production of IL-2 and with the expression of IL-2 receptors. An increase in PGE$_2$ production leads to lower T-cell function. This age-related reduced proliferation persists even when endogenous IL-2 is replaced at levels higher than normally observed. Several laboratories showed that the capacity of IL-2R to bind (219-222) and internalize (222) exogenous IL-2 decreases with age (235,236). In addition the fraction of cells that hyperexpress the high-affinity IL-2R complex decreased (220-221).

**Cytotoxicity**

Cytolytic T lymphocytes (CTL), natural killer (NK) cells, and lymphokine activated killer (LAK) cells represent groups of lymphocytes, with the common characteristic of selectively lysing target cells. Different types of cytotoxic cells appear to be differentially sensitive to the affects of aging. Human CTL effector cell activity declines with age (222). Virus-specific CTL decrease with advanced age (223). In contrast to the spleen, NK cell activity in marine peripheral blood does not decline with age (224). Also, the cytolytic response of murine spleen cells to culture with IL-2 is controversial. Bukenik (225) has observed a decline in IL-2-induced LAK activity with age in humans. IL-2 activated LAK cells seem to be derived predominantly from cells expressing NK
phenotypic markers, or from NK precursors (226-227). The ability of NK or NK precursors to respond to IL-2 with enhanced lytic activity suggests that they may, at least under some conditions, maintain their IL-2 responsiveness with advancing age.

What is the mechanisms responsible for decline of LAK cell activity? Most of the evidence suggests that the frequency of CTL precursors is reduced in aged mice (228,229). Also, much evidence supports the contribution of decreased T helper cell activity i.e. decreased IL-2 production as the causative factor for the decrease in CTL (240). However, Rosenberg (241) observed that MHC class II-restricted CTL response by CD4^{+} T cells cannot be reconstituted either by mitogen-stimulated lymphokine containing supernatant or by exogenous recombinant IL-2. Therefore, although an age-related decrease in IL-2 production undoubtedly contributes to the decline in CTL activity, it does not account for the entire decrease.

While the frequency of lytic cells among the target binding cells is diminished the frequency of antigen specific target-binding is not altered with age in each cell (242). Also the production of lytic mediators by CTL from young and old mice showed a diminished release of granzyme A, a granule-associated serine esterase, in sensitized spleen cells in the old mice (243).

Cytokine production

Cytokines are key regulators of virtually all physiological processes. The emerging information on the properties of individual cytokines and on cell lineage-specific
programs of cytokine production has helped to clarify how immune responses unfold, and how perturbations in the cytokine circuitry can have pathological consequences. Most of my study is about cytokines and their changes which occur with aging.

In general, cytokines are produced transiently after cell activation. They act locally as autocrine or pericrania regulators of cell function, and exert their effects by binding to specific high-affinity receptors on target cells. Cytokine production by T cells is Ag-driven, and it is regulated by the number, type and state of activation of local Ag-presenting cells (APCs), as well as by the clonal frequency and migratory properties of T cells with receptors (TCRs) specific for Ag. At another level, it is regulated by the partitioning of frequency among CD4^+ versus CD8^+ cells, and naive versus memory cells. Aging is accompanied by a diminishing reserve of naive cells and an increasing representation of effector memory or recently activated cells (244). Table 2-3 shows the changes in T-cell subsets during aging.
Table 2-3. Presentation of T-cell subsets during aging

<table>
<thead>
<tr>
<th>Species</th>
<th>Phenotype</th>
<th>CD4+ Cells</th>
<th>CD8+ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>CD45RA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>CD45RO&lt;sup&gt;-&lt;/sup&gt;</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>CD29&lt;sup&gt;-&lt;/sup&gt;</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>LFA-1&lt;sup&gt;-&lt;/sup&gt;</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Mouse</td>
<td>CD45RB&lt;sup&gt;-&lt;/sup&gt;</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>3G11&lt;sup&gt;-&lt;/sup&gt;</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>MEL-14&lt;sup&gt;-&lt;/sup&gt;</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>CD44&lt;sup&gt;-&lt;/sup&gt;</td>
<td>↑</td>
<td>↑</td>
</tr>
</tbody>
</table>

Naive CD4<sup>+</sup> and CD8<sup>+</sup> cells are generally defined as cells that have survived TCR selection in the thymus but have not yet responded to cognate Ag in the peripheral tissues. The initial activation of naive T cells results in a limited program of cytokine gene expression, including IL-2 (245-247), which could serve primarily as an autocrine factor to facilitate clonal expansion and differentiation. However, the cytokine program of stimulated T cells eventually diversifies (245,247) thus yielding more proficient primary effector cells (245,246). Memory T-cells are generated during the primary response and allow the immune system to recognize the antigen on subsequent
challenges. The naive to memory conversion of CD4^ cells is associated with an unchanged potential for IL-2, IL-6, TNF-α and TNF-β synthesis as well as an enhanced capacity for IL-1α, IL-3, IL-4, IL-5, IL-10, IFN-γ and GM-CSF synthesis (245-252).

Table 2-4 shows cytokine production in naive and memory T cells.

Table 2-4. Cytokine production in naive and memory T cells.

<table>
<thead>
<tr>
<th>Lymphokine</th>
<th>Species</th>
<th>Stimulus</th>
<th>Naive</th>
<th>Memory</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>Humans</td>
<td>PHA</td>
<td>+++++</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PHA</td>
<td>+++++</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PHA/calcium ionophore</td>
<td>+++++</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PWM</td>
<td>+++++</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Allogeneic cells</td>
<td>+++++</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD3</td>
<td>+++++</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD3</td>
<td>+++++</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD3</td>
<td>+</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SEA</td>
<td>+++++</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>Mice</td>
<td>PHA/ConA/PWM</td>
<td>+++++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD3</td>
<td>+++++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Allogeneic cells</td>
<td>+++++</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>Rats</td>
<td>ConA</td>
<td>+++++</td>
<td>+</td>
</tr>
<tr>
<td>IL-4</td>
<td>Humans</td>
<td>PMA/calcium ionophore, PHA, CD3</td>
<td>-</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Allogeneic cells</td>
<td>+</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>Mice</td>
<td>CD3, PHA, ConA, PWM</td>
<td>-</td>
<td>++++</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Human</td>
<td>PHA, CD3, Allogeneic cell, PWM, SEA</td>
<td>-</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD3</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>Mice</td>
<td>Mitogens and alloantigen</td>
<td>+</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>Rats</td>
<td>ConA</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>IL-3</td>
<td>Mice</td>
<td>Mitogens and alloantigen</td>
<td>+</td>
<td>++++</td>
</tr>
</tbody>
</table>
There is ample evidence supporting the fact that Th1- and Th2-derived cytokines are involved in a cross regulating network (253). For example, IL-4 and IL-10 can decline responses and along with IL-13, they can inhibit the production of inflammatory cytokines or the killing of intracellular and extracellular pathogens (by activating macrophages).

The dramatic age-related change in frequency of T cell subsets might alter the overall potential for cytokine production by the T-cell pool.

The unstimulated and LPS-stimulated production of interleukin-1β (IL-1) was significantly higher in subjects older than 55 years (254). Most studies of PBL from the elderly often report a marked heterogeneity in the capacity for inducible IL-2 synthesis among donors, with segregation of hypo-responsive and near-normal phenotypes (255). However, there still is not a consensus on the effects of IL-2 production by T cells in aging. Most protocols report that age-related deficits have employed Ag, allo-Ag, sAg, ConA, PHA or soluble anti-CD3 mAB as the cell activator (256-273,219). PHA or ConA preferentially stimulates naive T-cells from normal humans and mice to proliferate and secret IL-2 (257-259,246). In response to these activators, age-related deficits in IL-2 synthesis is expected since there is a deficiency in naive T-cells in older donors. In contrast, studies showing little or no effect of aging on IL-2 production employed
immobilized forms of anti CD3 MAb or PMA plus ionophore or other coactivators (250,252,256,258). IL-3 and GM-CSF are key regulators of hematopoiesis and can enhance the inflammatory response of mature myeloid cells. Both age-related decreases (257,267,269,271) and increases (250,252) in IL-3 production by mitogen-stimulated T-cells have been reported. Age-related decreases of IL-3 production were usually observed when using ConA, sAg, or soluble anti CD3 mAB were used, whereas strong polyclonal stimuli, such as immobilized anti-CD3 mAb or PMA plus ionophore usually reveal an age-related increase in IL-3 production. Flurkey et al (257) showed that increase in IL-3 production in response to ConA or sAg reside almost entirely in the naive T-cell pool. This explains why naive-deficient total T cells from old mice produce less IL-3 when cultured with these activators. The capacity of intact lymphoid tissues to produce GM-CSF decreases with age (267,271,274). These studies showed that dysregulation in cytokine production is due to an increase in memory cells in elderly and induction of Th2 cytokines.

CD4^+ cells are the major regulatory cells of the immune system. Recently, it has been recognized that CD4^+ T cells tend to differentiate, as a consequence of priming into cells that principally secrete IL-4, IL-5, IL-6 and IL-10 (Th2 cells) or into cells that mainly produce IL-2, IFN-\(\gamma\), and lymphotoxin (Th2 cells). Th2 cells are very effective in helping B cells develop into antibody-producing cells, whereas Th1 cells are effective inducers of cellular immune responses, involving enhancement in microbicidal activity of monocytes and macrophages and a consequent increased efficiency in lysing
intracellular microorganisms. The potent action of Th1 cells in enhancing microbicidal action, along with the capacity of the Th2 products, IL-4 and IL-10, to block these activities. Th-2 cells diminish the potency of the cellular immune response initiated by Th1 cells. Cells that mediate such effects are often referred to as suppressor T cells. Our study and other studies show that aging is accompanied by a shift from Th1 cells to Th2 cells and suppression of the immune response to new and common antigens in elderly.

Changes in cytokine production by aging: IL-4 gene expression by CD4+ cells is elevated as a consequence of the naive to memory/effector conversion. IL-5 functions as a growth and differentiative factor for B cells, and is a potent inducer of eosinophilia. IL-5 production is progressive with age and relates directly to the accumulation of memory cells, with an increased capacity for IL-5 synthesis (250,251). IL-6 is produced by many cell types, and it exhibits a very diverse range of activities including inductive roles in T-cell growth, B-cell differentiation, and the acute-phase response. Due to the great diversity of target cell types and various forms of biologic responses that can be stimulated by IL-6, the production of this cytokine must be under strict regulatory control. An unregulated production of IL-6 has been reported to be involved in a variety of chronic inflammatory diseases, lymphoid malignancies, chronic infections, osteoporosis and certain types of autoimmune conditions (275). Wei (276) reported that IL-6 production becomes unregulated as an apparently "normal" consequence of advancing age. Spontaneous production of IL-6 is consistently observed in culture
supernatant of lymphoid cells from both spleen and mesenteric lymph nodes in aged mice, but is absent from supernatant derived from peripheral lymph nodes (277). Dehydroepiandrosterone sulfate, a steroidal hormone, whose endogenous production is known to decline with advancing age can control IL-6 production in aged mice. For this reason, Daynes and Girasole concluded that steroidal hormones may be directly be responsible for regulating IL-6 production (277, 278). IL-10, also produced by many cell types, enhances Th2 responses and down-regulates the Th1 inflammatory responses.

Hobbs MV (251) reported an increase with age in the capacity of CD4+ cells to produce IL-10 in response to anti-CD3 mAb in vitro. He concluded that this change is directly related to the age-associated increase in of memory CD4+ cells. These studies show IL-4, IL-5, IL-6, and IL-10 increase with aging.

Interferon-τ (IFN-τ) is produced mainly by activated T cells and NK cells. Two reports show an age-related increase in IFN-τ. Synthesis of IFN-τ by T cell is caused by a shift in the T-cell subset frequencies (253, 279). Unfortunately, the result from studies on IFN-τ production in aging humans have been controversial. Conclusions have varied between the use of similar culture condition and assay methods (261, 280, 281) and a choice of stimuli (280, 281). Notably, one group observed an age-related decrease in IFN-τ gene expression even when strong polyclonal activators were used (274). Possible explanations could be related to the selection criteria for human donors or the use of blood in human studies versus spleen or lymph node in mouse studies.

TNF-α and TNF-β exhibit a broad range of properties, most notably are acting
as inflammatory mediators. In a study using mice, Hobbs MV (250) reported no change with age in the capacity of TNF-α and TNF-β synthesis by splenic CD4^+ cells activated with anti-CD3 mAb; Fagiolo (282) reported age-related increases in TNF-α production by mitogen-stimulated human pool.

TGF-β is a cytokine with potent suppressive effects on many cells of the immune system. Zhou D (283,284) found that constitutive and Con A-induced TGF-B release by mouse splenocyte increased with age. They observed that concomitant age-related increase in IL-6 production could result in enhanced TGF-B receptor expression by T-cell. This might be an explanation to the served increased sensitivity to TGF-B-mediated effects. With a better understanding of the regulatory interactions among lymphoid cells, it should be possible to improve the degree of immune responsiveness in elderly individual.

Based on the aging model presented in Figure 2-1 age-associated shifts in T cells subset lead to altered T cell lymphokine repertoire and immune dysregulation.
Fig 2-1. Model for age-associated shifts in T cell subset frequencies that lead to altered T cell lymphokine repertoires.
2. Humoral immunity

They found that cell-mediated immunity changes seem to precede humoral changes. As a result primary immunization with T-dependent antigens, is severely impaired (237,238) and rearrangements in immunoglobulin class and subclass levels have been observed that are associated with the appearance of autoantibodies and paraproteins. All these events are related to the development of pathological processes involving antibody production such as chronic lymphocytic leukemia and amyloidogenesis. The decreased antibody response also results in a propensity for infectious disease.

The interactions between B and T lymphocytes lead to the development of humoral responses. Experimental and epidemiological studies have shown that antibody production to common foreign antigens diminishes with age. At the same time, an increased prevalence of auto antibodies occurs. We know that the interaction between B and T lymphocytes leads to the development of T-cell dependent humoral responses. T-helper cells are represented by CD4\(^+\) lymphocytes. The events involved in providing help for B cells can be divided into two steps; T-B cell contact signaling and soluble cytokine-mediated signaling. The main molecule responsible for delivering the contact signal has now been characterized as gp39, the ligand of B cell-specific marker CD40. The CD40 is expressed mainly on CD4\(^+\) lymphocytes from 5-12 hours after activation (285). Binding of CD40 ligand with its counter part on B cells provides the first activation signal making these cells sensitive to soluble cytokines (285). Cytokines produced by T cells can induce immunoglobulin class switching and antibody production.
directly. In particular, IL-2 and IL-10 lead to increased production of IgM, IgG and IgA, whereas IL-4 and IL-13 induce IgE and IgG4 synthesis (286,287). Also IL-6 can amplify Ig synthesis by committed B-cells. IgG and IgA serum levels rise significantly with age, whereas IgM levels remain unchanged (288). Moreover, they observed that among IgG subclasses, IgG 1,2 and 3 showed significant increases, whereas IgG4 did not (288). This increased Ig production in aged individuals is also readily apparent in vitro (289). Serum and secretory IgA both increased in elderly persons, while IgA2 significantly decreased, compared to young subjects (290). Finally oligoclonal Ig was found in 20% of centenarians (288,291).

The number of B lymphocytes in the elderly was reported to increase. However, this increase was not significant (292). Paganelli R et al reported a striking decrease in CD19⁺ B cells both in the 70-83 year old group as well as centenarians (288). They found that the CD19⁺ cells coexpressing the CD5 molecules also decreased with age (288,293). Mariotti S et al found that healthy centenarians had a low incidence of organ-specific auto antibodies (293).

The combination of increased Ig levels with a decrease in the numbers of circulating B lymphocytes suggests that either B cells are confined to the bone marrow or mucosal areas, or that they produce more Ig per cell (possibly in response to stimuli provided by activated T lymphocytes). Even after booster immunization, levels of specific antibodies in the elderly did not reach those of young healthy volunteers in their primary response (294).
Along with other cellular and soluble factors, IL-4 plays an obligatory role in the production of IgE (286,295) as well as IgG4 (286,288). IgG4 levels are not increased in elderly subjects (288,291) and serum IgE levels, along with manifestation of IgE-mediated hypersensitivity (296), usually decreases with age (297).

Rowley MJ. and his colleagues (298) found that the serum antibody to foreign bacteria decrease with age while the serum autoantibodies increases with age. Weskeler MD. (195) found that old mice immunized with sheep RBC, just as humans immunized with influenza, make few antibodies to the nominal antigen. The total number of Ig-producing is comparable in old and young mice. However, the repertoire of antibody reactivities is different. Young mice make antibodies to foreign antigen while old mice tend to make auto antibodies. Hu A. (299) showed that CD5- B-cells, which produce antibodies to most foreign antigens, provoked a much lower antibody response in old compared to young mice. Antigens that stimulated CD5+ B cells, which produce many auto antibodies, provided an antibody response in old mice that was equal to or even higher than that in young mice.

B-cell function is also regulated by anti-idiotypic autoantibodies (300). Anti-idiotypic auto antibodies increase with age: These antibodies can down-regulate the production of antibodies to foreign antigens (300,301). The increased auto-anti-idiotypic response in old mice is regulated by their long-lived peripheral T cells.
3. Gut Mucosal Immunity

Statistical analysis exhibits a 400-fold increase in the mortality rate due to gastrointestinal infectious disease in Japanese people over the age of 75 (302). The incidence of colonic cancer also increases significantly in the elderly (303). One explanation for this increase is age-dependent failure of immune surveillance.

The mucosal immune system plays a dual role in the lymphoid system: It mediates the cell-mediated immune responses at the gut mucosal site, and also in the entire body. Since the gastrointestinal tract is an entry way to the body, it affects the quality and quantity of the antigens gaining access to the immune system as a whole.

Peyer's patches (PP) which are organized collections of small lymphoid follicles in the small intestinal mucosa and submucosa, serve as the first line of defense in gastrointestinal tract against offering antigens. The PP increase in numbers in the human small intestine during the first 20 years of life, reaching a peak of about 200 patches per intestine. The number of these patches levels off at 100 patches per intestine between 70 and 95 years of age (304). Lymphoid cells isolated from aged PP and mesenteric lymph nodes (MLN) show a greater reduction (40% greater) in magnitude than those from non gut mucosal lymphoid tissues, such as spleen (305). Kawanishi H showed prominent decrease in the number of Th in age PP and MLN, despite much lower decline in Tc (306). Age-associated changes in B cells of gut associated lymphoid tissue (GALT) however, are minor and not physiologically significant (306).

T lymphocytes isolated from PP and spleens of old mice have a markedly
decreased capacity to proliferate. Also, the production of antibody requiring T and B cell collaboration is significantly impaired in older animals (306,307). The synthesis and secretion of mucosal IgA is impaired (308). The excessively activated aged gut mucosal B cells evokes a decrease in the ratio of dimeric IgA to monomeric IgA. Total IgA becomes excessive, where the amount of dimeric IgA remains unchanged. In addition, the functional capacity of mucosal IgA appears to be diminished. The mechanisms are probably due to multistep defects: in mIgA synthesis; at an immunoglobulin heavy chain rearrangement level of \( \Gamma \) to \( \alpha \), a transcriptional level, regulating IgA mRNA synthesis, nucleocytoplasmic transcription of IgA mRNA and a ribosomal translational level in production of IgA immunoglobulin protein.

Oral tolerance such as systemic immunologic hyporesponsiveness to mycobacterium paratuberculosis Ag in aged mice is impaired. These age related changes, manifested as hyperactive humoral response to the enteric microbial Ag, are due, at least in part, to hyperfunction of Ag-specific Th cells, despite the reduction in the number of Th cell (309). In vivo IL-2 administration considerably restored the age-associated oral intolerance to whole mycobacterial Ag in mice, particularly by inducing the effector function of Ag specific Ts cells (310).

Cancer

In general, tumors occur more frequently in older people. However, many of the common tumors appear to be less malignant in older hosts. Weak immunogenic tumors
such as B16 melanoma or Lewis Lung Carcinoma (3LL) grow more slowly in old mice, but more immunogenic tumors such as methylcholanthrene-induced fibrosarcoma grow more rapidly (311). Breast, colon, prostate and lung carcinoma are less malignant in old people (311).

What are the mechanisms for the differences in tumor behavior with aging? Fidler demonstrated that tumor growth of weak immunogenic tumors is correlated with the level of immune competence and growth is slower in mice after sublethal irradiation or hydrocortisone treatment (312). He also found that the survival of these tumor cells decreased in immune-deficient mice (313). These observations are consistent with the hypothesis of Prehn and colleagues (313) which states that a loss in immunity, under certain circumstances, inhibits tumor growth. T-lymphocytes produce angiogenesis factors (314) in supernatant from cultured human mononuclear cells (315) or rodent splenocytes (316) and these factors stimulate normal or tumor cell proliferation. Also, human monocytes produce a factor that is heat stable, acid labile and stimulatory for tumor growth (315). An age-associated reduction in those factors could account for the observed reduced tumor growth.
CHAPTER III

Effect of long term antioxidant nutrient supplementation in the elderly

Sussan K. Ardestani, Steven M. Wood, Bailin Liang, Paula Inserra, Anabell Castro, Carla Backham, Cleamond Eskelson, Ronald R. Watson*

Arizona Prevention Center, University of Arizona Tucson Arizona

*To whom correspondence should be sent: Ronald R. Watson, Ph.D., Arizona Prevention Center, University of Arizona, Tucson, Az 85724, USA. Phone (520)626-4357, Fax (520)626-2030.
Abstract:

A randomized double-blind placebo-controlled trial was conducted to determine the effects of multiple antioxidant supplements (800 IU vitamin E, 30 mg β-carotene, 500 mg vitamin C, and 70 mg L-glutathione) on plasma lipid, lipid peroxidation and immune function. Subjects aged 67-82 years were randomly assigned to placebo (n=7) or antioxidant nutrient (n=10) groups. Lipid profile, lipid peroxidation and immune function were measured at baseline and monthly for 6 months (during antioxidant treatment) and 2 month after supplementation ceased. Although plasma triglyceride, phospholipid, and cholesterol levels were unchanged plasma thiobarbituric acid reaction levels was significantly (P < 0.05) decreased during treatment. The percentage of CD3+, CD4+ cells, CD8+, and CD56,CD16+ cells were unchanged during the study. T and B cell proliferation did not change. Interleukin-2 (IL-2) production by PHA activated T-cells significantly (P < 0.05) increased in month 3 and 4 of starting supplementation. IFN, and IL-6 production, by activated cells, and plasma concentrations of IL-6 did not change in the supplemented group.
Introduction:

The aging process is accompanied by a corresponding decline in immune function (317). It is thought that human disease contributes to making elderly persons more susceptible to infection (320).

The best documented evidence of age-related immune dysregulation is increased levels of autoantibodies that occur with age (318), while serum antibodies to foreign bacterial antigen decline (319,237,238). Cytokine production by Th1 cells, which stimulates cellular immunity, is compromised, while the production of cytokines attributed to Th2 cells is unaffected. Thus aging is associated with a progressive alteration in many physiological processes including those of the immune system.

Reactive oxygen molecules are formed as a natural consequence of biochemical and immunological activities. Reactive oxygen can damage nucleic acids, proteins (321,322) and unsaturated bonds of fatty acids in lipid membranes, thereby altering membrane structure and function (321). The most obvious consequence of membrane lipid peroxidation is the perturbation of various cellular and organelle membrane functions such as transport, maintenance of ionic gradients, receptor mediated signal transduction, plus many others (324,325). Membrane fatty acid modification is an age-dependent phenomenon that is thought to be related to the extent of lipid peroxidation (326,327). Lipid peroxidation can induce unregulated production of transcriptional activator proteins like Nuclear Factor-Kappa B (NF-KB), which increase interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and IL-10. This pattern of cytokine
dysregulation could be a consequence of Th1 cell suppression resulting in poor cell mediated cytotoxicity, and reduced antibody production and cell proliferation. This decline in immune function is associated with an increase in infectious disease and cancer, which is common in the elderly.

Reactive oxygen can also cause damage to DNA and proteins, which may also lead to cancer and increased cell death. Rapidly dividing cells show the least evidence of aging (328). On the other hand, non dividing cells such as nerve and lymphocytes undergo obvious signs of aging. The accumulation of DNA damage is associated with a decline in gene expression, as well as decline in cellular and organ function. An accumulation of DNA damage appears to increase the rate of aging. Lipid hydroperoxide can be enzymatically reduced to alcohol or fragmented to malondialdehyde (MDA) or other aldehydes. Yu (329) showed an age-related increase in MDA and other byproducts of lipid peroxidation.

Antioxidant defence and DNA repair mechanisms exist to protect cells, however, both of these mechanism are malfunctioning in elderly (323,239). Antioxidants, vitamin E, vitamin C, β-carotene and glutathione, can remove reactive oxygen species and protect the body from oxidative damage.

Vitamin E,C and β-carotene react with free radicals and singlet molecular oxygen ($1O_2$). Vitamin E is the major peroxyl radical scavenger in biological lipids, such as membranes or low-density lipoprotein. Vitamin C prevents oxidative damage in aqueous compartments and can reduce tocopheroxyl radicals enabling them to be recycled. β-
carotene exerts antioxidant functions in lipids by quenching free radicals and \( \text{LO}_2 \). Glutathione is directly involved in destruction of ROS because it participates in maintaining the reduced forms of ascorbate and tocopherol.

Cancer prevention by \( \beta \)-carotene, vitamin E and glutathione supplementation has been demonstrated in experimental animals (330-334). Recently, mixtures of \( \beta \)-carotene and vitamin C were found to be more effective than \( \beta \)-carotene or vitamin C alone in preventing cancer growth (335,336). A mixture of \( \beta \)-carotene, vitamin E, glutathione, and vitamin C significantly reduced oral cancer in hamsters (337). Antioxidants function by lowering DNA damage, malignant transformation, normal cell physiological function and improving immune surveillance.

The aim of the current study was to evaluate directly, and indirectly, the effect of antioxidant nutrients in the elderly. We directly measured proliferation, cytokine production and natural killer cell cytotoxicity of peripheral blood mononuclear cell cultures from old subjects after addition of different antioxidant nutrients. Indirectly, the effects of antioxidant supplementation on lipid peroxidation and immune function were measured in these subjects.
MATERIALS AND METHODS

Blood Collection and Lymphocyte Preparation

In Vitro Study: Blood was collected from 8 healthy volunteers in 10 ml heparinized vacutainer tube (Becton Dickinson, Ruthford, NJ). Peripheral blood mononuclear cells (PBMC) were isolated by placing whole blood with equal amounts of sterile phosphate buffer solution (PBS, 0.01 M phosphate and 0.15 M NaCl PH:7.4) and carefully layering it upon Lymphocyte Separation Medium (Organon Teknika Corp. Durham NC). The tubes were centrifuged at room temperature at 1900 rpm for 20 min. The PBMC were aspirated washed with PBS, centrifuged and resuspended in RPMI 1640, pH 7.2-7.4 (Gibco Laboratories, Grand Island NY) containing 10% fetal bovine serum (Intergen, Purchase NY), 100 units/ml penicillin, and 100 μg/mL streptomycin (complete medium, CM). Lymphocyte cell concentrations were adjusted to 4 X 10^6 cells/ml.

Nutrient Solution for in vitro application

Since β-carotene is not soluble in water, a novel technique utilized by Bertram JS. (338) and Cooney RV. (339) was applied in which 4 gr of β-carotene (Flunka Chemicka-Biochemika, Buchs/Switzerland) was solubilized in 35 ml of tetrahydrofuran (THF) (Fisher Chemical, Fair Lawn, NJ) and diluted to one liter in complete media, aliquated and frozen. A solution containing the same THF concentration was used as a control. During incubation, stock solution tubes diluted with complete medium yielding concentrations of 10, 1, 0.01, 0.002, and 0.001 μmoles/L. Final β-carotene cell culture
concentrations were 2.5, 0.25, 0.025, 0.0005, and 0.00025 /tmoles/L (0.330 \mu{mol}/L, normal plasma concentrations (249).

Vitamin E, 0.000189 \mu{g} of d-alpha-tocopherol (Sigma, St. louis, Mo) was dissolved in 1 ml 100\% ethanol. The solution was then diluted to 100 ml complete medium and filtered. Similar tubes containing identical ethanol concentrations were prepared to determine the effects of ethanol alone. At the time of incubation, stock solution tubes were thawed and diluted to give the final cell culture concentrations of 2.5, 0.25, 0.025, 0.0005, and 0.00025 \mu{moles}. These were based on 0.00001 \mu{mol}/l, normal plasma concentrations (340), 0.000043 \mu{mol}/L, plasma concentration of individuals supplemented with 800 mg vitamin E, and 0.002 \mu{mol}/l pharmacological concentrations (236). Also 2.46 gr L-glutathione was dissolved in water and filtered. Aliquots were frozen. On the day of the experiment, tubes were thawed, utilized, and discarded preventing solutional variations. At the time of incubation, stock solution tubes were thawed and diluted with complete medium to yield final concentrations 5, 2.5, 1.25, 0.5, 0.25, 0.125 and 0.025 mmol. A concentration of 2 nmol/ 10^7 lymphocyte was reported as the minimal intra cellular glutathione necessary to sustain a normal activation response (341).

Cell Culture for Antioxidant Application in vitro

Effectors cells (lymphocytes) were placed in U-bottom cell culture plates (Falcon 3077, Becton Dickinson, Lincoln Park, NJ) in triplicate at 1 x 10^5 cells/wells for NK cell
cytotoxicity analysis. Cells (2 x 10^5/well) were placed in flat bottom microtiter plates (Falcon, Lincoln Park, NJ) for cytokine stimulation. Appropriate wells received complete medium (Control, without addition of antioxidants), different concentrations of β-carotene, glutathione, or vitamin E. Cells were incubated for 24 hours at 37°C, 5% CO_2 in a humidified environment. NK cell cytotoxicity was then determined, in addition to IL-6, and IL-8 cytokine secretion and mitogenesis performed.

**Natural Killer Cell Cytotoxicity**

Natural Killer (NK) cell cytotoxicity was measured by fluorescence (342). This method measures the fluorescent dye, Calcein AM (Molecular Probes, Eugene OR), remaining in the live target cells, K562 myelogenous leukemia cells using the Pandex Fluorescence Concentration Analyzer (FCA) (IDEX, Portland, Maine). The NK sensitive cell line, K562, is highly sensitive as an in vitro target for NK cell cytotoxicity (343). Target cells were washed twice with PBS and labeled with Calcein AM. Cell concentrations were performed in triplicate. Six wells contained either CM (spontaneous release) or 2% Triton X 100 (maximum release). Target cells (K562 cells) were placed in each well (4 x 10^3 cells / well) and spun at 800 rpm for 3 minutes to facilitate cell to cell contact. Cell culture plates were incubated at 37°C, humidified, 5% CO_2 incubator for 3 hours. After incubation 10 μl of 1% inert fluoricon polystyrene assay particles were added to each well of a harvesting plate (IDEX, Portland, MN). Seventy μl aliquot from each well were transferred to a Pandex Harvesting Plate (IDEX, Portland MN).
Epifluorescence of each well was automatically determined at 486/533 nm excitation/emission wavelengths for Calcein AM using the Pandex FCA. Specific cytotoxicity was calculated as follows.

\[
\text{Mean Spontaneous Release} - \frac{\text{Mean Experimental Fluorescence}}{\text{Mean Spontaneous Release} - \text{Mean Maximum Release}} \times 100
\]

Cytokine Stimulation for in vitro supplementation studies

After overnight incubation with respective nutrients, 0.1 ml lipopolysaccharide (LPS) 20 ng/ml was added to stimulate production of IL-6 and IL-8 while 20\(\mu\)g/ml was added for the induction of TNF-\(\alpha\). Phytohemagglutinin (PHA) 10 \(\mu\)g/ml was added for induction of IL-2 and IFN-gamma. Certain wells were identified as background levels which received CM or solvents instead of mitogens. Cells were incubated at 37°C, humidified, 5% CO\(_2\) incubator for 24 or 72 hours. After incubation, the plates were centrifuged for 10 minutes at 1200 rpm. Supernatant fluids were collected and stored at -20°C until analyzed.

ELISA Assay for Cytokines

Kits for IL-6, TNF, IL-2, and IFN-gamma were purchased from Genzyme (Cambridge, MA). All cytokine detections were performed by ELISA assay as described below (344). Briefly, a 96 well tissue culture plate (Pynatech, Sallyfield Circle, Virginia) was coated
with capturing mouse monoclonal antibody specific for human (hu) IL-6, IL-2, IFN-\(\gamma\) or TNF-\(\alpha\). Antibodies were diluted in 0.1 M bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. After this and all subsequent steps, plates were washed three times with PBS containing 0.05% Tween 20 (PBS-Tween). Plates were then blocked with 2% bovine serum albumin (Sigma A7030) in PBS at 37°C, humidified, 5% CO\(_2\) for 2 hours. Supernatant fractions and serially-diluted recombinant (r) huIL-6, IFN-gamma, huIL-2, or huIFN-gamma standards were added to wells and incubated at 37°C, humidified, 5% CO\(_2\) for 1 hour. Plates were washed and 50 \(\mu l\) of diluted biotin-rabbit antihuman IL-6, IFN-gamma, IL-2 or TNF-\(\alpha\) polyclonal antibody detecting agent was added to all wells. Plates were incubated for 20 min and colors were developed with a substrate solution (2,2-azino-bis [3-thylbenzthiazaline]-6-sulfonic acid in citrate buffer, 0.1 M, pH 4.2, containing 0.03% H\(_2\)O\(_2\)). Optical density was measured at a wavelength of 405 nm by Titertek Multiscan (Flow Lab, Mclean, VA).

In vivo Study Populations: Blood Collection and Lymphocyte Preparation

Healthy adult volunteers over age 60 participated in this study. Subjects signed informed consent and the study was approved by the University of Arizona Human Subjects Committee. Participants were screened to determine eligibility by obtaining medical histories and nutrient supplementation information, as well as their own assessment of current physical well being. Participants who were taking high levels of nutrients in supplements, or who had chronic illnesses such as heart disease, kidney disease, cancer,
or diabetes were excluded from the study. Participants were randomly assigned to receive placebo, 1.8 mg β-carotene/day, or 30 mg β-carotene plus 70 mg glutathione plus 500 mg vitamin C plus 800 IU vitamin E/day. Participants were given a one month supply of capsules and instructed to take the supplements with meals. Toxicity was monitored monthly by clinical evaluations and monthly questionnaires. Any capsules not taken were to remain in pill packet and returned on monthly appointments, therefore allowing an approximate indication of compliance. Supplements were taken for six months and then discontinued for 2 months.

Blood was collected in a 10 ml vacutainer containing sodium heparin at initiation of the study (baseline) and then monthly for 6 months after initiation of supplementation. An additional blood sample was obtained two months post cessational supplementation.

**Dietary and Nutrient Analysis**

Plasma was placed in microcentrifuge tubes and kept in the dark at -70°C until analysis. Each subject completed an ACC Food Frequency Questionnaire that was developed and validated at the College of Medicine, Department of Family and Community, University of Arizona.

**Mitogenesis of lymphocytes**

Lymphocyte proliferation was determined by thymidine incorporation (346). Briefly, 0.1 ml of lymphocytes (1 x 10⁶ cell/ml) were cultured in a 96 well flat-bottom culture plates
(Falcon) with PHA (5 mg/ml) in CM at 37°C, and 5% CO₂ for 36 hours. They were then pulsed with ³H-thymidine (0.5 mCi/well, New England nuclear, Boston, MA). After 6 hours, they were harvested by a cell sample harvester (Cambridge Technology, Cambridge MA). Radioactivity was determined by a liquid scintillation counter (Tri-Carb, 2200CA, Packard, Laguanahills, CA). Data was presented as counts per minute (CPM).

**Cell Preparation for Flow Cytometry Analysis**

Peripheral blood mononuclear cell (PBMC) (1 x 10⁶ cells/tube) were placed in four sterile 3 ml plastic tubes. 20 μl of specific simultest monoclonal antibodies by Becton Dickinson, San Jose, CA Simultest T & B (Anti-leu-4 FITC & Anti-leu-12 PE); Simultest CD4/CD8 (CD4-FITC & CD8-PE) Simultest CD3/CD19 (CD3-FITC CD19 PE); or Simultest CD3/CD16+CD56 (CD3-FITC & CD16 PE + CD56 (leu-4/11c+19)-PE) was added to labeled tubes. The last tube in the series was the control. these cells received 20μl sterile PBS. Cells were slowly vortexes in the dark and placed on ice for 30 min. Cells were centrifuged and washed twice in cold PBS after which they were resuspended in 0.5 ml of 1% paraformaldehyde. Cells were analyzed on FACStar plus flowcytometer (Becton Dickinson, Mountain View, CA) and Consort 40 software (Becton Dickson, Mountain View, CA) in a bivariate fashion. Cells were gated for lymphocytes by size and granularity. Fluorescence was determined at 488 nm excitation and 530 or 575 nm emission for phycoerythrin (PE) and fluorescein isothiocyanate (FITC),
respectively. Data were presented as percent of each population.

**Phospholipid analysis:**

5 ml of folch solution (2:1 (v/v) chloroform: methanol) was added to 0.1 ml of plasma. The solution was centrifuged and supernatant (containing different lipids) was extracted.

Phospholipid was evaluated using method of the Raheja (347). Briefly, 1 ml lipid extract was dried in a steady flow of air at 50°C and was redissolved in 0.5 ml chloroform and 0.4 ml of a color reagent. We made color reagent by dissolved 16 g of ammonium molybdate in 120 mL of water, yielding solution I. Then we add 80 mL of solution I to 40 ml of concentrated HCl and 10 ml of mercury, shaken for 30 min and filtered yielding solution II. 200 ml of concentrated H₂SO₄ was added carefully to 40 ml of solution I. The resultant solution was added to solution II yielding solution III. 45 ml of methanol, 5 ml of chloroform and 20 ml of water was added to 25 ml of solution III yielding color reagent.

The tubes were then placed in boiling water for 1.5 min. After cooling to room temperature, 4 ml of chloroform were added and absorbance was measured at 710 nm against a blank. Phospholipid concentration was found by comparing samples to different concentrations of standards (diheptadecanoyl phosphatidyl choline).

**Cholesterol analysis:**

Cholesterol was determined using the method of Zak (348). Briefly, 1 ml of plasma lipid
extract was dried at 50°C in a steady flow of air. The samples were then redissolved in 3 ml glacial acetic acid then 2 ml of concentrated sulfuric acid was added and mixed. The solution was allowed to cool to room temperature, and the absorbance determined at 560 nm. Cholesterol concentration was determined using a standard-cholesterol-curve, obtained by least squares analysis. This derived linear equation was used for determinations the cholesterol-amounts of the unknown samples.

**Lipid peroxidation analysis:**

**fluorescence, and diene conjugated analysis**

Diene conjugates were analyzed by adding 2 ml folch reagent to 1 ml plasma folch extract. These solutions were washed twice with water, 0.5 ml of 100% ethanol was next added and the solution evaporated under nitrogen at 55°C. The residue was redissolved in hexan. The diene conjugates were measured at a absorbency of 232 nm. The results were recorded as absorbency per dl of plasma. The amounts of fluorescent products were expressed as the fluorescence amounts obtained at the emission wavelength of 470 nm after activation at 395 nm. The results were recorded as absorbency amounts of emission amounts per dl of plasma.

**Malondialdehyde (MDA) determination (thio barbituric acid reaction (TBR) analysis)**

The amount of plasma MDA formed due to lipid peroxidation (351), was quantify by
thiobarbituric acid reaction as described by Yagi (351). 4 ml of 1/12 N sulfuric acid was added to 20 µl plasma and mixed. 0.5 ml of 10% phosphotungstic acid was added and centrifuged. The supernatant was discarded, and the sediment was mixed with 2.0 ml of 1/12 N sulfuric acid and 3.0 ml 10% phosphotungstic acid. The mixture was centrifuged at 3000 rpm for 10 minutes. The sediment was suspended in 2 ml of distilled water and 1.0 ml of TBR reagent (a mixture of equal volumes of 0.67 % TBA and glacial acetic acid) was added. The reaction mixture was heated for 60 minutes at 95°C in a water bath. After cooling with tap water, 2.5 ml of n-butanol was added and mixed. After centrifugation at 3000 rpm for 15 minutes, the n-butanol layer was aspirated for fluorometric measurement at 553 nm with 515 nm activation. Fluorescence intensity of different concentrations tetra methoxy propane which breaks down to MDA was used as a standard.

STATISTICAL ANALYSIS:
Statistical analysis was performed using Number Cruncher Statistical System (345). Statistical differences (p<0.05) were determined by one-way analysis of variance (ANOVA) with respect to percent changes at each concentration. When statistical differences were determined, Fisher Least Significant Differences was employed to identify those concentrations of nutrients added in vitro which were significantly different.

The effects of supplementation were presented as a percentage change from pretreatment
levels. The differences were then determined for each time point and compared with the placebo group. Statistically significant differences (p < 0.05) were determined by one-way analysis of variance (ANOVA) with respect to treatments at each time point. Also, ANOVA was employed to note differences within groups over the time course of the study. When statistical differences were determined, Fishers Least Significant Differences was used to identify those groups which were significantly different from the placebo group.
Results:

Subject characteristics at time of drawing blood are shown in table 3-1 for in vitro and in vivo study.

Table 3-1. Characteristics of participants by groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Age</th>
<th>BMI</th>
<th>Smoke</th>
<th>Alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>6</td>
<td>71.0±3.21</td>
<td>24.94±2.</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>antioxidant</td>
<td>10</td>
<td>70.6±6.15</td>
<td>24.96±4.</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>nutrient</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

BMI, Body Mass Index = The weight (kg) / height^2 (m)

Alcohol, some of them had one drink per week.

16 elderly women completed the study. Subjects took all of the supplements each month.

No toxicity or adverse effects were reported by subjects for treatment or placebo group. But unfortunately not all subjects were available every month. The number of individual in the placebo and supplemented group in each month is shown in Fig.3-1.

Modification of lymphocyte function in vitro by application of antioxidants:

Incubation with 0.1-20 μmol of L-glutathione did not change NK cell cytotoxicity, IFN-γ,
or IL-2 production significantly (data not shown).

0.001-10 μmol of d-α-tocopherol did not change NK cell cytotoxicity, mitogenesis, IL-2, or IFN-τ production (data not shown).

0.05-10 μmol of β-carotene increased NK cell cytotoxicity in comparison to the THF control. No changes were observed when compared to untreated cells. Mitogenesis, IFN-τ, and IL-2 production remained unchanged.

**Effect of antioxidant nutrients on lipid profile and lipid peroxidation**

A change with time occurred for phospholipid and cholesterol in both treated and placebo groups. The antioxidant nutrient supplements however, did not differ from placebo triglycerides, phospholipid or cholesterol levels (Fig. 3-2). A significantly (P < 0.05) decrease was observed in the amount of lipid peroxidation, as measured by TBRs after one month treatment as compared to baseline levels (Fig. 3-3) and stayed low until the end of the study. These data show antioxidant supplementation has a short term effect. However it did not change two other lipid peroxidation parameters, conjugated diene and fluorescence (fig. 3-4). Plasma triglyceride, phospholipid, and conjugated diene levels were significantly lower in young (25-35 years old) subjects as compared to older subjects.

*Modification of lymphocyte function in vivo by application of antioxidants:*

**Effect of antioxidant nutrients on T-cell population**
The percentage of CD3⁺, CD4⁺, CD8⁺, and CD56 / CD16⁺ cells did not significantly change at 2 or 6 months compared to placebo group and baseline (Fig. 3-5,3-6,3-7.3-8).

**Effect of antioxidant nutrients on T-cell function**

T- and B-cell mitogenesis did not change with supplementation (Fig. 3-9,3-10).

Production of IL-2 by activated T-cells are increased after 3 months and stayed higher than placebo and pretreatment values for two months (Fig. 3-11). However supplementation had no effect on IFN production (Fig. 3-12).

Production of IL-6 by activated PBMC did not change (Fig. 3-13), also plasma concentration of IL-6 did not decrease in supplemented group (Fig. 3-14).

NK cell cytotoxicity was higher in elderly subjects than young adults and did not change with supplementation (Fig. 3-15).

**Discussion**

Antioxidant nutrients in vitro did not change mitogenesis or cytokine production by lymphocytes. Other authors (158-163) have found that 2000-10000 μmol of glutathione can increase mitogenesis and 5000 μmol can increase IL-2 production and decrease PGE₂ production, or 0.002 μmol of α-d-tocopherol can increase mitogenesis and 0.1-1 μmol of β-carotene can increase cell cytotoxicity (81,113,121,122). These results, however, were not confirmed in the current study. Different forms of antioxidant supplement preparations or varying study lengths may have contributed to these differences.
Additionally, different mitogens, and varying age of subjects may have also contributed to these discrepancies. However, the best concentration of antioxidants may have decreased mitogenesis because cells require small amounts of free radicals for proliferation and cytokine secretion. In elderly people the majority of immune cells are memory cells which may respond differently than those from young adults.

In vivo antioxidant supplementation did not affect fluorescence or conjugated diene but it decreased the amount of plasma MDA. It did not change subpopulations of lymphocytes and it did not significantly increase Th-1 cytokines or decrease Th-2 cytokines. Epidemiologic studies show a high incidence of cancer and cardiovascular disease to be associated with low concentrations of antioxidant nutrients (352,349). High intakes of foods rich in β-carotene, vitamin E, or vitamin C have been leaded to a reduction the risk of some cancers and cardiovascular disease (353). These studies, however, do not distinguish between the effects of antioxidant nutrients or naturally occurring compounds in foods that may contribute the protective effects.

The cancer prevention activity of β-carotene, vitamin E and glutathione has been demonstrated in experimental animals (330-334). Also the concentration of vitamin E in liver, muscle, and heart of mice infected by retrovirus, decreased. Vitamin E supplementation in these mice delayed death by increasing immune function (354). Thymic involution is a consequence of aging. In addition elderly persons have a change of naïve cells to memory cells. Immune dysfunction in the elderly appears to be related to these occurrences. The immunological decline mainly occurs in the T cell-dependent
immune system and is generally associated with an increase in susceptibility to infections and autoimmune diseases (288,293). A pronounced age-related decrease in CD8^+ T cells as compared to CD4^+ T cells was observed (217,218,244). Th1 cells are decreased, while Th2 and NK cells increased (346). Age-related changes in functional heterogeneity of CD4^+ T cells may account for the underlying deficits in immune function that accompanies aging (356). Cell mediated immunity, IgA and IgG production decrease and humoral immunity and Th-2 cytokines like IL-6, IL-10, TNF-α, IgM, IgE and autoantibodies increase. As we demonstrated, serum IL-6 and NK cell cytotoxicity, are increased in our elderly population as compared to young adults. IL-2 and IFN-γ production by activated T cells are decreased in the elderly as compared to young subjects. Antioxidant nutrient supplementation did not change the repertoire of T cells in our study. Supplementation after puberty may, in fact, prevent thymic involution. However, supplementation can not change the activity of each T-cell population and cannot shift cytokine production from Th2 to Th1 sub populations. No effects on T-cell proliferation were detected. Therefore, the increase in IL-2 production during antioxidant supplementation by activated T-cell was not sufficient or, perhaps, response of these cells is atypical. Proliferation of old impaired T cells can not fully recover by administration of excessive amounts of IL-2 (357). Formation of second messengers such as IP3 and DAG are lower in old as compared to young subjects. T cells and signals from T-cell receptors cannot be efficiently transmitted to form phospholipase C (which is responsible for the formation of 2nd messengers) (358). The modification of
membrane exchange (Ca\(^{2+}\)) in lymphocytes of elderly subjects may also be responsible for decreasing cell proliferation (350). By trapping reactive oxygen species (ROS), antioxidants prevent cell membrane damage. Although antioxidant supplementation decreased the amount of lipid peroxidation products, no changes in mitogenesis of mitogen-stimulated T cells was detected. Moreover, macrophage secretions can play a positive (IL-1) or negative (PGE\(_2\)) role in the regulation of T cell proliferation. IL-1 secretion does not change in the elderly (359-360), while production of PGE\(_2\) increases in aged mice (361). Moreover, lymphocytes from elderly subjects are more sensitive to exogenous PGE\(_2\) than those of younger persons (362). Antioxidant nutrients can decrease PGE\(_2\) production and improve T cell function. PGE\(_2\) interferes with the production of IL-2 and expression of the IL-2 receptor (363). Thus, antioxidant nutrients can alter antigen presenting cells.

An unregulated production of IL-6 has been reported in aged subjects (276). Aging is accompanied by an unregulation of some nuclear factors such as NF-KB (necessary for cytokine secretion of IL-6, and TNF-\(\alpha\)). Antioxidant nutrients did not significantly decrease IL-6 production. No significant changes were observed which may be due to the small sample size which could not control for the large variation among human subjects (different genetics, environment, and previous immunizations).
Fig. 3-1: The number of individuals in the placebo and supplemented group in each months.

Fig. 3-2: Effect of antioxidant nutrient supplementation on plasma concentration of lipid before during and 2 month after supplementation. The values are mean±SD.

Fig. 3-3: Effect of antioxidant nutrient supplementation on plasma concentration of TBRs before during and 2 month after supplementation. The values are mean±SD. (b) P value compare to the control in the same group. *P < 0.05.

Fig. 3-4: Effect of antioxidant nutrient supplementation on plasma concentration of Conjugated diene and fluorescence before during and 2 month after supplementation. The values are mean±SD.

Fig. 3-5: Effect of antioxidant supplementation on percentage of CD3+ cell before and after 2 and 6 months of supplementation. The values are mean±SD.

Fig. 3-6: Effect of antioxidant supplementation on percentage of CD4+ cell before and after 2 and 6 months of supplementation. The values are mean±SD.

Fig. 3-7: Effect of antioxidant supplementation on percentage of CD8+ cell before and after 2 and 6 months of supplementation. The values are mean±SD.

Fig. 3-8: Effect of antioxidant supplementation on percentage of CD3/CD56CD16+ cell before and after 2 and 6 months of supplementation. The values are mean±SD.

Fig. 3-9: Effect of antioxidant supplementation on PHA-induced T cell proliferation before, during and 2 month after supplementation. The values are mean±SD.

Fig. 3-10: Effect of antioxidant supplementation on LPS-induced B cell proliferation
before during and 2 month after supplementation. The values are mean±SD.

Fig.3-11: Effect of antioxidant supplementation on IL-2 production by PHA-activated T cell before during and 2 month after supplementation. The values are mean±SD. (a) P value compare to the same supplementation in young mice. (b) P value compare to the control in the same group. *P < 0.05. **P < 0.005.

Fig.3-12: Effect of antioxidant supplementation on IFN-γ production by PHA-activated T cell before during supplementation. The values are mean±SD.

Fig.3-13: Effect of antioxidant supplementation on LPS-induced PMNC IL-6 production before during and 2 month after supplementation. The values are mean±SD. (b) P value compare to the control in the same group. *P < 0.05.

Fig.3-14: Effect of antioxidant supplementation on Plasma concentration of IL-6 before during and 2 month after supplementation. The values are mean±SD.

Fig.3-15: Effect of antioxidant supplementation on NK cell cytotoxicity before during and 2 month after supplementation. The values are mean±SD.
Number of subjects in each month:

- Antioxidant
- Placebo

Figure 3.1
Fig: 3-2

Cholesterol Placebo
Cholesterol Antioxidant
Phospholipid Placebo
Phospholipid Antioxidant
Triglyceride Placebo
Triglyceride Antioxidant

Cholesterol, Phospholipid, and Triglyceride (mg/dl)

Months

0 1 2 3 4 5 6 7 8

0 100 200 300 400 500 600 700 800
Fig 3-3

- TBR Placebo
- TBR Antioxidant
Fig: 3-4

- Conjugated diene Placebo
- Conjugated diene Antioxidant
- Fluorescence Placebo
- Fluorescence Antioxidant

Conjugated diene, Fluorescence (unit/ml)

Months

Conjugated diene Placebo and Antioxidant show a decrease over the 9 months. Fluorescence Placebo remains relatively stable, while Fluorescence Antioxidant shows a slight decrease.
Fig: 3-5

- PLACEBO
- ANTIOXIDANT

CD3+ (%)

<table>
<thead>
<tr>
<th>Months</th>
<th>PLACEBO</th>
<th>ANTIOXIDANT</th>
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<tbody>
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<tr>
<td>6</td>
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</tbody>
</table>
Fig: 3-6

![Graph showing CD4+ levels over months for PLACEBO and ANTI-OXIDANT groups.](image-url)
Fig: 3-7

![Bar chart showing CD8+ (%) over months for PLACEBO and ANTIOXIDANT groups.](image)
Fig: 3-8

PLACEBO
ANTIOXIDANT

CD16 CD56+ (%)

0 2 6

Months
Fig: 3-9

Tcell proliferation(cpm)

PLACEBO
ANTIOXIDANT

Months

Young 0 1 2 3 4 5 6 8
Fig: 3-10

B cell proliferation (cpm)

MONTHS

PLACEBO
ANTIOXIDANT
Fig: 3-11

The graph illustrates the IL-2 levels in young individuals over the first 8 months of treatment with PLACEDO and ANTIOXIDANT. The bars represent the median IL-2 levels, with error bars indicating the interquartile range. The groups are distinguished by different symbols: a* and b**. No significant differences are observed between the placebo and antioxidant groups. 

- **PLACEBO**
- **ANTIOXIDANT**
Fig: 3-12

PLACEBO
ANTIOXIDANT

IFN-gamma (pg/ml)

Months

0 1 2 3 4 5 6
Fig: 3-14

Serum IL-6 (pg/ml)

- PLACEBO
- ANTIOXIDANT

Months

young 0 1 2 3 4 5 6 8
Fig: 3-15

![Graph showing NK cell cytotoxicity 25:1(%) over months with placebo and antioxidant treatments.]

- **PLACEBO**
- **ANTIOXIDANT**

*Note: Young group comparison.*
CHAPTER IV

Modulation of immune function and cytokine production by
Dehydroepiandrosterone (DHEA) and Melatonin (MLT) in old mice

Sussan K. Ardestani, Zhen Zhang, Paula Inserra, Mohsen AraghiNiknam, Bailin Liang, Shuguang Jiang, Don Shaw, Mark Molitor, Kerry Elliott, Ronald R. Watson*

* To whom correspondence should be sent: Ronald R. Watson, Ph.D., Arizona Prevention Center, P.O.Box 245052, University of Arizona, Tucson, AZ 85724, USA. Phone (520)626-6001, Fax (520)626-2030.
Abstract:
Two immunoregulatory hormones Dehydroepiandrosterone (DHEA) and Melatonin (MLT) decline as immune dysfunction develops with age. The simultaneous replacement of these hormone may be immune restorative. C57BL/6 female mice, young 6 weeks or old 16 months were fed control, DHEA, MLT or both DHEA and MLT. After 12 weeks of supplementation the number of splenocytes in young mice was significantly (P < 0.05) decreased. DHEA, MLT and DHEA plus MLT significantly (P < 0.005) increased B cell proliferation in young mice. MLT and MLT plus DHEA significantly (P <0.05) increased B cell proliferation old mice. MLT alone was found to increase T cell proliferation in young mice and decreased natural killer cell cytotoxicity in old mice. Production of several cytokines, IL-2, IFN, IL-4, and IL-10 was significantly (P < 0.05) decreased in old mice. Supplementation significantly (P< 0.005) increased IL-2 production by activated T cells in both young and old mice. Also, supplementation significantly (P< 0.005) increased IFN-γ production by activated T cells in young mice. But only MLT significantly (P < 0.005) increased IFN-γ production in old mice. DHEA, MLT and DHEA plus MLT significantly (P < 0.05) increased IL-4 production in young mice and significantly decreased IL-6 production in young (P < 0.0005) and old (P < 0.005) mice. MLT significantly (P < 0.05) decreased IL-10 in young and old mice. In conclusion, DHEA, MLT and MLT plus DHEA all improved immune responses in old mice. Both DHEA and MLT have a significant role in normalizing cytokine
production in older mice. Splenocyte from hormone supplemented mice produced more Th1 cytokines when activated with mitogen therefore, cell mediated immunity may have been improved.

**Introduction:**

Immunological functions peak near puberty and gradually decline thereafter. The thymus is the center for growth and differentiation of T cells with intrinsic factors, including both growth and inhibitory factors. Physiological thymic involution is the major causes of immune dysfunction in the elderly. Chronological changes in the level of these factors contributes to age-related changes in the micro environment with regulation by various hormones and neuropeptides.

DHEA and MLT are both involved in immune regulation and the decline with age (364,365). Neuroimmune-modulation by the hypothalamic-pituitary system involves secretion of adrenocorticoids in response to antigenic or inflammatory stimulation. The consequent activation of the pituitary adrenal axis reduces the intensity of an immune response, because virtually all components of an immune response are inhibited by cortisol (366,374,382). The effect of corticotropin-releasing hormone (CRH) on the pituitary gland induces the release of ACTH. ACTH acts on the adrenal cortex and induces expression of 21- and 11-hydroxylase and increases cortisol production. When CRH is present in low amounts ACTH is also low, thus, allowing 17-hydroxypregnenolone to be converted to DHEA by action of 17,20-lyase.
Aging results in an impaired endocrine systems as a decrease in DHEA while cortisol production remains unchanged, this should produce a relative increase in cortisol compared to DHEA. Thus suppresses the release of interferon-gamma (IFN), granulocyte-monocyte colony-stimulating factor (GM-CSF), interleukin (IL) 1, 2, 3, and 6, tumor necrosis factor-alpha (TNF-α), inflammatory mediators such as eicosanoids, bradykinin, serotonin and histamine (367). On the other hand, inflammation and sepsis inhibit thyrotropin secretion in part through the action of cytokines. The hypothalamus then inhibits the production and secretion of thyrotropin-releasing hormone (TRH) (368) and enhances somatostatin secretion (369), which enhances immune responses. In vitro DHEA can overcome the suppressive effects of cortisol on IL-2 and IFN-τ production (370-371). These changes should enhance disease resistance by down regulation of IL-6 and other T helper 2 (Th2) cytokines. DHEA or its metabolites may block the action of cortisol thereby regulating cytokine production. Supplementation with DHEA in aging humans can improve immune function (372). DHEA replacement may prevent or even reverse, some age-associated conditions (diabetes, heart disease, cancer, immunosenescence) (373).

A second potential route of the neural regulation of immune function is thought the sympathetic nervous system. The sympathetic nervous system releases catecholamines and the adrenal medulla also release catecholamines. Activation of the sympathetic nervous system, or the injection of epinephrine, causes leukocytosis,
lymphopenia and inhibition of natural-killer (NK) cell activity (374,375). Epinephrine triggers the adenylate cyclase cascade by binding to the β-adrenergic receptor. One important neuropeptide that influences the immune system, is melatonin (MLT). MLT is synthesized and released by the pineal gland in response to the nocturnal, post synaptic activation of pineal β-adrenergic receptors (376). MLT modulates natural cytotoxicity of NK cells, modifies the antibody response, inhibits proliferation of lymphocytes activated by mitogen, and inhibits production of IFN by human T cells (377,378,379). It also activates monocytes and induces their cytotoxic properties (380).

DHEA and MLT both decline with age and are both have immunoregulatory function. Decline in immune function is also observed with age. Therefore the aim of the current study is to measure immune function in old mice supplemented with these hormones.

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). Mice were housed in transparent plastic cages with stainless steel wire lids (3-4 mice per cage) 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 12h. light:dark cycle. Water and diet were freely available. After 2 weeks of housing (on control diet), the mice were randomly assigned to the following treatments. Group A-D were young mice (8 mice / group) fed (A) control chow diet and 0.05% ethanol in drinking water (B) 0.02% DHEA diet for the first three weeks (6.2 μg/mouse/day); and then 0.06% DHEA diet for next nine weeks (18.66 μg/mouse/day) and 0.05% ethanol in drinking water (C) control chow diet and 10 μg/ml melatonin (MLT) dissolved in 0.05% ethanol drinking water (49.8 μg/mice/day) (D) 0.02% DHEA diet for the first three weeks and then 0.06% DHEA for the other next weeks and 10 μg/ml MLT in 0.05% ethanol drinking water (for twelve weeks). Four groups of old mice (4 mice / group) were provided with the same diets and water as described for young mice. DHEA was purchased from Sigma (St. Louis, Mo.), and 0.02% DHEA diet and 0.06% DHEA diet were prepared by Diets Inc. (Bethlehem, Pennsylvania). MLT was also purchased from Sigma, dissolved in ethanol and diluted in distilled water. The final concentration of melatonin in the drinking tap water was 10 μg/ml (0.05%) ethanol. The treatment period was twelve 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 biotinulated antibodies, and recombinant murine IFN-gamma, IL-2, IL-4, IL-6, IL-10 were obtained from Pharmingen (San
ELISA for Cytokines

IFN-gamma, IL-2, IL-4, IL-6, and IL-10 were produced by splenocytes as described previously (354). Briefly, spleens were collected after sacrifice under ether anesthesia. Mononuclear cells were obtained by gently teased 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 minutes. 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 µg/ml, 0.1 ml/well, Sigma) for induction of IL-2, IL-4 and IL-10 with 24 hours incubation, IFN-gamma with 72 hours incubation at 37°C, 5% CO₂ incubator. Splenocytes were also stimulated by lipopolysaccharide (LPS, 10 µg/ml, Gebco, Grand Island, NY) for 24 hours induction for IL-6 and TNF-α production. After incubation, the plates were centrifuged for 10 min at 800 xg. Supernatant fluids were collected and stored at -70°C until analysis. They were determined by sandwich ELISA (344), and as we have described
Mitogenesis of Splenocytes

Splenic T and B cell proliferation was determined by $^3$H-thymidine incorporation as described previously (387). Briefly, splenocytes in 0.1 ml of CM (1x10^7 cell/ml) were cultured in 96-well flat-bottom cultured plates (Falcon) with Con A and LPS (10 $\mu$g/ml). They were incubated at 37°C, 5% CO$_2$ incubator for 44 hours for Con A and LPS-induced T and B cell proliferation respectively, and then pulsed with $^3$H-thymidine (0.5 uCi/well, New England Nuclear, Boston, MA). After 6 hours, 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 (342). 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 4x10^4 target
cells/100 μl. The plate was centrifuged (90 xg) for 3 minutes to facilitate cell to cell interaction. The cells were then incubated at 37°C in a humidified atmosphere of 5% CO₂ for 3 hours. After incubation, 20 μl of 1% inert fluoricon polystyrene assay particles was added to each well of plate (Pandex Harvesting Plate, IDEX, Portland, Maine), and 70 μl 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:

\[
\text{Specific Cytotoxicity} = \frac{\text{Spontaneous Release} - \text{Experimental Fluorescence}}{\text{Spontaneous Release} - \text{Maximum Release}} \times 100
\]

**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 reminding cells were washed twice with cold CM. The number of viable cells was determined by trypan blue exclusion, and cell concentration adjusted between 1-2 x 10⁵ / 0.1 ml/ tube for subsequent lymphocyte surface marker determination as described by Lopez (381). 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 Sandieg 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:

Spleen Cell Numbers

Spleen weight was significantly (P < 0.001) higher in untreated old mice (4 mice) with 106 ± 13 mg for old mice compared to 66 ± 14 mg / spleen of untreated young mice (8 mice). No significant difference in spleen weight of supplemented old or young mice compared to untreated, age-matched controls was observed (data not shown). Splenocyte count was significantly (P < 0.001) higher in old mice (4.2 x 10^7 / spleen) compared to young mice (2.27 x 10^7 / spleen). Supplementation with DHEA or MLT did not change spleen cell numbers in young or old mice, However DHEA plus MLT decreased splenocyte (3.3 x 10^7 in old mice and 1.73 x 10^7 in young mice)(Fig. 4-1).
Percentage of thymus CD3\(^+\) and CD8\(^+\) cells of old and young mice was not significantly different and did not change with supplementation (data not shown). The percentage of autoreactive CD5\(^+\) cells was not quite significantly (\(P = 0.06\)) higher in old mice (19.3 ± 2.88) than young mice (13.8 ± 3.5). Supplementation had no effect on percentage of CD5\(^+\) cell.

**Spleen Cell Functions**

B cell proliferation in response to LPS did not differ between old and young mice. DHEA, MLT and DHEA plus MLT significantly (\(P < 0.001\), 0.001, and 0.05 respectively) increased B cell proliferation in young mice. MLT and the combination of DHEA and MLT significantly (\(P < 0.03\), and 0.04 respectively) increased B cell proliferation in old mice (Fig. 4-2). T cell proliferation in response to ConA was similar for both old and young mice. However MLT alone significantly (\(P < 0.05\)) increased T cell proliferation in young mice (Fig. 4-3).

Natural killer cell cytotoxicity in old and young mice was not significantly different and supplementation did not change NK cell cytotoxicity in both group of mice (data not shown).

Splenocytes from old mice in response to ConA produced significantly (\(P < 0.05\)) lower amounts of IL-10. MLT and MLT plus DHEA significantly (\(P < 0.05\)) decreased IL-10 production in old and young mice and only MLT significantly (\(P < 0.05\)) decreased IL-10 production in old mice (Fig. 4-4).
Splenocytes from old mice in response to ConA produce significantly (P < 0.05) lower amounts of IL-4. MLT and MLT plus DHEA, significantly (P < 0.05, 0.00005, and 0.005) increased IL-4 production in young mice, but had no effect in old mice (Fig. 4-5).

Splenocytes from old mice in response to ConA produced significantly (P < 0.05) lowered the amount of IFN (Fig. 4-6). Supplementation with DHEA, MLT, and DHEA plus MLT significantly (P < 0.005) increased IFN production in young mice and only MLT significantly (P < 0.005) increased IFN production in old mice. The difference in IFN production was not significant in supplemented old and young mice (Fig. 4-6). Splenocytes from old mice in response to ConA produced significantly (P < 0.05) lower amounts of IL-2 (Fig. 4-7). Supplementation with DHEA, MLT, and both significantly (P < 0.01) increased IL-2 production in old and young mice (Fig. 4-7).

Discussion:

Immune senescence involves an increased level of many autoantibodies, and a decreased antibody response to most foreign antigens (383,384), impaired function of CD5+ B (382) and Th1 cells (376). However, in this study, we showed that the response of splenocytes from old mice to B and T cell mitogens was increased as compare to young mice. The capacity of activated lymphocytes to synthesize and secrete IL-2, IL-3 and GM-CSF was significantly reduced in cells derived from aged...
animals and humans, whereas the capacity of these same cells to produce IL-4, IL-5, IL-6, IL-10, and IFN was increased (385-389). A decrease in IL-2 IL-4, IL-10 and IFN in old mice was observe as compare to young mice. Supplementation with DHEA and also MLT improves this defect in old mice. IL-10 can suppress Th1 cells and, only MLT regulated IL-10 production in old mice. MLT, and DHEA plus MLT significantly decreased IL-10 production in young mice, also MLT significantly decreased IL-10 production in old mice. IL-10 possesses a broad range of reported activities. Many of the age-associated changes in T cell, macrophage, and B cell are linked to dysregulated control over endogenous IL-10 production. IL-10 can directly inhibit IL-2 gene expression by activated T cells (396), reduce expression of class II major histocompatibility complex molecules (397), and depress B7 costimulatory molecule expression on activated macrophages (398). Actually CD5⁺ B cells, are the major producers of IL-10 following cellular activation (399), and number of CD5⁺ B cells are increased with advance aging (400). Our study is in agreement with these results; however, supplementation did not decrease CD5⁺ cells in old mice.

The dysregulated production of IL-6 is so great in aged subjects, that cytokine presence can be readily detected in the plasma of aged animals (390-393). IL-6 appears to be involved in T cell activation, growth, and differentiation. It also serves as an inducer of both B cell proliferation and maturation (394) and it is also important for the development of mucosal immunity system (395). But unregulated IL-6 production is life threatening.
DHEA is one of the principal adrenal steroids. It has been known for some time that serum levels of DHEAS (the circulating sulfated form of DHEA) decreases with age (364). Daynes (401) reported that, chronic or acute administration of DHEA to old animals restored antibody response and induced the production of Th1 cytokines. Th1 cytokines increased expression of MLT receptor on spleen from mice and rats (402). MLT treatment increased mitogen responsiveness of splenocytes in aged mice (403), and increased IL-2 and IFN production (404,405). We found MLT increased IL-2, IFN, IL-4, T and B cell proliferation by activated splenocytes. Also, we found that MLT and DHEA stimulate each other effects. However, C57BL/6J mice do not have a detectable amount of MLT in their pineal glands. Genetic analysis suggests that melatonin deficiency in C57BL/6J results from a mutation in two independently segregated, autosomal recessive genes (406). Our model is a good model for studying the effects of MLT in the elderly.

Aging is related to fuel use, specifically to the generation of oxygen free radicals (168). It is accompanied by increasing DNA, protein, and lipid damage due to defects in repair mechanisms and decreases in antioxidant enzymes and compounds. Beier (407-409) showed that the liver of aged rats had reduced numbers of peroxisome and reduced peroxisomal β-oxidation activity. Chronic administration of DHEA to rodents caused a massive increase in peroxisome number and peroxisomal enzyme activity in liver (410,412).
Fig 4-1. Effect of DHEA, MLT and DHEA plus MLT on splenocyte count in old and young mice. The values are mean ± SD for 4 or 8 mice in each group. The number of young mice was 8 per group. The number of old mice was 4 per group. (a), P value compare to the same supplementation in young mice. (b), P value compare to the control in the same group. * P < 0.05. ** P < 0.005.

Fig 4-2. Effect of DHEA, MLT and DHEA plus MLT on B cell proliferation by LPS-stimulated splenocyte in old and young mice. Every sample was determined in triplicate. The values are mean ± SD for 4 or 8 mice in each group. The number of young mice was 8 per group. The number of old mice was 4 per group. (a), P value compare to the same supplementation in young mice. (b), P value compare to the control in the same group. (c), P value compare to DHEA supplementation. * P < 0.05. ** P < 0.005.

Fig 4-3. Effect of DHEA, MLT and DHEA plus MLT on T cell proliferation by ConA-stimulated splenocyte in old and young mice. Every sample was determined in triplicate. The values are mean ± SD for 4 or 8 mice in each group. The number of young mice was 8 per group. The number of old mice was 4 per group. (a), P value compare to the same supplementation in young mice. (b), P value compare to the control in the same group. * P < 0.05. ** P < 0.005.

Fig 4-4. Effect of DHEA, MLT and DHEA plus MLT on T cell IL-10 production by ConA-stimulated splenocyte in old and young mice. Every sample was determined in triplicate. The values are mean ± SD for 4 or 8 mice in each group. The number of
young mice was 8 per group. Mouse number of old mice was 4 per group. (a), P value compare to the same supplementation in young mice. (b), P value compare to the control in the same group. * P < 0.05. ** P < 0.005.

Fig 4-5. Effect of DHEA, MLT and DHEA plus MLT on T cell IL-4 production by ConA-stimulated splenocyte in old and young mice. Every sample was determined in triplicate. The values are mean ± SD for 4 or 8 mice in each group. The number of young mice was 8 per group. Mouse number of old mice was 4 per group. (a), P value compare to the same supplementation in young mice. (b), P value compare to the control in the same group. * P < 0.05. ** P < 0.005.

Fig 4-6. Effect of DHEA, MLT and DHEA plus MLT on PMNC IL-6 production by LPS-stimulated splenocyte in old and young mice. Every sample was determined in triplicate. The values are mean ± SD for 4 or 8 mice in each group. The number of young mice was 8 per group. Mouse number of old mice was 4 per group. (a), P value compare to the same supplementation in young mice. (b), P value compare to the control in the same group. * P < 0.05. ** P < 0.005. *** P < 0.0005.

Fig 4-7. Effect of DHEA, MLT and DHEA plus MLT on T cell IFN-γ production by ConA-stimulated splenocyte in old and young mice. Every sample was determined in triplicate. The values are mean ± SD for 4 or 8 mice in each group. The number of young mice was 8 per group. Mouse number of old mice was 4 per group. (a), P value compare to the same supplementation in young mice. (b), P value compare to the control in the same group. * P < 0.05. ** P < 0.005.
Fig 4-8. Effect of DHEA, MLT and DHEA plus MLT on T cell IL-2 production by
ConA-stimulated splenocyte in old and young mice. Every sample was determined in
triplicate. The values are mean ± SD for 4 or 8 mice in each group. The number of
young mice was 8 per group. Mouse number of old mice was 4 per group. (a), P
value compare to the same supplementation in young mice. (b), P value compare to
the control of the same group. * P < 0.05. ** P < 0.005.
Fig: 4-3

T-cell proliferation (cpm)

Control  DHEA  MLT  DHEA+MLT

[Bars representing young and old groups with error bars]
Fig 4-4

IL-10 production (pg/ml)

Control  DHEA  MLT  DHEA+MLT

- young
- old

Legend:

- a**
- a*
- b*
- c*
Byoung

Control DHEA MLT DHEA+MLT

Fig: 4-5

IL-4 production (pg/ml)

- young
- old

DHEA

MLT

DHEA+MLT

0 100 200 300 400 500 600 700 800 900

a* b*

a** a** b**
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