The Hong Kong Polytechnic University

Department of Health Technology and Informatics

IMMUNOMODULATORY, ANTI-INFLAMMATORY AND ANTI-MICROBIAL EFFECTS OF SELECTED DIETARY AGENTS:

a study using a biomarker approach

By

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CERTIFICATE OF ORIGINALITY

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ABSTRACT

There is a well established age-related decline in the protective action of the immune system, and this increases not only risk of infection but risk of cancer. It has been shown that the absolute number of several immune parameters decreases with ageing. These include the absolute number of T lymphocytes subsets and B lymphocytes. Other changes that also occur with ageing can directly affect cells of the immune system. Oxidative stress and glycation for instance are related to the ageing process and these affect DNA, proteins and lipid structures of cells. There is also a worrying increase in antibiotic resistance in both hospital and community acquired infections. Therefore, there is an *urgent need* for strategies to prevent the age-related decline in immune status, and to boost the compromised immune system. In addition, novel antimicrobial therapies are needed to treat antibiotic resistant infections. Many herbs and dietary agents are reported to have immunomodulatory or anti-inflammatory actions. Some herbs have also been reported to have direct or indirect antimicrobial effects. Multi-dimensional approaches for validating beneficial effects of different herbs/ supplementation on health or body systems are needed. Many studies of well known herbs have been widely carried out in *in vitro* and animal studies and there is a wide range of anecdotal evidence available. However, there is lack of systematic scientific evaluation showing the effect of herbs on human immune status related to antioxidant balance, and thus controlled intervention trials are needed to determine if the claims of health benefits for these herbs/dietary supplements are justified, and if they have a role in prevention and treatment of increasingly difficult-to-treat infectious disease and age-related immune decline.

This study investigated the effect of selected herbs/dietary agents on cellular immunity and its relationship with antioxidant status. The direct and indirect antimicrobial effects (against MRSA strains and VISA strain) of these herbs were also studied. Dietary agents/herbs of interest in this study were Lingzhi (*Ganoderma lucidum*), bilberry (*Vaccinium myrtillus*) and green tea (*Camellia sinenis*). Experimental work included *in vitro* and controlled human intervention trials.

The methodology for assessing cellular immunity included measurement of immunologic phenotype of lymphocyte sub-sets using monoclonal antibodies against surface antigens of white blood cells by flow cytometry and complete white blood cell count. Plasma antioxidant status was determined by measuring plasma biomarkers [including total antioxidant power, ascorbic acid (FRAP assay) and alpha tocopherol (HPLC)]. Antimicrobial effects were investigated by determining the minimal inhibitory concentration (MIC) of the herbs of interest (alone and in combination with antibiotics) by using the spiral gradient endpoint and the agar dilution method.

In the supplementation trials, some potential interesting results were observed in terms of inter-relationships between changes in antioxidant status and changes in some white cell subsets. No convincing evidence of supplementation-related positive effects on white cell numbers and subsets was seen. However, in the green tea study, there was some indication of an anti-inflammatory effect; however variation in the response of hsCRP (inflammation biomarker) was wide. There was some indication of improvement in cytotoxic T cells (CD8+) cells after bilberry supplementation, which could have implications for immune surveillance. In addition, lingzhi was shown to have some potential effect in counteracting the side effects of radiotherapy on cellular immunity. At last, this study provides supporting data showing the potential effect of these natural products as antibacterial agents. Direct antimicrobial effect was found in bilberry (MIC= 0.6 mg/ml), screw-shaped green tea (MIC= 0.6 mg/ml) and loongjin green tea (MIC= 1.2 mg/ml) against MRSA, MSSA and VISA strains. Synergistic effects of lingzhi, bilberry and green tea were found when these were used in combination with oxacillin and vancomycin against VISA and MRSA strains. The outcome of this study relates to the potential health benefits of the dietary agents studied in relation to immune status, antioxidant balance and antimicrobial effects. These data may provide evidence to justify the use of food/herbal supplements for immunomodulation and health maintenance in our ageing population.

LIST OF OUTPUT

Publications

A.R.W. Lau, S.W. Choi,T. Chu, B.Tomlinson, I.F.F. Benzie. Possible immuno-enhancing effects of bilberry in subjects under increased oxidative stress: results of a controlled human intervention study. Progress in Nutrition volume 12 (Number 1; 2010) Page 80.

Conference Papers

ARW Lau, SW Choi, WM Lo, KC Wong, IFF Benzie. The immunomodulatory & anti-inflammatory effects of green tea: results from a controlled human intervention study. Hong Kong-Macau Symposium on Chinese Medicine (August 2009)

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LIST OF ABBREVIATIONS

Ala	Alanine
ADP	Adenosine diphosphate
APC	Antigen presenting cell
BB	Bilberry
CLSI	Clinical and Laboratory Standards Institute
CNS	Central nervous system
CSF	Colony stimulating factor
CVD	Cardiovascular disease
D	Diversity
DHPPP	7,8-dihydro-6-hydroxymethylpterin-pyrophosphate
DHPS	dihydropteroate synthase
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
EC	Extracellular
ECG	Epicatechin gallate
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin gallate
ELISPOT	Enzyme linked immunospot
ELISA	Enzyme linked immunosorbent assay
ER	Endpoint radius
ESBL	Extended spectrum β-lactamase
FDA	Food and Drug Administration
FITC	Fluorescein isothio-cyanate
FRAP	Ferric reducing antioxidant power
FRASC	Ferric reducing ascorbate
G. lucidum	Ganoderma lucidum
HbA1c	Glycosylated hemoglobin
HIV	Human immunodeficiency virus
HK	Hong Kong

HPLC	High-performance liquid chromatography
hsCRP	high-sensitivity C-reactive protein
IFN	Interferon
Ig	Immunoglobulin
iGln	Isogulutamine
IL	Interleukin
IMRT	Intensity-modulated radiotherapy
J	Joining
L	Lingzhi
LCMS	Liquid-chromatography mass spectrometry
LGT	Loonjin green tea
LPS	Lipopolysaccharides
Lys	Lysine
MDA	Malondialdehyde
MDR	Multidrug resistant
MIC	Minimum inhibitory concentration
MHC	Major histocompatability complex
mRNA	Messenger ribonucleic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
MSSA	Methicillin-sensitive *Staphylococcus aureus*
M. Tuberculosis	Mycobacterium Tuberculosis
NF- κB	Nuclear factor-κB
NK	Natural killer
NPC	Nasopharyngeal carcinoma
P. *aureginosa*	*Pseudomonas aureginosa*
PABA	p-aminobenzoic acid
PBP	Penicillin-binding proteins
PCR	Polymerase chain reaction
PE	Phycoerythin
PFGE	Pulsed field gel electrophoresis

PGE_2	Prostaglandin E2
PRC	People's Republic of China
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
RND	Resistance-nodulation-cell division
ROS	Reactive oxygen species
RT	Radiotherapy
SARS	Severe acute respiratory syndrome
SCC	Squamous cell carcinoma
Ser	Serine
SD	Standard deviation
SEM	Standard error of the mean
SGE	Spiral gradient endpoint
SMART	Study for Monitoring Antimicrobial Resistance Trends
SSGT	Screw-shaped green tea
TCR	T cell receptor
TER	Tail ending radius
TGF	Transforming growth factor
Th	T helper
TNF	Tumour necrosis factor
UK	United Kingdom
V	Variable
VISA	Vancomycin-intermediate *Staphyloccoccus aureus*
VRE	Vancomycin-resistant enterococci
VRSA	Vancomycin resistant *Staphylococcus aureus*
WBC	White blood cell
WHO	World Health Organization
8-oxodG	8-Hydroxyguanine

Chapter 1

LITERATURE REVIEW

Introduction

The human immune system is a highly complex network of defense which overall plays a vital role in protecting the body from infectious microorganisms, responding to injury and trauma, and identifying and eliminating changed and foreign cells. Various factors can affect the immune status in an individual and, in particular, ageing is associated with immune decline. All developed countries are facing the challenge of the advancing age of the population. In addition, there is a marked rise in antibiotic resistant infections. In Hong Kong it is expected that the proportion of elderly aged 65 and over will be 14% by the year 2016 (according to the HK Department of Health Population Projections). Prevalence of many diseases is increased in older adults, creating a huge socio-economic burden. Many age-related disorders are related to immune decline or to inflammation. Therefore, there is an *urgent need* for strategies to prevent the age-related decline in immune status, and to boost the compromised immune system. Novel antimicrobial therapies are also essential to treat antibiotic resistant infections. Herbs and dietary agents have been found to have immunomodulatory or anti-inflammatory actions. Also, direct or indirect antimicrobial effects have been reported. The immunomodulatory effects of herbs or foods could be related, at least in part, to their antioxidant activity. In preliminary study our group found a significant correlation between white cell subsets and the antioxidant activity of the plasma in healthy subjects leading us to hypothesise that increased intake of antioxidant-rich foods or beverages could be beneficial to the immune system. In addition, increased intake of antioxidant-rich agents, such as berries and teas, is associated with decreased inflammation. Further, our group has also found that lingzhi, a traditional 'herb', appears to act in combination with known antibiotics to lower the minimum inhibitory concentration (MIC). Other groups have reported direct antimicrobial effects of herbs, including linghzi and green tea. These findings form the basis of this study, as dietary agents that enhance the

immune system and/or decrease inflammation would have significant health promoting effects. Moreover, direct or complementary/synergistic antimicrobial effects of herbs could offer useful adjunct therapies for infectious disease. Evidence for such effects is *the desirable outcome* of this research, in which effects of three selected dietary agents, namely lingzhi, green tea and bilberry, were studied.

This chapter presents an overview of the human immune system, how it is assessed and the factors affecting it. A review of the literature on immune decline and antibiotic resistant bacterial infections and how these relate to human health, is then presented. This is followed by a discussion on the role of herbs and dietary agents for the promotion of health and healthy ageing in relation to immunomodulation and antibacterial therapy.

An Overview of the Immune System: what it is, what it does and how it does it

The immune system is a highly complex biological system which starts to function, albeit immaturely, at a very early stage of life and it serves as a major protector against disease throughout the lifespan [Provinciali and Smorlesi, 2005]. However, immunity is less than optimal at both ends of life (in the newborn and the elderly) [Levinson, 2008]. The different parts of the immune system protect the body by killing or inactivating infectious agents such as bacteria, viruses, parasites and fungus, as well as identifying and eliminating changed, damaged or foreign cells and material [Frederic, 2006]. The immune system is comprised of lymphoid tissues and organs (Table 1.1), and can be divided into two main component systems, the innate and the adaptive [Parkin & Cohen, 2001; Roitt and Delves, 2001]. The innate immune system involves non-specific physical and chemical barriers that prevent entrance or invasion of foreign pathogens into the body, and immune cells that recognize and destroy foreign cells. The adaptive immune system involves recognition and removal of specific antigen-bearing cells and has the ability to retain antigenic ‘memory’ [Rao, 2006; Moore, 2007]. Both the innate and adaptive systems are needed, and they work in a coordinated and tightly regulated manner, comprising a network of genetic and signaling pathways supporting a network

of interacting cells [Chaplin, 2006; Heng et al., 2008; Christensen and Thomsen, 2009]. Both arms of the immune system provide armor for defense against the invasion of pathogens at any site in the body. It is of paramount importance for health for the immune system to be maintained and appropriately modulated since a weak immune system increases the risk of infection and cancer, whereas over-reactivity leads to autoimmune diseases, hypersensitivity and chronic inflammation [Rao, 2006].

Table 1.1

Organs and Tissues of the Immune System in Adult Humans

Organ/Tissue	Function
Bone marrow	The site of initial formation of all white cell (leukocyte) types.
Thymus	Major site of T-cell maturation.
Spleen	Major site of removal of opsonized microorganisms. Main site for blood borne antigen response and B-cell activation
Lymph nodes	Site of antigen concentration for helping in antigen presentation to T cells. Site of removal of foreign antigens that have entered the circulation
Mucosal-associated lymphoid tissue	Site of first line defenses in sites exposed to the exterior, e.g. respiratory, gastrointestinal, urinary, female reproductive tracts; site of specialized antigen-transporting epithelium cells (M cells) that take up inhaled or ingested antigens.
Barriers	-Mechanical: Sneezing, coughing and washing action of tears and urine. -Physical: Intact skin is impermeable to microbes and inhibits bacterial growth by the secretion of lactic acid and fatty acids which generate low pH. Cilia and mucus of respiratory tract block adherence of bacteria. -Chemical: Chemical factors that destroy bacteria such as gastric juice, spermine and zinc in semen, lactoperoxidase in milk, lysozyme in tears, nasal secretions and salivary substances. -Biological: Normal bacterial microflora (commensals) of the gastrointestinal tract suppresses the growth of many potentially pathogenic bacteria and fungi.

The Innate Immune System

The innate immune system is a natural 'inborn' defense against infection and it consists of cells with phagocytic action and a family of proteins known as the complement system. The different cells of the innate immune system and their functions are presented in Table 1.2. Innate immunity is found in all multi-cellular organisms. It is characterized by its immediate readiness and, in relation to its cells, activation that is not antigen-specific. Unlike the adaptive system, no antigenic memory is needed and there is no requirement for a large array of cells each designed to respond to a specific antigen for pathogen recognition [Si-Tahar et al., 2009]. However, the innate immune system plays an important role in the activation of the cells of the adaptive immune system.

Table 1.2

Cells (Leukocytes) of the Innate Immune System

Cells	Function
Mast cells	They are associated with allergy and anaphylaxis. They contain granules rich in histamine and heparin which are released when activated.
Phagocytes	They engulf particles, pathogens and dead cells. They are important for the healing process after tissue injury. Phagocytic cells include neutrophils, macrophages and dendritic cells.
Basophils	They are important during parasitic and allergic infections. Basophils have receptors on their cell surface that bind IgE (an immunoglobulin involved in macroparasite defense), which confers a selective response to helminth antigens.
Eosinophils	They are associated with allergic responses and are capable of inducing tissue damage and dysfunction. When activated, eosinophils degranulate to release an array of cytotoxic granules.
Natural Killer (NK) cells	They destroy tumor or viral-infected cells by inducing apoptosis.

The cells of the innate immune system work in cooperation with the complement system and with the adaptive immune system to phagocytose and destroy pathogens efficiently and quickly [Gennery and Cant, 2006]. Phagocytic cells recognize and adhere to molecular patterns on the surface of pathogens (e.g. lipopolysaccharides of Gram-negative bacteria, lipopeptides of Gram-positive bacteria and double-stranded RNA of certain viruses), which are then engulfed and killed by the generation of a 'respiratory burst', causing the release of reactive oxygen species (ROS) [Appelberg, 2007]. Pathogen-associated molecular patterns, which are highly conserved structures present on microorganisms, are recognized by Toll-like receptors that are expressed on antigen presenting cells such as macrophages and dendritic cells [Bauer er al., 2009; Si-Tahar et al., 2009]. Activated phagocytes can also synthesize and release biologically active molecules which include cytokines, chemokines and chemotactic lipids [Si-Tahar et al., 2009]. These chemicals released by activated phagocytes attract other immune cells in the region to the infected or damaged site and also act as chemical messengers within the innate system and between the innate and the adaptive immune systems [Tosi, 2005]. Phagocytes also express engulfed microbial antigens on their surface, which are then 'presented' to the T cells of the adaptive immune system, as described in a later section.

Apart from phagocytes, Natural Killer (NK) cells are also considerably important in innate immunity. These eliminate changed (for example pre-cancerous or infected) cells by inducing apoptosis [Orange and Ballas, 2006; Nairn, 2007]. NK cells have receptors for antibodies (immunoglobulins) and adhere to antibody-coated cells or microbes, and they also express an identifying molecular label known as CD56. NK cells when activated release perforins, which creates holes in the membrane of an antibody-coated changed cell. Protease enzymes (granzymes) are then released by the NK cells, and enter the target cell through the holes, inducing apoptosis by various mechanisms, fragmenting DNA and inactivating cellular repair process, and thus inducing death of the antibody coated target cell [Parkin & Cohen, 2001; DeFranco et al., 2007]. NK cells also have receptors for the major histocompatibility complex (MHC-I) antigen, which is expressed on the surface of normal cells. NK cells are activated when a cell is encountered and no MHC-I antigen is detected. Foreign, malignant and infected cells

have low levels or lack MHC-I, triggering their destruction by NK cell-induced apoptosis [Parkin and Cohen, 2001].

The complement system is a family of over 30 proteins and glycoproteins [Duncan et al., 2008] which, in a proteolytic cascade of reactions, are involved in the direct killing of pathogens as well in attracting phagocytic cells and facilitating the engulfing process through opsonization (coating of a foreign cell by antibodies or complement). There are three pathways of complement activation, the Classical, the Alternative and the Lectin pathway, each with different triggers. For example, the Alternative complement pathway is directly activated by the presence of microorganisms, the Classical pathway is activated by antibody-coated microorganisms and the Lectin pathway is activated by binding mannose-binding lectin to mannose residues on the pathogen surface [Gennery and Cant, 2006]. However all pathways converge at the cleavage of complement protein C3 into C3a and C3b fragments [Zipfel et al., 2005]. The complement cascade results in production of a membrane attack complex which eliminates foreign microorganisms by osmotic lysis, phagocyte recruitment, and stimulation of the adaptive immune response involving T and B cells [Nairn, 2007; Duncan et al., 2008].

If the innate immune system fails, bacterial and fungal infections develop. For instance, neutropenia (low neutrophil count) results in frequent bacterial infections, often with organisms that have low pathogenicity under normal circumstances. Children born with neutropenia suffer from bacterial infections almost immediately after birth [Actor, 2007]. Defects in the complement system can also lead to life-threatening infections with pyogenic bacteria [Roitt and Delves, 2001].

The innate immune system comprises various cells and substances. With reference to this study, the cell of major interest is the C56+ NK cell, as it has an important role in immunosurveillance and also in aged-related diseases such as cancer, which is related to poor immunosurveillance [Ostrand-Rosenberg, 2007].
In summary, the innate immune system is associated with immediate host defense which is very important in survival. However, if the host response is excessive or uncontrolled,

it may lead to inflammation that in extreme cases may become pathological, such as in the case of hypersensitivity.

A note on inflammation

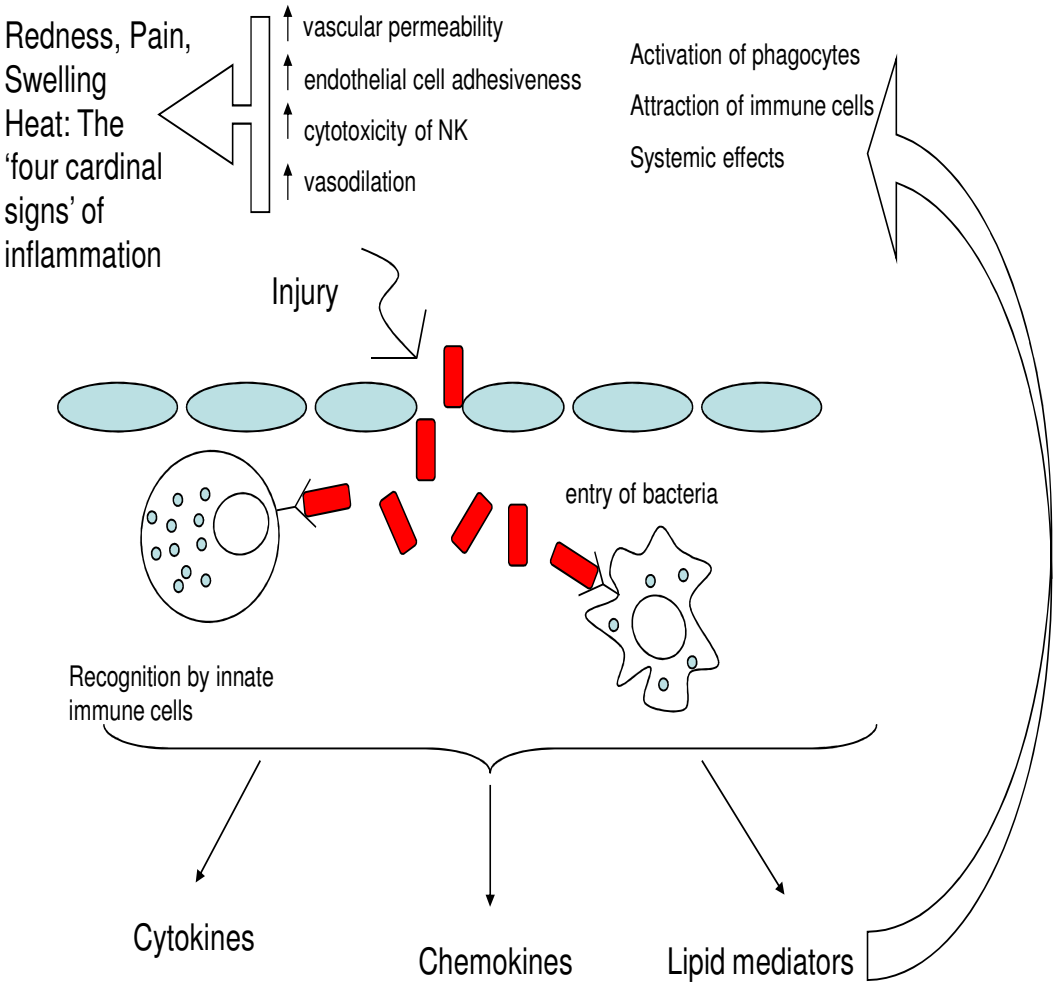
Inflammation is a process that produces changes in the both the innate and adaptive immune systems. Indeed, the association is so close that inflammation can be regarded as part of the immune response. Inflammation is characterized by the 'four cardinal signs', which are pain, swelling, redness and heat in the inflamed area [Marceau and Bachvarov, 2008]. Inflammation is a normal response to injury and a crucial component of normal tissue repair as well as being essential to the body's defenses against infection and other damaging agents [Franceschi, 2007; Gauldie, 2007]. Inflammation is a vascular response to injury, and it has several generalized effects on the adaptive immune response and innate immunity [Medzhitov and Janeway, 1997] (see Figure 1.1). Tissue macrophages, mast cells and dendritic cells induce the production of lipid mediators, cytokines and chemokines after being activated by pathogens leading to inflammation [Si-Tahar et al., 2009]. Inflammatory cells migrate to the site of infection by expressing cell-surface adhesion molecules that bind to the activated endothelial cells. The first cells to migrate to damaged or infected areas are neutrophils [Barton, 2008]. Monocyte/macrophages constitute the second wave of inflammatory cells and are characteristic of chronic inflammation [Sorg, 1991].

Efficiency of cellular influx to the inflammatory site is mediated by substances found in plasma as well as secreted by inflammatory cells, endothelial cells and degranulating cells which have been activated. These inflammatory mediators include anaphylatoxins, kinins, leukotrienes, prostaglandins and neuropeptides which are responsible to induce vascular permeability, change the adhesive properties of the endothelium and activate phagocytes and NK cells [DeFranco et al., 2007]. A coordinated and controlled inflammatory response is essential to destroy the invading pathogens and downregulating the response for resolution. However, under certain conditions inflammation might be harmful. For example, in major trauma or allergy, an excessive acute inflammatory response may develop, blocking airways or affecting blood pressure

[Headley et al., 1997]. Other problems might also develop if the initial inflammatory response is inadequate or if the initiating agent or process is ongoing [Gabay and Kushner 1999]. In this case a state of chronic inflammation occurs. Laboratory tests of inflammation usually include markers such as high-sensitivity C-reactive protein (hsCRP), cytokines, fibrinogen, acute-phase reactants and total white blood cell count in venous blood [Pearson et al., 2003].

Figure 1.1

Inflammatory Response and the Immune System



The Adaptive Immune System

Adaptive immunity is also referred to as specific acquired immunity. The major evolutionary innovation in this system is the antibody, which recognizes and interacts with a specific antigen. An antibody (immunoglobulin) is a protein produced by B cells, also known as plasma cells, of the adaptive system, and its major role is to bind the molecular complex (the antigen) that evoked its production. The response that causes the production of antibodies and that helps in eliminating pathogens is called humoral immunity. [DeFranco et al., 2007]. The general functions of antibodies include the binding to and neutralization of antigen and toxins, fixation of complement, binding to various cell types for enhancing phagocytosis, opsonization and agglutination of pathogens, and cell (NK and macrophage) activation [LaRosa and Orange, 2008]. Therefore, there is a strong and vital link between the adaptive and the innate parts of the overall immune system.

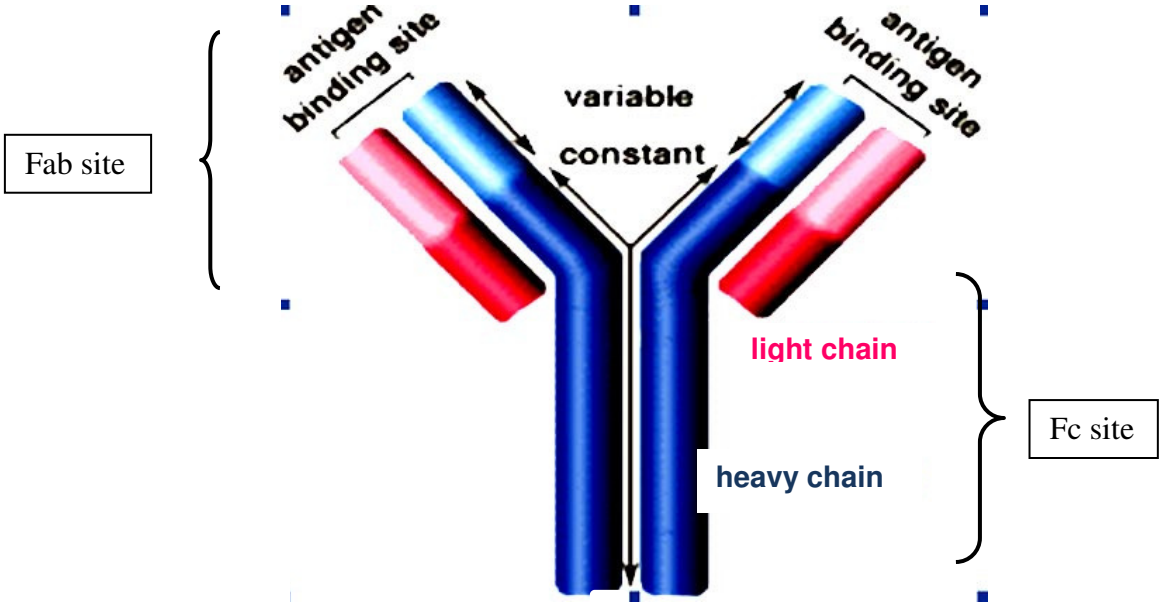
Antibodies are displayed on the surface of the B lymphocytes that produce them, but they exist also as free proteins in the blood, secretions or intracellular spaces. Each antibody contains four protein chains, two identical large 'heavy' chains, and two identical, smaller 'light' chains. Each of the four chains contains a variable and a constant region (or domain). In each antibody there are two 'Fab' sites, which bind to antigen, and one 'Fc' site, which is associated with various functions of the antibody (Figure 1.2). Heavy chains can be made up of proteins of five classes, μ, δ, ε, α or γ. Light chains are designated as κ and λ. The variable regions of the heavy and light chains consist of 100-110 amino acids that show high variation among different antibodies [Rao, 2006]. Thus, an antibody displays a unique amino acid sequence in its variable region. This is transcribed and translated by several segments in the antibody gene. The variation is generated by several processes [Gennery and Cant 2006]:

1. Gene rearrangement of the three gene segments called V (variable), D (diversity) and J (joining) segments.
2. Heavy and light chain variable region combinations.
3. Imprecise splicing and rejoining of different gene segments

Figure 1.2

Diagrammatic Representation of Antibody Structure

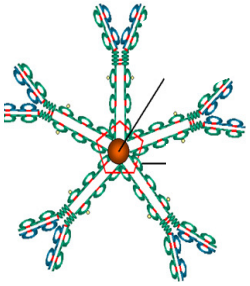
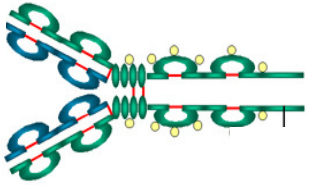
[image from http://www.biology.arizona.edu/IMMUNOLOGY/.html**]**

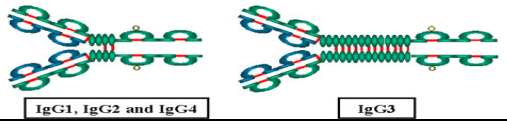
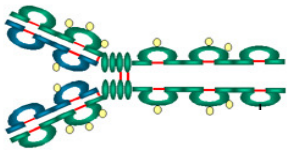
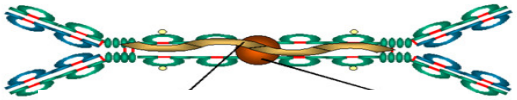


The class of the antibody is determined by the heavy chain of a particular antibody molecule. Classification into five classes of antibodies, each with their functional properties is shown in Table 1.3. The five classes are: IgM, IgD, IgG, IgE and IgA, which have, respectively, two μ, δ, γ, ε, and α heavy chains as well as either two κ (kappa) or two λ (lambda) light chains [Roitt and Delves, 2001; Nairn, 2007].

Table 1.3

Characteristics and Properties of the Immunoglobulin (Ig) Classes

Class of Ig	Structure	Properties
IgM	IgM 	• Exists as a pentamer (a complex of 5 immunoglobulins), linked by a 'J chain' with high molecular weight. • Heavy chains are all μ; light chains are all either κ or all λ. • High efficacy in agglutinating bacteria and fixing complement. • The first Ig to appear in a primary response to an antigen. • Produce antigen-antibody complexes efficiently with both bacteria and viruses
IgD	IgD 	• A membrane bound antibody attached to the B cell that produces it • Functions as a receptor • Heavy chains are both δ; light chains are both κ or both λ • Elevated levels seen in chronic inflammation

Class of Ig	Structure	Properties
IgG	IgG 	• Occurs in four subclasses distinguished by the disulfide linkage between the heavy chains and the length of the hinge region. • Constitutes about 75% of total plasma immunoglobulins. • IgG1, IgG3 and IgG4 can cross the placenta • Heavy chains are both γ; light chains are either κ or both λ.
IgE	IgE 	• Mediates hypersensitivity reactions • Protects the external mucosal surface (mouth, gut) by triggering acute inflammatory reactions • Heavy chains are both ε; light chains are either κ or both λ. • Triggers the release of mast cell granulocytes
IgA	IgA 	• Mainly found in secretions and as a dimer. • Prevents bacterial and viral surface antigens from binding to mucosal surfaces • Heavy chains are all α; light chains are all κ or all λ. • It is critical in preventing the entry of microorganisms through portals of entry

Each B-cell makes only one class and type of antibody, which is carried like a label on its outer surface. However, although each individual B cell produces only one specific antibody, the B cells of the adaptive immune system have the ability to make millions of different antibodies (10^8 or more) overall. This allows for the recognition of and response to almost every possible antigen that could exist even before the antigen is ever encountered [Roitt and Delves, 2001]. B-cells express co-receptors such as CD19, which is important in signaling pathways and which is also used as a marker for identification of B cells [Ollila and Vihinen, 2005].

Naïve B cells (resting mature B cells that have not been stimulated to produce antibody) recirculate from blood to lymphoid tissues and back, otherwise they die within few weeks. These naïve B cells are carried to the lymphoid organs such as spleen, lymph nodes and mucosa-associated lymphoid tissue. When the specific antigen (for example on the surface of a pathogenic microbe or on an antigen-presenting cell) is bound to its specific antibody on the B-cell, signals are triggered (phospholipase C, the Rho family of GTPases, Ras and phosphatidylinositol-3-kinase pathways activation), and the B cell divides to form plasma cells and memory cells [Rao, 2006]. Three important events occur during this differentiation process. First, somatic hypermutation alters the antigen binding properties, leading to the formation of high affinity clones. This is the result of deletions, insertions and point mutations into the V, D and J segments in the heavy and light-chain variable region genes coding for the antibody. Second, class switching, which is due to the substitution of the appropriate C domain on the heavy and light chains encoding for the different types of Igs, changes the effector activities (i.e. alteration of how B cells are recognized by effector cells or isotype switching on B cells) of the molecule without changing the specificity of the antibody [Ollila and Vihinen, 2005; Gennery and Cant, 2006]. Thirdly, there is the formation of memory cells which generate long-lived plasma cells which can respond quickly to generate immunogloblins if that specific antigen is encountered again [Gennery and Cant, 2006].

The antibody produced is determined by the specific antigen that stimulated its production, and it will bind the same antigen when encounters it in the circulation or tissue spaces [Engelke et al., 2007]. After antibodies bind to antigens on pathogens, they act to promote agglutination and enhance phagocytosis of pathogens by the process of opsonization. Moreover, antibodies can induce direct killing of the pathogen by activating the complement system [Actor, 2007].

In addition to B cells and their antibody products, T cells also form a crucial part of the adaptive immune system. These express antigen-specific receptors on their surface, the T cell receptors (TCR), which recognize specific antigens but only if presented on a cell membrane and bound to MHC [Chapplin, 2006; Rubin, 2009]. All T cells express a cell surface marker called CD45. But there are two well defined subpopulations of T cells that express additional surface markers: the CD4+ (T helper cell) and the CD8+ (T cytotoxic cell). Both CD4 and CD8 are protein markers that are expressed on the surface of these cells and can be used to identify and quantify these two sub-types of T cells [Calder, 2007]. T helper cells (CD4+) recognize and bind antigens complexed to MHC class II on antigen-presenting cells (dendritic cells, and macrophages). In addition, the CD4+ T cells are further classified as T helper 1 (Th1) and T helper 2 (Th2) cells, depending on the type of cytokines they produce [Harrington et al., 2005]. Th1 cells secrete cytokines (i.e. Tumor necrosis factor and interferon-α) that activate cytotoxic T cells and macrophages and are also involved in delayed-type hypersensitivity [Zabriskie, 2009]. Th1 cells mainly cause cell-mediated inflammatory response, as the cytokines produced activate macrophages and NK cells to kill cells infected with (and so expressing antigens from) pathogens such as mycobacteria, fungi and protozoa. Th2 cells secrete cytokines (i.e. IL-4, IL-5, IL-10 and IL-13) that activate B cells. Th2 response mainly favours antibody production and it is associated with allergic responses. This is because the cytokines Th2 cells produce induce B cell activation, class-switching, and growth of eosinophils [Chapplin, 2006; Zabriskie, 2009].

T cytotoxic cells (CD8+) recognize MHC Class I-bound antigens, which are presented on virus-infected cells or cells that have been changed in some way [LaRosa and Orange, 2008]. T cytotoxic cells kill their target cell by activating programmed cell death (apoptosis). The cytoplasm of T cytotoxic cells contains perforin and granzymes which are released onto the target cells without damaging neighboring healthy cells. These proteins are released by calcium signaling from the T cell receptor (TCR). Perforin disrupts the cell membrane by forming pores and facilitates the entry of granzymes to induce apoptosis [LaRosa and Orange, 2008]. Therefore, while the initiating event is different, the mechanism by which T cytotoxic cells kill infected or changed cells is the same or similar to that of NK cells.

The cells of the adaptive immune system and their functions are summarized in Table 1.4.

Table 1.4

Cells (Leukocytes) of the Adaptive Immune System

Cell Class or Sub-set	Characteristic Surface Marker	Function
B-lymphocytes	CD19+	Produce and secrete antibodies, each of which recognizes a different foreign antigen; Provide humoral immunity against extracellular pathogens.
CD8+ T-lymphocytes 'Cytotoxic' T cells	CD3+, CD8+	Eliminate foreign cells or transformed cells and cells that have been infected by viruses or intracellular pathogens.
T-lymphocytes CD4+ 'Helper' T cells Th1, Th2, Th17 cells	CD3+, CD4+	Th1 produce cytokines such as IFN-γ, lymphotoxin and TNF-β in response to viruses and microbes that infect or activate macrophages and NK cells. Th2 produce cytokines such as IL-4, IL-5 and IL-13 in response to helminthes, allergens and extracellular microbes and toxins. Th17 produces IL-17 to stimulate stromal cells to produce inflammatory cytokines.
T-lymphocytes CD4+ 'Regulatory'	CD4+, CD25+	Suppress T cell responses and prevent autoimmunity

Failure in the adaptive immune system can have severe consequences, but these depend on whether B cells, T cells or both are affected [DeFranco et al., 2007]. In B cell immunodeficiency, which affects antibody production, patients suffer frequent pyogenic infections caused by encapsulated bacteria, as these can resist phagocytic digestion by the cells of the innate system. Patients usually require lifelong immunoglobulin replacement therapy as well as antibiotic treatment. However, they still have normal immune response against viruses and fungal infections as T cells and the innate immunity are unaffected [Ollila and Vihinen, 2005]. In T cell deficiency, both the cell-mediated and humoral immune responses are affected. This is because T cells are the main players in cell-mediated and humoral immune response. Therefore, patients with T cell deficiency usually have decreased antibody production as well as increased infections caused by intracellular pathogens such as bacteria (e.g. *Mycobacterium*), protozoa, viruses and fungi [Zabriskie, 2009].

The integrated immune system

The immune system is divided into two components: adaptive and innate. However, both components work within an integrated system to eliminate foreign materials and damaged cells [Christensen and Thomsen, 2009]. In the course of an immune response, the innate immune system is the front-line of defense which blocks, recognizes and destroys invading pathogens through barriers, phagocytes and NK cells. The activation of adaptive immunity requires a signal from the antigen presenting cells of the innate immune system. Furthermore, it is by the function of components of the innate system, such as complement and cytokines, that cells of the adaptive immune system are attracted to sites of infection and activated [Chabalgoity et al., 2007]. In turn, the activities of the adaptive system affect cell activity in the innate system. The cross-talk between the innate and adaptive systems is via cytokines produced by all the cells of the immune system.

Cytokines are molecules that act as messengers to link the adaptive and innate immune systems and are named according to their function and the type of cells they are secreted from. There are many cytokines and these are secreted by many types of

cells in the immune system, and there is both overlap and diversity in their properties and effects. [Chabalgoity et al., 2007]. Leukocytes secrete cytokines called interleukins (IL). Chemokines have chemo-attractant activity. Interferons (IFN) interfere with (suppress) viral replication and cytokines that cause differentiation and proliferation of stem cells are called Colony Stimulating Factors (CSF). Cytokines bind to immune cell receptors in order to carry out their effect on cells. There are three types of receptors: cytokine receptor family, chemokine receptors and the TNF (tumour necrosis factor) receptors [Roitt and Delves, 2001]. These receptors usually are not expressed in high levels, but are upregulated following cell activation [Haddad, 2002]. Cytokines are multifunctional and can have antagonistic as well as synergistic effects when working together. Their functions can be summarized into four broad categories [Rao, 2006]:

- Stimulation of growth and differentiation of immature leukocytes
- Mediation of innate immunity response
- Regulation of activation, growth and differentiation of lymphocyte
- Regulation of immune-mediated inflammation.

Cytokines play an important role in various elements of the immune response, including acute inflammatory response, development of specialized T-cell response and inhibition of the immune response. For instance, IL-1, IL-6 and TNF stimulate local inflammation after being secreted by macrophages. Cytokines IL-10 and TGF-β (transforming growth factor-β) have an inhibitory effect on the immune response, and IL-2 induces T-cell proliferation [Cebo et al., 2002]. A simplified view of the role of a few cytokines is shown in Figure 1.3, and a summary of the innate and adaptive systems interacting within an integrated immune system is shown in Figure 1.4.

Figure 1.3

Simple Schematic Diagram of the Roles of Selected Cytokines in the Immune Response

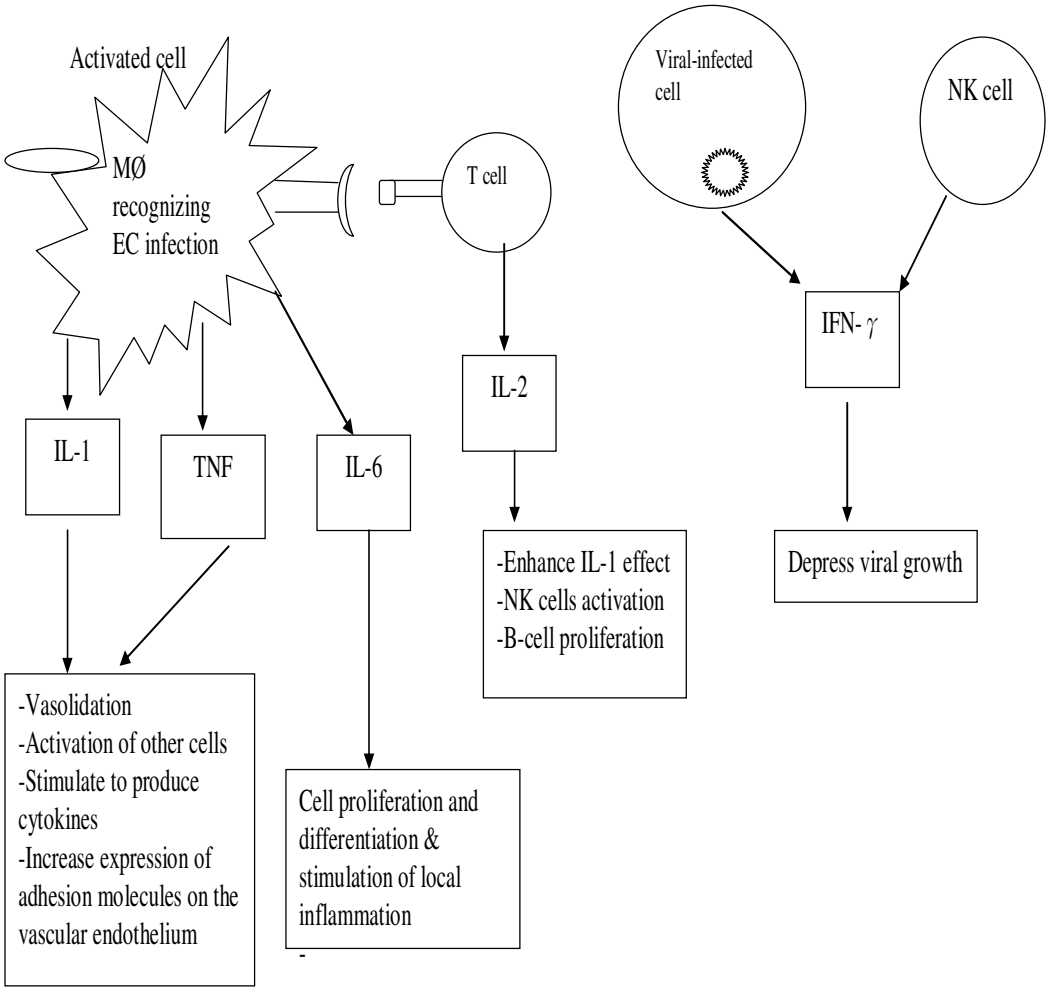
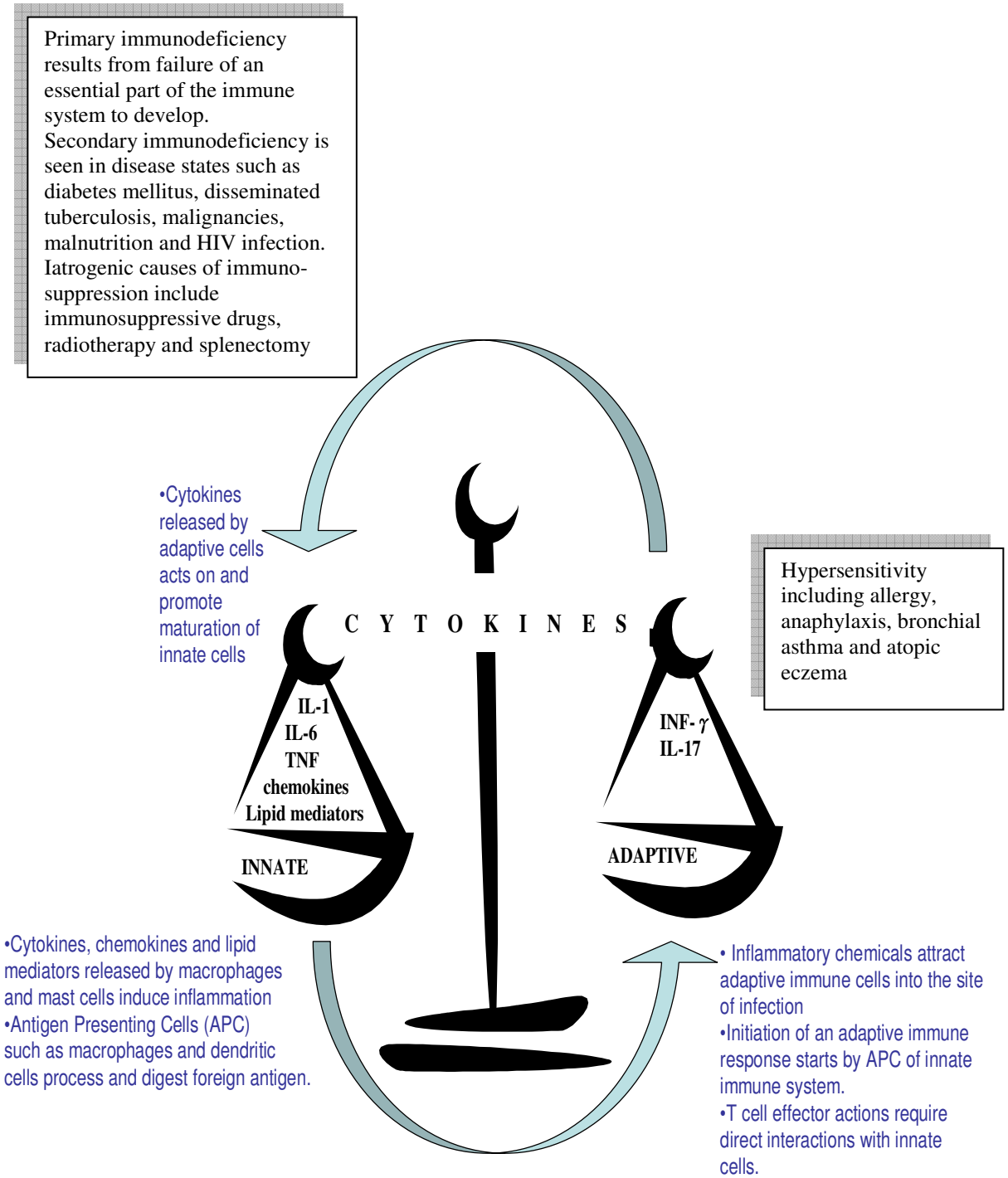


Figure 1.4

The Integrated Immune System and Immune Disorders



The immune system in action

The immune system acts through different levels of interaction from a variety of activating stimuli, including lipopolysaccharides on the bacterial wall, MHC molecule-antigen complex on antigen presenting cells and on foreign (transplanted) cells. Several signals which are activated when the immune system is challenged include cytokines, complement system, chemokines and antibodies [Calder, 2007].

Response to infection

A pathogen that has breached the barriers of first line defense mechanisms and which has successfully entered the body finds a suitable microenvironment for replication. In immune defense, the innate immune system is firstly triggered. Chemokines released from infected cells and complement components attract leukocytes to the site of infection. Complement also mediates antibody-independent opsonization and enhances phagocytosis. Afterwards cells such as macrophages and dendritic cells (antigen-presenting cells) process soluble protein antigens into peptides and express them on MHC molecules on their surface, which activate the adaptive immune response by interacting with T cells [Rao, 2006]. This interaction between antigen presenting cells (APC) and T cells is mainly determined by co-stimulators on the cell membrane. For instance, CD80 (B7-1) and CD86 (B7-2) on the APC interact with CD28 and CTL4 on the T cell. The importance of these co-stimulators is emphasized by the fact that lack of these molecules does affect immune response. For example, CD28 (-/-) mice, which lack CD28 expression, have severely impaired adaptive immune response [Marks et al., 2007]. The adaptive immune response is characterized by the proliferation of antigen-specific T and B cells after their receptors have interacted with antigens presented on cells from the innate system [Gennery and Cant, 2006].

Response to changed or infected cells

Cell-mediated immune response is responsible for the clearance of infected cells and their intracellular pathogens, as well as removing changed cells. The MHC molecule complexed with antigens is displayed on virus infected cells and altered cells. T-

cells recognize these specific antigens by the TCR and are then activated. This is followed by proliferation and differentiation into memory and other effector cells [Chapplin, 2006; Rao, 2006]. Cyototoxic activity by T cytotoxic cells is brought about by secreting proteins such as perforin and granzymes on the target cells without damaging neighboring cells. Similarly, NK cells induce apoptosis by recognizing changed or altered cells lacking MHC I molecule. Other effector cells such as T helper cells produce different cytokines to activate other cells including B cells and cytotoxic T cells [LaRosa and Orange, 2008].

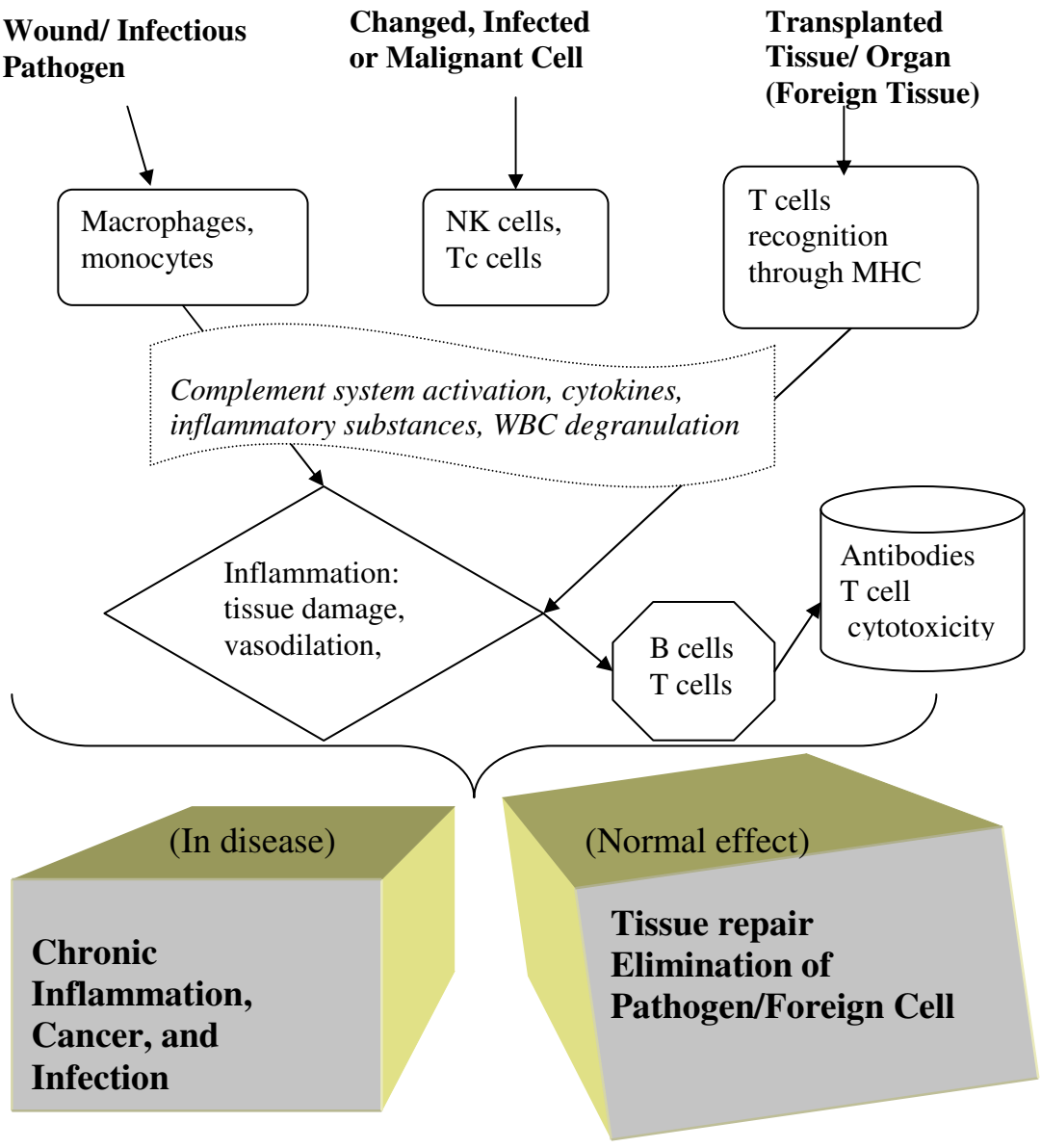
Response in transplantation

Transplantation is the replacement of a failed organ/tissue by a transplant of healthy tissue from a different site or a different body. If recognized as foreign the transplant ('graft') is rejected by the immune system. There are different types of transplants: autograft, in which tissue is relocated within same donor (e.g skin graft); isograft, in which tissue or an organ from a genetically identical individuals is transplanted; allograft, in which tissue or an organ from a different individual of the same species is transplanted; and xenograft, in which the transplanted material is from a different species [Lynch and Platt, 2009]. Here the mechanism of graft rejection will be related to allograft only. In this case graft rejection is principally by cell-mediated immune response. MHC is the major cause of graft rejections and T cells are implicated in the process [Lynch and Platt, 2009]. The process of graft rejection is divided into two stages: sensitization phase and effector phase. In the sensitization phase, lymphocytes (CD4+ and CD8+) recognize the alloantigens on the cells of the graft and become activated and undergo proliferation. In the effector phase cytokines released by lymphocytes play an important role in allograft rejection and various effector mechanisms are involved. These include a cell-mediated reaction, antibody-dependent cell-mediated cytotoxicity or antibody-plus-complement lysis [Rao, 2006].

A summary of the immune system response to different stimuli or initiating events is presented in Figure 1.5.

Figure 1.5

Overview of the Immune System Response to Different Stimuli



The human immune system is an extremely complex system which is crucial for health and prevention of diseases, in particular infectious diseases and cancer. Therefore, assessment of immune status is needed to diagnose immune system defects and decline, as well as assessing the effects of immunomodulatory treatments.

Assessing Immune Status: a biomarker approach

In the last decade the complexity and sensitivity of tests used for the assessment of immune status have expanded tremendously and scientific knowledge in clinical immunology has increased rapidly. Laboratory methods range from simple and routine procedures such as total white blood cell (wbc) counting and white cell differential testing to complex research techniques that can evaluate immune defects using cell culture and analysis of white cell subsets using surface markers and flow cytometry [Folds and Schmitz, 2003; Corbett, 2008]. In addition, plasma levels of specific immunoglobulin classes and cytokines can also be measured [Wallach, 2007]. Inflammatory markers are also often assessed.

Enumeration of cells by flow cytometry (immunophenotyping)

Flow cytometry has become useful in the evaluation and diagnosis of many diseases and offers a means to detect cell surface antigens by a technique called immunophenotyping. This is most often achieved through the use of fluorochrome-conjugated monoclonal antibodies directed against lineage-specific/associated antigens, the characteristic surface markers, on white cells. Immunphenotyping is used to reveal information on the absolute and relative amounts of white cell subsets in blood plasma [Macey, 2007].

Flow cytometric immunophenotyping for determining immune status has become the standard laboratory practice in the study of cellular immune response [Henry, 2001]. Recent advances in instrumentation, computer technology and flurochrome chemistry have made flow cytometry the most important tool in the evaluation of immunological status. Moreover, using flow cytometry reduces variability in

lymphocyte counts caused by differences in white cell count estimation by haematology analyzers [O'Gorman, 2000]. It has been reported that this 'single-platform' flow cytometric approach provides better coefficients of variation and a lower probability to generate inaccurate results [Chng et al., 2004]. Therefore, this method has been suggested as the gold standard for lymphocyte subset cell counting [Brando et al., 2000].

Immunoglobulin measurement

The level of serum or plasma immunoglobulins reflects humoral immunocompetence, and a number of factors such as sex, age, ethnicity and genetic factors affect the normal immunoglobulin (Ig) levels [Folds and Schmitz, 2003]. There are various laboratory techniques available for determining immunoglobulin levels and most of them rely on the reaction between soluble antigen and antibody which leads to the formation of visible precipitate. The radial immunodiffusion test is performed in an agarose gel that contains an antibody to an Ig class, and the diameter of the precipitin ring formed is proportional to the concentration of the Ig [Rao, 2006]. Nephelometry is a technique that uses light scattering by soluble immune complex formation [Folds and Schmitz, 2003]. Enzyme Linked Immunosorbent Assay (ELISA) is another relatively simple, specific and highly reproducible method used for measuring the common Ig classes and subclasses in cell culture supernatants and other biologic fluids such as serum [Nutman, 1991]. The principle in this assay relies on an enzyme and substrate reaction and the formation of coloured substrate complexes. Firstly, antibody to an Ig class is adsorbed on the well surface of microtiter plate. The fluid containing the Ig of interest is added to the well and is captured by the antibody. Then, a second antibody to a different region of the same Ig and conjugated to an enzyme (typically horseradish peroxidase or alkaline phosphatase) is added, and binds to the captured Ig. Substrate solution is added and the intensity of colour developed is dependent upon the amount of enzyme labeled 2^{nd} antibody bound, which is proportional to the amount of Ig captured by the 1^{st} antibody, and therefore to the amount of Ig of interest in the test fluid [Nutman, 1991; Rao, 2006].

Cytokine measurement

The assessment of cytokines is of value because these molecules have an important role in immunity, inflammation, apoptosis and hematopoiesis [House, 2001]. There are several types of assays available to assess the level of cytokines. The choice of method depends on the source of test sample (circulating plasma, fluid or effusion from an inflamed site, or intracellular fluid). Bioassays measure the effect of cytokines in living materials such as cells and isolated tissues by inducing processes such as proliferation, maintenance of viability, migration and inhibition of function, which release IL-1, IL-2 and IL-10 cytokines respectively [House, 2001]. Immunoassays such as ELISA are used to measure cytokines in cell culture supernatant fluid, plasma or serum. Molecular biology techniques, which are extremely specific and sensitive, examine the genetic material responsible for cytokines production at a single cell level. Finally flow cytometry can also be used to analyze cytokine production at a single-cell level, simultaneously with other cell phenotypic markers [House, 2001].

Complement system measurement

The complement system is a major component of the innate immune system and it is important as it provides first line of defense for the host [Folds and Schmitz, 2003]. In order to assess complement activity the total haemolytic complement (CH50) is the basic screening test. This measures the complement activity in plasma by measuring haemolysis of sheep erythrocytes that have been coated with anti-sheep erythrocyte antibody. ELISA is another important assay to assess the Mannan-binding lectin pathway as well as C1 inhibitor function. Also, the individual components of the complement system can be quantified by immunochemical methods such as immunoprecipitation assays (i.e. nephelometry, radial immunodiffusion and radioimmunoassay) [Wen et al., 2004].

Other tests of immune status

More specialized tests to study and evaluate the function of the immune system cells and immune status include:

- Delayed type hypensensitivity skin testing: This is used to evaluate cell-mediated immunity *in vivo*. It is performed by inoculating a defined amount of standardized antigen intracutaneously and monitoring for the presence of induration (hardening of the skin) after 48 to 72 hours [Folds and Schmitz, 2003].
- Lymphocyte proliferation assay: a standard *in vitro* test is used to determine lymphocyte function by assessing the ability of lymphocytes to proliferate in response to different stimuli [Folds and Schmitz, 2003].
- Enzyme linked immunospot (ELISPOT): quantifies the secretion of cytokines by lymphocytes. This assay is performed by incubating white blood cells or T lymphocytes, which are stimulated by a mitogen or specific antigen, in wells containing anti-IFNγ capture antibody on a membrane. The stimulated cells produce IFNγ which is captured on the membrane and the cytokine produced is quantified by counting the number of spot-forming cells per input cell number [Schmittel et al., 1997].
- Respiratory burst activity of neutrophils: assessed by nitroblue tetrazolium dye reduction test, chemiluminescence and flow cytometry [Folds and Schmitz, 2003].

Reference ranges of immune system parameters and inflammatory biomarkers

In the following Table 1.5, the indicative normal values of different parameters for the assessment of the immune status are shown.

Table 1.5

Expected Values for Biomarkers of Immune and Inflammatory Status in Healthy Adults (data from Kam et al., 1996; Henry, 2001; Sertic et al., 2007).

Cell type	**Reference range in adult population**		**Laboratory methodology**
WBC Differential	**% of total wbcs**	**number/µL**	Manual or automated cell counting and scoring by appearance or staining differences of different types of white cells
Neutrophils	56	1800-7800	
Eosinophils	2.7	0-450	
Basophils	0.3	0-200	
Lymphocytes	15-46	1000-3200	Flow cytometry Automated blood count analyzer And Manual blood count
Monocytes	3-14	0-800	
Granulocytes	45-80	2200-7800	
Total white blood (WBC) count	-	5,000-10,800	
	% out of total lymphocytes	**number/µL**	
CD3+ CD4+	23-51	290-1400	
CD3+ CD8+	18-48	240-1000	
CD4/CD8 ratio	0.5-1.8		
CD19+ CD3-	5-21	82-560	
NK cell	7-38	130-938	
Immunoglobulins: IgM IgG IgA IgD IgE	 54-222 mg/dL 800-1801 mg/dL 133-563 mg/dL 0.5-0.3 mg/dL 0.01-0.04 mg/dL		Commercial kit methods employing nephelometry or ELISA
Cytokines: IL-1 TNF IL-6 IL-8	 0.1-374 pg/mL 0.1-188 pg/mL 0-30 pg/mL 2.5-314 pg/mL		ELISA
	Risk of cardiovascular disease		
hsCRP	Low risk <1.0 mg/L Average risk 1.0 - 3.0 mg/L High risk>3.0 mg/L		Commercial kit method using immunoturbidimetry or chemiluminescence

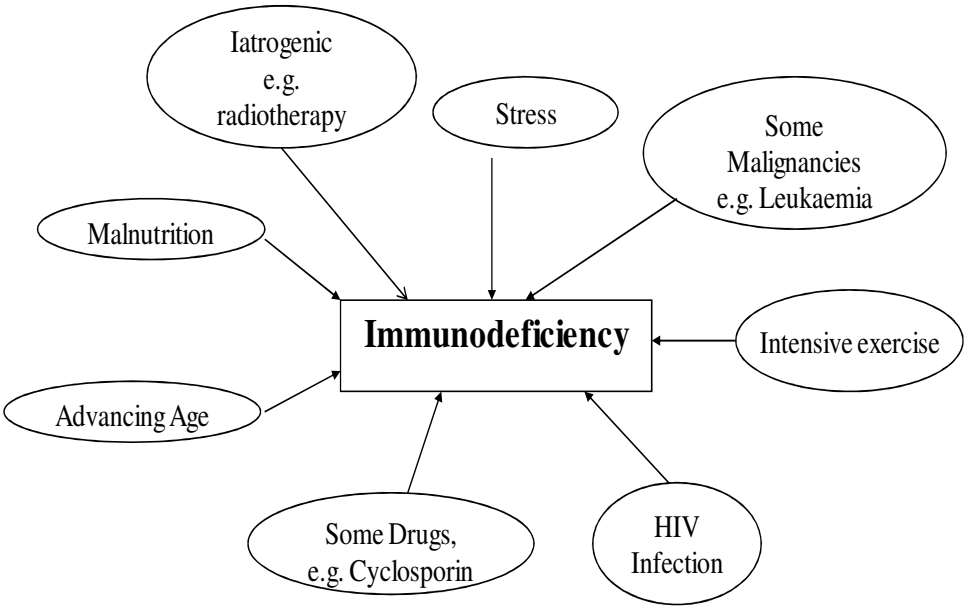
Factors Affecting Immune Status

If either part of the immune system is compromised, health is threatened. The problem may arise from a genetic or developmental problem in the immune system (primary immunodeficiency) or it can be acquired (secondary immunodeficiency). Acquired disorders of immunity are common and have many causes, and can be divided into two categories [Sompayrac, 2003]:

1. Conditions caused by an immune response that is excessive, as seen in hypersensitivity reactions, or inappropriate, for example autoimmunity.
2. Diseases due to an immune response that is inadequate, as seen in drug-induced immunosuppression, or immunodeficiency associated with HIV infection, malnutrition, stress, intensive exercise, and advancing age [Nairn, 2007] (Figure 1.6).

Figure 1.6

Immunodeficiency and Its Causes



In the rest of this review the focus will be on the effect of age, exercise, stress and nutritional deficiency on immune status and how these effects may be prevented or reversed by dietary and herbal strategies. The role of oxidative stress as a modulator of immune status will also be described briefly.

Ageing

Ageing has a marked influence on the immune system and it has been observed that there is a decline as well as dysregulation of the immune function as we age [Hakim and Gress, 2007]. As reviewed by Chandra, according to many longitudinal studies that followed young individuals for several years, the impairment of immunocompetence starts around 35-40 years of age [Chandra, 2004]. It has been shown that the absolute number of several immune parameters decreases with ageing. These include the absolute number of T lymphocytes subsets and B lymphocytes [Sansoni et al., 1993]

The most prominent change in the ageing immune system is involution of the thymus, which is related to loss of T-cell progenitor population, loss of self-peptide expressing thymic epithelium, defective gene associated with T cell receptors and ageing of thymic microenvironment [Aw et al., 2007; Gruver et al., 2007]. The thymus is the place where differentiation, positive and negative selection of 'naive' T cells occurs. After selection, the resulting mature T-cell populations are self MHC-restricted and self-antigen-tolerant [Hughes et al., 2004]. These T-cell populations are responsible for recognizing foreign antigens and responding to mitogens, important aspects of adaptive immunity. Therefore, thymic involution leads to a significant decrease in naïve T cells [Gruver et al., 2007]. A shift in T lymphocytes subpopulations and alteration of cell surface receptors leads to malfunction of T-cell mediated and T-cell dependent functions [Karasek, 2006]. As a result of T-cell malfunction with age, incidence of infections with new antigens increases as well as delayed-type hypersensitivity (type IV hypersensitivity) [Hankim and Gress, 2007]. It has also been shown that the function of B-cells decreases with age which results in alterations in the quantity of antibodies produced,

as well as specificity, isotype and affinity of antibodies [Lazuardi et al., 2005; Frasca et al., 2005]. As a result, older people have less effective specific antibody response to vaccination with a new antigen such as influenza or tetanus vaccine [Karasek, 2006].

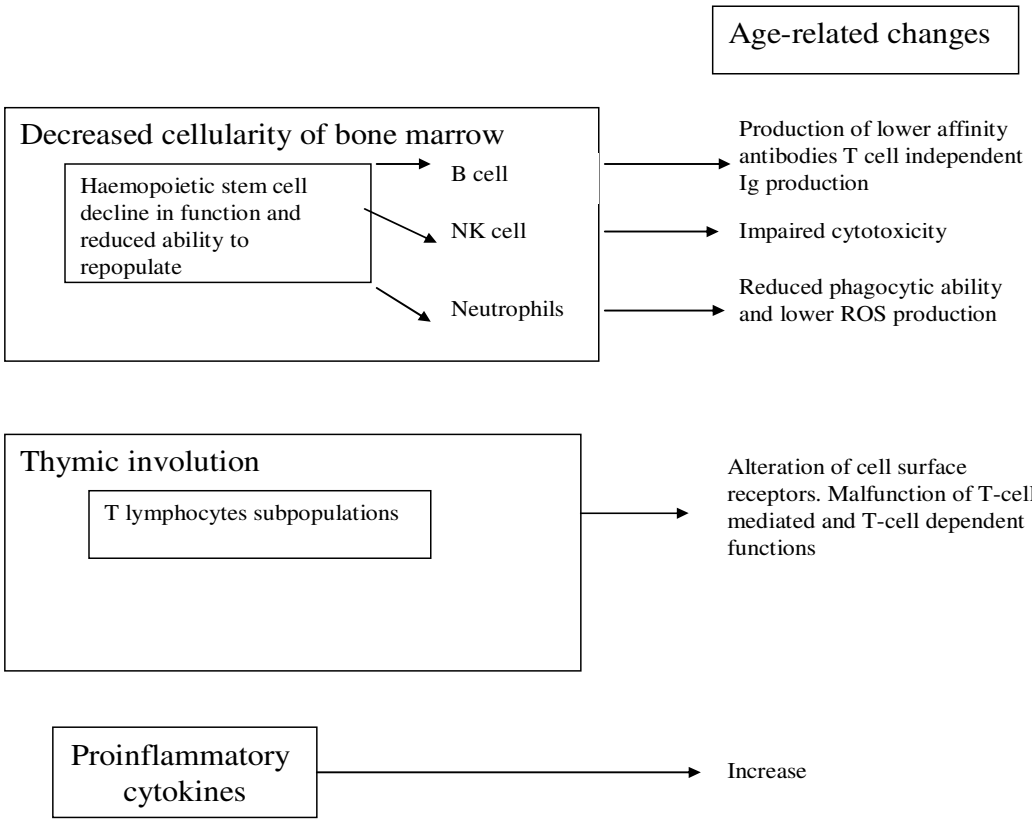
Innate immunity is also affected by ageing and it has been reported that neutrophil function decreases with age making older people more vulnerable to infections [Perskin and Cornstein, 1992]. Other changes that also occur with age can directly affect cells of the immune system. Oxidative stress and glycation for instance are related to the ageing process and these affect DNA, proteins and lipid structures [Hipkiss, 2006]. These changes might be expected to affect the functioning of cells, including immune cells. In a study where the DNA breaks and oxidized purines in lymphocytes (markers of oxidative stress) from 57 old subjects (63-82 years) and 40 young subjects (20-35 years), assessed by the comet assay, it was found that in the old age group there was higher oxidative base DNA damage ($P<0.001$) than in the younger group [Humphreys et al., 2007]. In another study, vegetarian diet was shown to prevent increase in oxidative damage in aging. Indeed, lower oxidative stress and higher antioxidant status was found in old vegetarian group (60-70 years) compared with age-matched nonvegetarians (n=34). Moreover, the old vegetarian women did not show higher oxidative stress compared to the young vegetarian women (20-30 years) [Krajcovicova-Kudlackova et al., 2008]. However, it is not clear whether increased oxidative stress might be due to ageing itself or vice versa. A poorer diet in the elderly or undiagnosed disease might also contribute to increased oxidative stress [Benzie and Wachtel-Galor, 2009].

Alterations in lipids affect the lipid raft in the membrane of cells as a result of increased level of oxidized lipids [Karasek, 2006]. Furthermore, the decline of immune responsiveness is also largely affected by the cumulative effects of ageing in the bone marrow and thymus which directly affect the haematopoietic stem cell and lymphoid progenitor pools [Lazuardi, 2005]. Furthermore, there is an association between dysregulated cytokine production and ageing [Kahman et al.,

2008]. In a study where the cytokine production by monocytes and T cells in elderly subjects was compared with younger women and men, it was observed that cytokine production was altered with ageing. The production of IFN-γ, IL-2, IL-4, IL-10 and IL-13 by different subset of T cells was higher in the elderly than in the young subjects, whereas several cytokines produced by monocytes was lower in elderly compared to young subjects [Pietschmann et al, 2003]. However, contrasting results have been reported as reviewed by Rink et al. [1998]. They described that in various studies lymphocytes from the elderly produce less IL-2 and IFN-γ and related these findings with the impaired immune response observed in the elderly [Rink et al., 1998]. These age-related changes in the immune system are summarized in Figure 1.7.

Figure 1.7

Age-related Changes in the Immune System



Immune decline increases risk of chronic degenerative diseases, and it is suggested that age-related diseases and conditions such as increased susceptibility to infection, lower response to vaccinations, atherosclerosis, Alzheimer's disease, and cancer can be prevented by optimal functioning of the immune system [Hughes et al., 2004; Sansoni et al., 2007]. In this regard, nutrition has been shown to play a crucial role in the immune system and it has been suggested that nutritional status can affect immune function as well as immunity development [Calder at al., 2006]. Exercise is also a lifestyle factor that can affect immune status, as can psychological stress.

Nutrition

Diet has an important impact on the immune function [Bengmark, 2006].The adverse effect of protein energy malnutrition on immunity has been shown in numerous studies [Gershwin et al., 2004; Fock et al., 2007; Xavier et al., 2007]. Protein calorie malnutrition can result in immunodeficiency, leaving the individual at risk of infection [Chadra, 1983]. In a study in which 44 children with different degrees of infection and malnutrition were evaluated, it was found that malnutrition and infection affect the proportion of lymphocyte subsets when compared with well-nourished non-bacterium-infected children, with a significant decrease in the proportion of T-lymphocytes seen in malnourished children [Najera et al., 2004]. Moreover, effects of deficiencies in micronutrients such as copper, zinc, iron, folate and vitamin B12 have been found to affect the immune function [Bogden et al., 1999; Samman, 2000]. For instance, zinc supplementation has been associated with a reduction in the prevalence of pneumonia and malaria [Hambidge, 2000]. Also zinc deficiency is said to affect facets of the immune system such as activation of lymphocytes, Th1 production and B lymphocyte development and antibody production [Sharkar and Prasad, 1998]. Indeed, micronutrient deficiency has been shown to suppress the immune system by affecting the responses of innate system, T-cell mediated and adaptive antibody responses. In particular, supplementation with vitamin B12, iron and copper has been shown to support the protective activities of the immune cells and thus immune function [Maggini et al., 2007]. Therefore, a lot of attention has been put in the effect of nutrition on the immune

system and many research studies have focused in the effect of nutritional supplements or agents on the prevention or reversal of immune decline. Good nutrition is suggested to prevent immune decline. For instance, in a study in which the immune status of well-nourished 75 older women (62-88 years) and 35 younger women (20-40 years) were compared, it was found that the total T-cell (CD3+), T-helper (CD4+) and T-cytotoxic (CD8+) cell number of the older group did not differ significantly from the younger group, and it was suggested that few age-related effects on cell-mediated immunity were shown in women with adequate nutritional status [Krause et al., 1999]. In another study, zinc supplementation has been suggested as an anti-ageing agent and inducer in the immune system [Putics et al., 2008]. It was reported that zinc supplementation in healthy old subjects (n=20) safely and efficiently induced the stress response in lymphocytes. The stress response involves various stress proteins (e.g. Hsp70) which confer cytoprotection and promote survival due to cellular adaptation to various stresses [Putics et al., 2008]. The results of Putics et al. [2008] were consistent with other studies reporting the positive effect of zinc supplementation on the immune function in the elderly [Bogden et al., 1994; Chandra 1992].

Some nonessential (non-nutrient) components of human diets, including several phytochemicals, have been shown in *in vitro* study to have significant immunomodulating potential [Matthias et al., 2007]. Plant-based polysaccharides found in lingzhi (*Ganoderma lucidum*) mushrooms were found to increase the activity of immunological effector cells in immunosuppressed mice. The effect was reported with low-dose polysaccharides of *G. lucidum*, resulting in accelerated recovery from leucopenia, myelosuppression, and all common conditions associated with cancer therapy [Zhu et al., 2007]. Efficacy of *G. lucidum* in stimulating various immune effectors has been demonstrated in several *in vitro* and animal studies [Wachtel-Galor et al., 2004]. In human study, an immunomodulatory effect of lingzhi was shown in an uncontrolled clinical trial on patients with advanced colorectal cancer [Chen et al., 2006]. In this study, various immune markers were reported to increase, though not significantly, after treatment with *G. lucidum*

polysaccharides [Chen et al., 2006]. However, the immunomodulating effect of *G. lucidum* polysaccharides is yet to be elucidated and further studies in well designed controlled trials are needed.

Exercise

Exercise can have both positive and negative effects on immune function. For instance, it has been suggested that a moderate level of exercise may enhance immune function, whilst excessive amounts of prolonged, high intensity exercise may impair immune function [Gleeson, 2007]. As reviewed by Brolinson and Elliot and Gleeson [2007], a substantial number of studies have reported that intensive exercise is associated with an increased risk of upper respiratory tract infection as well as having a detrimental effect on immune cell functions [Brolinson and Elliot, 2007; Gleeson, 2007]. In a study in which 21 males underwent 3 weeks of training followed by a 5-day combat course, it was reported that the incidence of upper respiratory tract infection was increased during the trial, and the salivary IgA decreased after the 5-day course [Tiollier et al., 2005]. Also, bouts of prolonged, continuous heavy exercise have been shown to affect cell function. The NK cell counts as well as NK cell responsiveness significantly decreased in athletes (n=9) after a second bout of high-intensity exercise [Ronsen et al., 2000]. In contrast, moderate physical activity has been suggested to have many beneficial effects on the immune system. For instance, the positive effect of exercise was shown in a study in which a two-hour judo session in 22 males (19.1±0.8years) significantly increased the neutrophil count, the ratio of neutrophil and leukocytes count and serum IgG count after the session when compared with the values immediately before the session [Umeda et al., 2007]. Also, it has been suggested that routine exercise is associated with a lowered risk and reduction in recurrence of colon and breast cancer [Lee, 2003]. In an animal study, exercise enhanced mucosal T-cell proliferation, cytokine production and IFNγ production in the spleen in female C57BL/6 mice [Rogers et al., 2007]. The authors suggested that exercise may be an effective intervention used alone or in combination with other cancer-prevention strategies in order to enhance lymphocyte function [Rogers et al., 2007].

Psychological Stress

Stress has a suppressive action on the immune system and there is a substantial body of evidence that indicates that stress affects the immune system in various ways [Elenkov and Chrousos, 1999; Kiecolt-Glaser, 1999; Kiecolt-Glaser and Glaser 2002]. Stress results in the release of glucocorticoids and catecholamines (stress hormones released by the neuroendocrine system) and these have been associated with the inhibition of lymphocyte proliferation, migration and cytotoxicity and decreased secretion of certain cytokines (i.e. IL-2 and IFNγ) [Elenkov and Chrousos, 1999]. Moreover, in many studies the effect of stress such as that experienced by students during examinations, people with low quality of personal relationships, and family carers of dementia patients, has been shown to affect immune function. It was reported that people with high levels of stress have lower immune response to hepatitis B vaccine, lower NK cell cytotoxicity and higher susceptibility to infection [Kiecolt-Glaser, 1999]. More severe is the combined effect of chronic stress and depression. For instance, depression has been associated with increasing morbidity and mortality due to dysregulation of proinflammatory cytokine production [Kiecolt-Glaser and Glaser, 2002] whilst chronic stress may result in prolonged immunosuppression [O'Leary, 1990].

Oxidative stress

Oxygen-derived free radicals are important for the normal functioning of both natural and acquired immunity. Production of reactive oxygen species (ROS) by macrophages during their respiratory burst kills bacteria, but ROS can be detrimental to the immune system [Benzie, 2000; Knight, 2000a]. Free radicals are reactive species that contain one or more unpaired electron and they can attack DNA, proteins and lipids [De Zwart et al., 1998; Benzie, 2003; Seifried et al., 2007]. High concentrations of free radicals can cause oxidative stress and lead to impaired immune response, loss of cell membrane integrity, altered membrane fluidity and alteration of cell to cell communication, leading to degenerative disorders such as cancer and cardiovascular disease [Maggini et al., 2007].

There is a link between oxidative stress and the immune system function. For instance, *in vitro* studies have shown that oxidative stress decreases T cell function and impairs TCR signaling [Gringhuis et al., 2002; Larbi et al., 2007]. Heavy exercise, which is associated with a substantial production of ROS [Packer, 1997] has been shown to affect the immune function. A study, in which 19 endurance-trained athletes performed treadmill exercise until volitional exhaustion showed that the population of activated CD4+ and CD8+ as well as the total concentration in blood of CD3+, CD4+, CD8+ and NK cells were decreased [Vider et al., 2001]. Decreased lymphocyte mitogenic response to concanavalin A and phytohemagglutinin were also observed and there was a significant increase in the lipid peroxidation, indicating increased oxidative stress caused by the exhaustive exercise [Vider et al., 2001]. Moreover, HIV-patients have altered antioxidant defense, which includes depletion in plasma glutathione, cysteine, ascorbic acid, tocopherols, carotenoids, and selenium leading to chronic oxidative stress, and this oxidative stress observed in HIV-patients has been associated with alterations in immune function [Pace and Leaf, 1995]. The authors suggested that oxidative stress contributes to HIV disease pathology [Pace and Leaf, 1995].

Antioxidant defenses have evolved to protect organisms against the damaging effects of free radicals. These include endogenous antioxidants such as superoxide dismutase, catalase and glutathione peroxidase and molecules from the diet such as ascorbate (vitamin C), α-tocopherol ('vitamin E'), β-carotene and flavonoids, which are important to protect the structural integrity of cells and tissues [Benzie, 2003; Gershwin et al., 2004; Seifried et al., 2007].

Consequences of Immune Decline

A summary of the effects of age, nutrition, exercise, psychological stress and oxidative stress in the immune system is presented in Table 1.6. The consequences of immune system decline with age and in association with nutritional deficiencies, exercise, psychological stress and oxidative stress, are increased risk of infection, more severe infection, lower response to vaccination, and increased risk of cancer

[Karasek, 2006]. There is also increased risk of autoimmune reactions and chronic inflammatory diseases with age [Wick and Grubeck-Loebenstein, 1997]. These alterations in immune functioning have serious clinical implications in relation to morbidity and mortality, in particular in the older population. It is worth noting that old age is often accompanied by depression, malnutrition, lack of exercise, and these may exacerbate age-related decline in immune function. Consequently, maintenance and restoration (modulation) of immune function are important therapeutic targets for promotion of healthy ageing. It is noted that, in Hong Kong, 33% of deaths are due to cancer (according to the HK Department of Health figures), and many cases of cancer would at least be delayed if immune surveillance was maintained. However, while cancer is the main cause of death in Hong Kong, and affects mainly older persons, there is intense attention being paid to the dangers of new infectious disease. In the 2003 SARS outbreak in Hong Kong, most cases were in older people, and mortality in those SARS cases aged 65 years and older was >50% , much higher than in younger persons with SARS [Tse et al., 2003]. Currently, the world is on alert due to H1N1, and high risk cases, such as the frail and elderly, are being offered priority vaccination (according to WHO Global Alert and Response report). However, vaccination will not have the desired effect if the required immune response cannot be mounted. In addition, current therapies for infectious disease often do not work because of emergence and rapid spread of resistance to antibiotics. This is a key issue in healthcare today [Woodford and Livermore, 2009].

In the next section, a brief introduction to bacterial infection and its treatment will be presented. Then, a section on how bacteria become resistant to antibiotics will be discussed followed by the consequences of infection with antibiotic resistant bacteria.

Table 1.6

Summary of Effects of Age, Nutrition, Exercise, Psychological Stress and Oxidative Stress on the Immune System

Immune parameters	**Ageing**	**Nutrition**		**Exercise**		**Psychological Stress**		**Oxidative Stress**
		Micro nutrient deficiency	**Protein Energy Malnutrition**	**Moderate**	**Intensive**	**Acute**	**Chronic**	
Granulocytes	↓			↑				
Monocytes	↓							
Lymphocytes	↓	↓	↓	↑				↓
CD4+	↓		↓	↑				↓
CD8+	↓		↓	↑				↓
B cells	↓	↓	↓					
NK cells	↑				↓↓	↓		↓
Cytokines (IL-1, IL-6, TNF, IL-8)	↑					↓	↑↑	
Immunoglobulins (Ig)	↓	↓		↑↑ (IgG)	↓ (IgA)	↓		

Bacterial Infection and its Treatment

The human immune system, which consists of barriers, cells and substances, works to protect the body against pathogenic microbial agents and their damaging effects by working in a complex but integrated manner. Apart from the natural mechanisms, presented above, there are additional factors that can induce or suppress host immunity, affecting susceptibility to bacterial infection. For instance, in the case of vaccination, administration of antigenic material, which has been inactivated or weakened, produces immunity against a specific infectious disease [Salisbury, 2006]. Vaccines are routinely administered in developed countries as part of public health measures to control (or eradicate) infectious disease such as polio, tuberculosis, smallpox, measles and influenza. Although vaccines can reduce the severity and spread of infections, there are not vaccines available for every microbial agent; moreover the emergence of new strains of pathogens also makes existing vaccines inadequate and ineffective. In addition, and as noted above, those with compromised immune systems, such as the malnourished and elderly, may not respond well to vaccination. Therefore, treatment of infectious diseases, in particular bacterial infections, relies heavily on the use of antibiotics.

Antibiotics can be classified according to several schemes. For instance, they can be broad or narrow according to their bacterial spectrum, or bactericidal (which kill bacteria) or bacteriostatic (which arrest bacterial growth). Antibiotics can also be classified according to their chemical structure, which determines their mechanism of action. This is summarized in Table 1.7.

Table 1.7

Antibiotic Class by Chemical Structure, Mechanism of Action and Year Developed (from Kohanski et al., 2007; Schwalbe, 2007)

Antibiotic class	Examples	Mechanism of action (Activity)	Year made
Sulfonamides	Sulfaisodimidine Sulfamethoxazole	Inhibit folic acid synthesis	1930
β-lactam	Penicillin Methicillin Cephalosporin Carbapenem	Disrupt cell wall (bactericidal)	1943
Aminoglycosides	Streptomycin Gentamicin	Affect protein synthesis (bactericidal)	1943
Streptogramins	Synercid Pristinamycin	Affect protein synthesis (bacteriostatic)	1943
Chloramphenicol	Amphicol Biomicin	Affect protein synthsis (bacteriostatic)	1947
Cephalosporins	Ceftazidime	Disrupt cell wall	1948
Tetracyclines	Doxycycline Demeclocycline	Affect protein synthesis (bacteriostatic)	1948
Macrolides	Erythromycin Clarithromycin	Affect protein synthesis (bacteriostatic)	1952
Glycopeptides	Vancomycin Telavancin Ramoplanin	Disrupt cell wall	1956
Rifamycins	Rifampin Rifabutin	Inhibit RNA synthesis	1957
Lincosamides	Clindamycin Licomycin	Affect protein synthesis	1962
Quinolones	Ciprofloxacin Nalidixic acid	Inhibit DNA synthesis (bactericidal)	1987
Oxazolidinones	Cycloserine Linezolid	Affect protein synthesis	2000

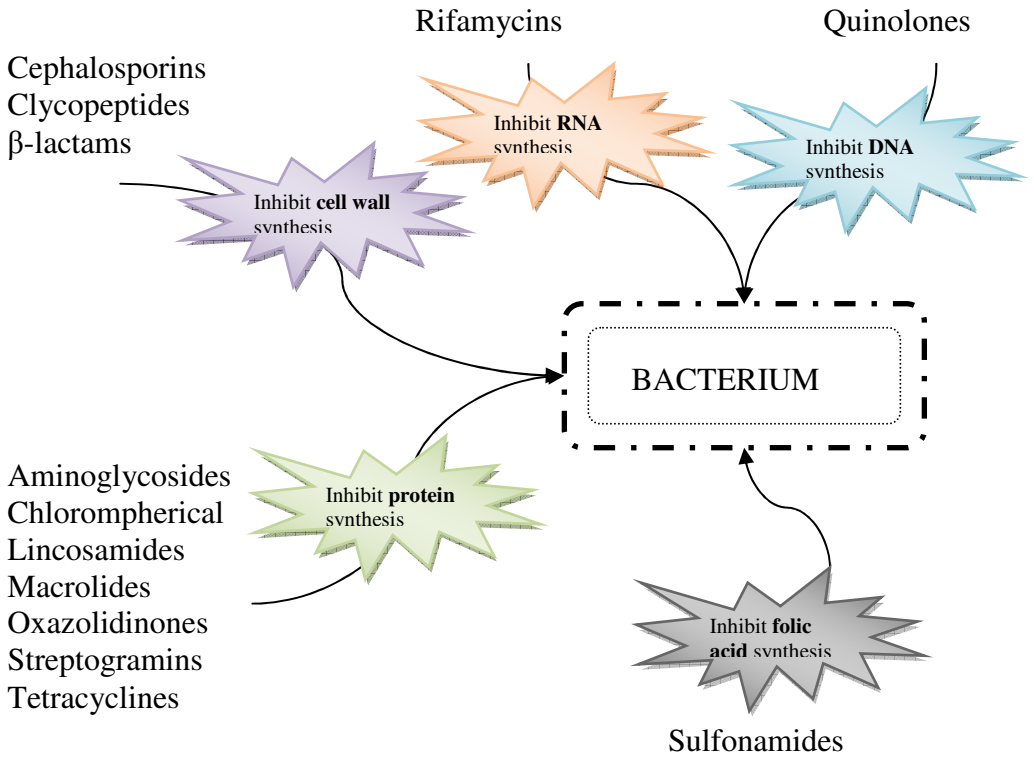
Antibiotics work by targeting structures or processes found in bacterial cells. Many of these are also found in human cells, and some antibiotics can have serious toxic effects if taken in too high dose [Theopold, 1977]. The most common targets for antibiotics are the bacterial cell wall, ribosome, DNA and RNA synthesis and specific enzyme pathways. Many antibiotics target different steps in the biosynthesis

of the bacterial cell wall. For instance, β-lactam antibiotics, such as penicillin, irreversibly acylate the active sites of enzymes such as penicillin-binding proteins (PBP). These enzymes catalyse vital transpeptidation reactions in cell wall biosynthesis, and when acylated, the enzymes are inactivated, with death of the bacterium ensuing due to collapse of the cell wall [Azucena and Mobashery, 2002]. Antibiotics can also interfere with the movement of ribosomes and binding of ribosomal protein, and as a consequence protein synthesis is inhibited. For instance, aminoglycosides exert bactericidal action by interacting with the bacterial ribosomal RNA which leads to the mistranslation of mRNA and thus nonfunctional proteins are produced [Walsh, 2000]. Quinolones are bacteriocidal and they inhibit the bacterial DNA gyrase enzyme which is needed for DNA replication and bacterial cell division [Walsh, 2000]. Rifampin binds to bacterial RNA polymerase and thus inhibits RNA synthesis. As a result, new protein synthesis is prevented causing the death of the bacteria [Walsh, 2000]. Sulfa antibiotics such as sulfonamides bind to and disable an enzyme that is required to convert p-aminobenzoic (PABA) acid to tetrahydrofolic acid, thereby inhibiting the folic acid synthesis in bacterial cells and leading to their death [Walsh, 2000].

In summary, antibiotics have many pathways of anti-bacterial action such as loss of membrane permeability and cell wall integrity, and inhibition of biochemical pathways. These effects and killing mechanisms are summarized in Figure 1.8. However, resistance to these agents is a constant and increasing challenge [Wright, 2005]. This is discussed in further detail in the next section.

Figure 1.8

Antibiotics Types and Killing Mechanisms



Mechanisms of Bacterial Resistance to Antibiotics

There are many different mechanisms by which antibiotic resistance is created, but they are all genetically encoded and so represent an evolutionary process. The nature of resistance depends on the nature of the antibiotic and its action, its target site, the bacterial species and whether it is mediated by a resistance plasmid or by a chromosomal mutation [Azucena and Mobashery, 2001]. The organism may develop genes encoding enzymes, such as β-lactamases or for structures such as efflux pumps that extrude the antibacterial agent from the cell before it can reach its target site and exert its effect. Also, bacteria may evolve several genes for a metabolic pathway which ultimately produces altered bacterial cell walls or they may acquire mutations that limit access of antimicrobial agents to the intracellular target via

downregulation of porin genes [Tenover, 2006]. Once these mutations developed in a bacterium, these innovations can spread through a bacterial population by cell division and by a phenomenon of plasmid exchange [Lorenz and Wackernagel 1994].

Antibiotic modification and inactivation

Antibiotic inactivation can be due to changes in its structure by bacterial antibiotic-modifying enzymes [Sheldon, 2005]. Biological activity of many antibiotics depends on their tertiary structure, which is determined by chemical bonds. The strategies which render different antibiotics inactive can be due to hydrolysis, modification, oxidation, reduction and cleavage of the antibiotics by enzymes produced by resistant bacteria. Hydrolysis of antibiotics is a common mechanism against β-lactams. For instance, β-lactams antibiotics are inactivated by β-lactamases, which hydrolyse the β-lactam moieties of antibiotics such as penicillins and cephalosporins [Wright, 2005]. Other enzymes include macrolide sterases, which break the ester bond in erythromycin, and epoxidases, which destroy the reactive epoxide ring of fosfomycin. Moreover, enzyme efficiency is further augmented with other mechanisms that affect the expression of porin channels and drug efflux. For instance, resistance in clinical isolates of P. *aureginosa* to carbapenem was shown to be due to a combination of AmpC β-lactamase, decreased production of the OprD porin channel for entry of antibiotics and activation of MexAB-OprM and other efflux systems [Jacoby, 2009]

Chemical group transfer to an antibiotic structure impairs target binding of antibiotics due to modification and alteration of their structure. The enzymes responsible for this alteration are group transferases. Acetyltransferases covalently modify the hydroxyl and/or amine groups of antibiotics, which render them inactive as antibiotics lose their ability to bind target sites. Other important group transfer enzymes are phosphotransferases, thioltransferases, nucleotidyltransferases, ADP-ribosyltransferases and glycosyltransferases [Wright, 2005].

The oxidation or reduction of antibiotics is another way in which bacteria protect themselves. For example, the oxidation of tetracycline antibiotics by the enzyme, TetX, produced by *Bacteroides fragilis* [Wright, 2005]. Moreover, antibiotic resistance lyases cleave carbon-carbon, carbon-oxygen, and carbon-sulfur bonds by non-hydrolytic or non-oxidative routes forming double bond formation or ring closure [Wright, 2005].

Remodeling of cell wall

Cell wall remodeling is another mechanism in which bacteria become resistant to antibiotics which target the cell wall. It has been found that cell wall peptidoglycan of susceptible strains contained monomeric and oligomeric forms of primarily (70% or more) linear stem peptides with the sequence of L-Ala-D-iGln-L-Lys-D-Ala (where iGln is isoglutamine). In contrast, the major peptide species (70% or more) of resistant cell walls were abnormal branched-stem peptides carrying Ala-Ser or Ala-Ala dipeptides on the E-amino groups of the stem peptide lysine residues. [Garcia-Bustos and Tomasz, 1990]. The peptidoglycan structural alteration showed strong correlation with penicillin resistance. In this study, it was suggested that the remodeling of the PBP in the resistant bacteria reduced affinity for penicillin and these changes were due to genetic transformation [Garcia-Bustos and Tomasz, 1990].

Antibiotic pumps

Active drug efflux involves certain bacterial transport proteins which pump out toxic antimicrobial compounds from the cell. Drug efflux pump proteins in bacteria fall into five distinct protein super families [ATP binding cassette super family, major facilitator super family, small multidrug resistance super family, multidrug and toxic compound extrusion super family and resistance-nodulation-cell division (RND) super family] and are mostly encoded by chromosomal genes. RND type multidrug efflux proteins usually function together with an outer membrane canal protein and a membrane fusion protein to pump out drugs. AcrAB-TolC of *Escherichia coli* and MexAB-OprM of *Pseudomonas aeruginosa* are the typical examples of these tripartite systems [Hasdemir, 2007].

Enzyme/substrate overproduction

Biochemical pathways require enzymes and co-factors as well as substrates. Under or over supply of co-factors can affect enzyme action. Antibiotics such as sulfonamides target an enzyme required for the chemical reaction such as for the folic acid synthesis in order to kill bacteria. The target of sulfonamides is the enzyme dihydropteroate synthase (DHPS) catalyzing the condensation of p-aminobenzoic (PABA) acid and 7,8-dihydro-6-hydroxymethylpterin-pyrophosphate (DHPPP) to form dihydropteroic acid, which is the next to last step in the formation of dihydrofolic acid. The resistance mechanism involve in sulfonamides is the overproduction of the enzyme substrate PABA, which make the antibiotic difficult to reach their target as excess PABA will occupy the enzyme active site, which is the same targeted by the antibiotic [Skold, 2000]. Resistance to sulfonamide in *Streptococcus agalactiae* isolates where shown to be due to the amplification of five genes required for tetrahydrofolate biosynthesis [Brochet et al., 2008].

Reduced uptake of drug

Reduced uptake or permeability is another resistance strategy for bacteria to prevent the entry of antibiotics into the bacteria. Gram-negative bacteria possess an outer membrane containing lipopolysaccharides with lipid A moiety. This outer membrane is impermeable and porin proteins that form water-filled channels are required for the transportation of many substrates across the outer membrane. Mutations in LPS as well as the porins have been found in species such as P. *auruginosa*, which make them resistant to antibiotics as their membranes become more impermeable and prevent the passage of many antibiotics [Kumar and Schweizer, 2004].

Modification of enzymes that activate the antibiotic

Certain antibiotics or prodrugs require an enzyme to activate their toxic effects on cellular targets. Isoniazid is an antibiotic that is used to treat tuberculosis and it has to be converted to a different compound in order to become active. Resistance to isoniazid develops due to the failure to activate the drug. This is because of a

mutation in the *katG* gene, which encodes the catalase-peroxidase KatG enzyme (an endogenous mycobacterial enzyme) essential for the activation of the antibiotic [Barry et al., 1998].

In summary, there are many different mechanisms which render bacteria resistant to a wide range of antibiotics. Therefore treatment of serious or life-threatening infections and management of critical patients requires targeted therapy where the choice and dosage have to be optimized. Thus, it is essential for antibiotic susceptibility testing to be done in order to determine whether the organism is resistant or not to the commonly used antibiotics. Antibiotic sensitivity testing used an index called the minimum inhibitory concentration (MIC). This is discussed further in the next section.

Antibiotic Susceptibility Testing: the MIC

Antimicrobial susceptibility testing is one of the most valuable laboratory tests performed for the management of infectious diseases. The index of susceptibility of bacteria to an antibiotic is known as the minimum inhibitory concentration (MIC). This is defined as the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a pathogen after overnight incubation [Andrews, 2001]. MIC is considered the gold standard method to test for susceptibility of organisms to antimicrobials and the range of antibiotic concentrations used is, in doubling dilutions steps up and down, from 1mg/L as required [Andrews, 2001]

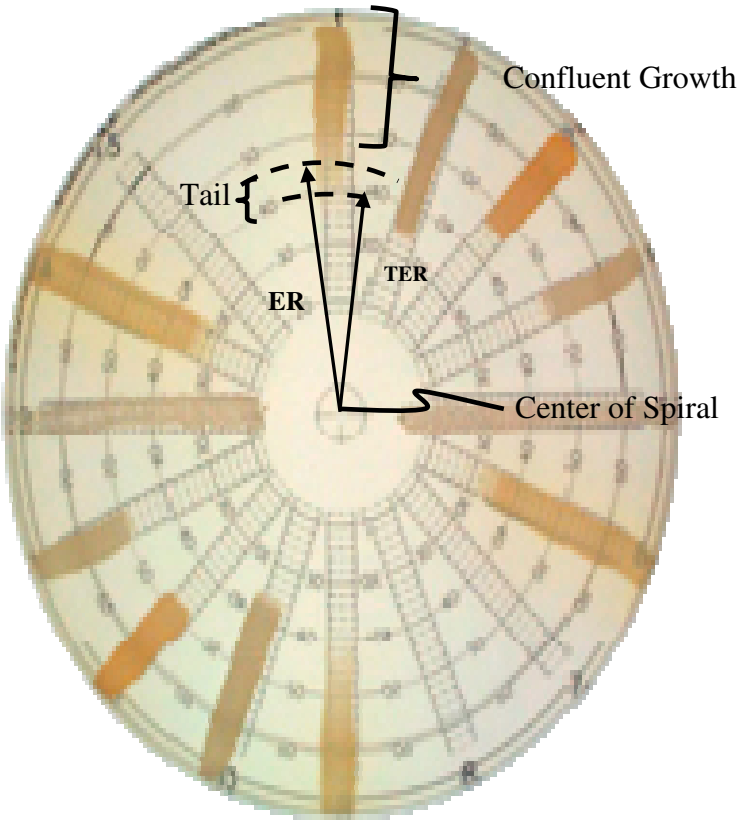
Conventional methods for measuring the MIC, such as diffusion methods, have many limitations. For instance it is only suitable for rapidly growing aerobic bacteria and it only provides categorical results and not quantitative on-scale MIC values [Schwalbe, 2007]. The disk diffusion method is always suitable for fast-growing bacteria, but some bacteria such as M. *tuberculosis* take about 10 days to give a positive or negative result.

A quantitative gradient was developed to determine concentrations of antibiotics in a reproducible manner. The gradient technique can be applied in the spiral gradient endpoint (SGE) test, which uses a spiral plater to dispense a continuously diluted antibiotic solution as closely spaced concentric rings on an agar surface. An antibiotic gradient is produced with a high concentration in the middle and decreasing progressively toward the rim of the agar plate. The organism is streaked from the center to the outward as a radius on the agar plate and the MIC of the antibiotic is calculated by measuring the distance from the center to the point of inhibition of the organism [Guilfoile, 2007; Schwalbe, 2007]. This concept is presented in Figure 1.9.

Figure 1.9

Spiral Gradient Endpoint Concept

ER is measured as the radius from the center of the plate to the endpoint of growth. The ER is entered into a software program; the program uses the molecular weight and diffusion characteristics of the antimicrobial agent to calculate the corresponding concentration. The ER is used to calculate the MIC.



ER= endpoint radius
TER=Tail ending radius

There are many tests available in order to identify pathogenic organisms that are resistant to frontline antibiotics; however there is the challenge of testing these organisms quickly, efficiently, accurately and at a relatively low cost. A wide range of tests are being developed as antibiotic resistant microbes are constantly emerging. In addition, there may be antagonism between different drugs, there may co-operation, and there may be synergistic action between drugs, all of which will affect the dose requirement for management [Acar, 2000]. There is no standard methodology to quantify the level of interaction between antibacterial agents and possible adjunct therapies. However, interaction affects the MIC, and measuring this using the SGE approach has several advantages.

SGE for determining MICs of antibacterial agents was introduced in 1990 and it has been used in clinical studies [Paton et al., 1990]. SGE testing minimizes time and material needed compared to the National Committee for Clinical Laboratory Standards reference agar dilution method, because the antibiotic concentration gradient on one plate typically spans eight twofold dilutions. SGE can be also very useful to determine synergistic activity of natural agents impregnated in the agar in a fast, reproducible manner when compared with the microdilution broth method, which is labor intensive and time consuming [Wexler et al., 1996].

Molecular methods have also been developed to overcome certain limitations of other tests. In the treatment of serious bacterial infections, choosing the correct antibiotic or combination of antibiotics is important as multidrug-resistance is found in many pathogens. Polymerase Chain Reaction (PCR) can provide critical information for resistance determinant identification by targeting resistance determinants that are encoded by genes with highly conserved DNA sequences [Hujer et al., 2009]. PCR is a technique for producing many copies of a single segment of DNA. Pathogens that are likely resistant to a particular antibiotic will contain a specific resistance gene that can be amplified using PCR. The advantage of this method is that a bacterial strain which is resistant to a specific antibiotic or a group of antibiotics can be rapidly determined [Schwalbe, 2007].

Consequences of Antibiotic Resistant Bacterial Infection

According to the World Health Organization, infectious disease remains a significant cause of morbidity and mortality worldwide, accounting for 16.2% of deaths worldwide and remaining the world's second top killer (World Health organization website). The problem of antimicrobial resistance has become a serious threat to public health, with high economic, social and medical impact. In fact, infections that are resistant to all current antibacterial options now occur. According to surveys such as the SENTRY antimicrobial surveillance programme and the Study for Monitoring Antimicrobial Resistance Trends (SMART) surveillance programme a consistent increase in Gram-positive and Gram-negative resistant pathogens has been recorded [Isturiz, 2008]. The emergence of resistant species such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) extended spectrum β-lactamase (ESBL) and multidrug-resistant *Pseudonomas aeruginosa* has been found around the globe [Isturiz, 2008; Song, 2008; Boucher et al., 2009]. VRE represents the fourth leading cause of nosocomial infection in the USA. In 2004, ESBL production was detected globally in 10% *Escherichia coli*, 17% of *Klebsiella spp* and 22% of *Enterobacter* species clinical isolates according to the study for monitoring antimicrobial resistance trends [Isturiz, 2008]. In Asia, MRSA has a prevalence rate of >50% in many hospitals. Moreover, it was found that multi-susceptible MRSA collected from patients in health care settings in Hong Kong increased from <2% to around 10% between 1995 to 2005 [Ho et al., 2009].

Antibiotic resistant infections are associated with greater morbidity, mortality and healthcare costs than susceptible bacterial infections. Twenty five percent of patients who carry MRSA will have an episode of infection, compared to 4% of those who are colonized with susceptible *Staphylococcus aureus* [Neu, 1992]. However, the community colonization with MRSA and VRE has been reported to remain low in Hong Kong, although continued monitoring was suggested as isolated enterococci strains in hospitalized subjects were shown to be resistant to other antibiotics such as fluoroquinolone and aminoglycoside [Boost et al., 2004; O'Donoghue and Boost

2004]. In a study, in which the direct clinical impact of multidrug resistant *Pseudomonas aeruginosa* (MDR P.*aeruginosa*) infection was investigated among affected patients (n=82), it was found that MDR P. *aeruginosa* was associated with more severe outcomes compared to controls, including increased mortality, hospital stay and requirement for procedures [Aloush et al., 2006]. Also, increased mortality and morbidity as well as length of stay in the intensive care unit were found in severely ill patients who were infected with carbapenem-resistant *Acinetobacter* species [Maragakis and Perl, 2008].

The future for the use of antimicrobial drugs is still uncertain. The cost for pharmaceutical research and development of a new drug is extremely high and also the current research and development has rather shifted towards agents that treat chronic diseases. It is estimated that pharmaceutical research and development of antimicrobial agents is $400-$800 million per approved agent [Spellberg et al., 2004]. Therefore, pharmaceutical companies are discouraged from research and development due to high direct costs, risk, and the time associated with animal and *in vitro* studies [Song, 2008]. Over the past 25 years, the number of new antimicrobials that can complete the process of development and receive the Food and Drug Administration (FDA) approval has decreased markedly. During the period from 1983 to 2007, a 75% decrease in systemic anti-bacterial agents approved by the FDA was found. It is foreseen that the number of approved antibacterials will plateau at a level similar to that of the past, which is around 1 drug per year [Boucher et al., 2009]. Only a few new classes of antimicrobial for the treatment of Gram-positive infections were approved since 2000, which include oxazolidinones (linezolid), cyclic lipopeptides (daptomycin), and glycylcyclines (tigecycline) [Song, 2008]. According to the Pharmaceutical Research and Manufacturers of America report, only 83 antibacterial drugs were in development, in which most of them are preclinical or phase 1 compound. The drugs in the report, which are already approved agents, were being studied for new indications and did not focus on new molecular entities [Boucher et al., 2009].

Moreover, there is the challenge of multi-drug resistant bacteria which have been accumulating during years. It has been postulated that antibiotics select for those resistant bacteria via mutation or DNA transfer. Antibiotics are not only used in human medicine but also for treatment, mass prophylaxis and growth promotion in animals, which in turn facilitate the emergence of further resistance by selection pressure [Livermore, 2007]. The efficacy and the amount of the existing antibiotics which are active against resistant bacteria are also becoming a serious problem. To date, there is no agent in advanced development for treating carbapenemase-hydrolysing β-lactamases *Klebsiella pneumoniae*. Only a few antimicrobials are active against resistant enterobacter species (colistin and maybe tigecycline). Low efficacy and tolerability of new agents such as linezolid, doptomycin and tigecycline against VRE remains questionable and no drugs in clinical development focus on combating carbapenem resistance or MDR P.*aeruginosa* or offer a less toxic alternative to the polymyxins [Boucher et al., 2009)]. As a result, the development of new antibiotics, drug agents and vaccines are of most importance to keep ahead of resistant infections.

Prevention and Cure: a two-pronged approach to healthy ageing in relation to immunomodulation and antibacterial therapy by natural products

Immunomodulation is a term used to describe upward or downward regulation of the immune system. It can be brought about by the administration of a drug or compound in order to suppress or activate the immune response. For example, in autoimmune diseases, drugs such as corticosteroids and non-steroidal anti-inflammatory drugs are used to suppress the immune response [Burt et al., 1998]. However, in this review, the term “immunomodulation” is used to describe the process of maintaining or restoring the immune system, and the down regulation of immunity will not be discussed in detail here.

There are many therapies available to modulate the immune system. For instance, in the treatment of HIV the use of low dose IL-2 increases immune function by increasing circulating NK cells, eosinophils, monocytes and CD4+ T cells [Jacobson et al., 1996]. Hormonal products such as thyroid hormones and thymic peptides have been shown to modulate and restore deficient NK function in old mice [Karesek, 2006]. Moreover, prolactin has been shown to restore the immune function on hypophysectomized rats [Reber, 1993]. As noted in an earlier section, diet, lifestyle and physical exercise can affect immune status. Dietary agents, including herbs, teas, spices, berries, and other natural products are of particular interest as immunomodulators, and there is intense research activity on the beneficial effects of natural products for the promotion and maintenance of health. In the following part of this review, selected agents with potential immunomodulatory effects will be described. In additional, dietary agents that have been reported to have direct or indirect antimicrobial action will be discussed.

Examples of functional foods and herbs with immunomodulatory and antimicrobial potential

A functional food is "any modified food or food ingredient that may provide a health benefit beyond that of the traditional nutrients it contains" [Milner, 2000]. Herbs are related to the use of products containing ingredients of exclusively plant origin, which may involve seeds, roots, leaves, bark or flowers. However, herbal medicine in some systems such as in traditional Chinese medicine, Yoruba medicine and Unani, often include animal or insect products and medicinal mushrooms (fungi) as "herbal medicines" [Blumenthal, 2000]. In this review, herbs are regarded to be of exclusively non-animal or insect sources, i.e. plant or fungal materials.

Medicinal products from herbs are usually consumed as decoctions, powders or supplement pills, where production consists of the concentration and purification of herbs resulting in extracts, tinctures, fatty/essential oils or juices [World Health Organization, 2003; Packer et al., 2004]. The use of plants for maintaining health and improving the quality of life has been witnessed for thousands of years, and it is

estimated that around 80% of the world's population rely on traditional medicine involving usage of plant extracts for their basic health care needs [World Health Organization, 2003]. There is huge interest in natural products for health promotion, and herbs and specialized supplements are of intense research interest. The exponential growth in the market of herbal medicines can be observed all around the world, with US$ 60 billion being spent annually [World Health Organization, 2003]. Many herbal supplements have been introduced with claims of boosting the immune system. However, for most herbs scientific evidence is lacking, and there are reports of fraud, adulteration and toxicity [Kumar, 2007; Rader et al., 2007]. Therefore, it is becoming extremely important to produce valid scientific evidence based on controlled studies to determine the benefits of herbs and natural products, identify active components, and establish safe dosage. Nonetheless, because of the immense threats of age-related decline in the immune system and of new infectious diseases at a time when the development of new antibiotics is almost at a standstill [Helfand and Bonomo, 2005], the potential benefits of herbs and functional foods for maintaining immune function and for treatment of infectious disease is a worthwhile area of research.

The effect of many herbs and functional foods on the immune function has been associated with their active compounds, which include flavonoids, vitamin C, and carotenoids [Craig, 1999]. Experimental findings have demonstrated that nutritional antioxidants enhance both adaptive and innate immunity [Gershwin et al., 2004]. As reviewed by Bendich, many studies have demonstrated that supplementation with antioxidants such as vitamins C, E and A, α-tocopherol and β-carotene have immune-enhancing effects [Bendich, 1993]. Vitamin E supplementation in aged-mice has been shown to reverse the age-associated defects in naïve T cells by increasing immune synapse formation [Marko et al., 2007]. Dietary supplementation of vitamin C and E, zinc, selenium and β-carotene in prematurely aging mice was shown to improve leukocyte function and restore redox balance [Alvarado et al., 2006]. However, conflicting results have also been reported such as in the use of vitamin C to reduce the incidence of common cold. In a review in which a large

number of controlled trials were compared, the authors concluded that vitamin C failed to lower the incidence of colds in the normal population [Douglas et al., 2007]. Nonetheless, there is evidence to support dietary antioxidants having an effect on the immune system, and a better understanding of antioxidants in relation to immunity and infection can be of great significance for the development of new therapeutic agents and promotion of healthy ageing. Dietary agents rich in antioxidants are excellent candidates that deserve increased research effort, given their abundant availability and low cost. As mentioned by Gershwin et al. [2004], both developed and developing countries will benefit from increased research in the field of antioxidant nutrition and immunology [Gershwin et al., 2004]. Furthermore, some herbal products have been shown to have effects on the immune system in clinical study. For example, *Echinacea purpurea, Astragalus membranaceus* and *Glycyrrhiza glabra*, have been reported to activate immune cells [Brush, 2006]. A herbal medicine, KY88, produced from *Fructus schisandrae* was shown to possess immunomodulatory property by lowering circulating monocyte count [Yip et al., 2007]. A Chinese mixed herbal formula was shown to enhance the anti-inflammatory cytokine IL-10 and decrease IFNγ, IL-5 and COX-2 mRNA expression in allergic patients [Yang et al., 2001]. A summary of the effects of the mentioned herbs on the immune system in human clinical trials are presented in Table 1.8.

Table 1.8

Summary of Effect of Selected Herbs on the Immune System in Clinical Trials

Herb	Effect on immune system/clinical effect	No. of subjects	Duration	Study Design	Source
Echinacea purpurea, Astragalus membranaceus and Glycyrrhiza	Stimulation of CD4 and CD8 T cells. The combination of the three herbs showed additive effect on CD69 expression but no additional increase proliferation	16 healthy individuals	7 days (twice daily)	Placebo-controlled, double-blinded investigation	Brush et al., 2006
Ganoderma lucidum (lingzhi)	Increased mitogenic reactivity to phytohemagglutinin, counts of CD3, CD4, CD8 and CD56 lymphocytes, increased plasma concentrations of interleukin (IL)-2, IL-6 and interferon (IFN)-gamma, and NK activity with no statistical significance	47 patients with advanced colorectal cancer	12 wks (3 times daily)	Uncontrolled (no placebo) study, non-randomized, no blinding	Chen et al., 2006
	Promoted recovery from *Herpes simplex* and shortened the time to obtain symptom relief.	28 patients with *Herpes genitalis* and *Herpes labialis*	A single daily dose until complete relief was obtained	Uncontrolled study	Hijikata et al., 2007
Fructus Schisandrae	Decrease in circulating monocytes	23 healthy individuals infected with HBV	2 wks (twice daily)	Uncontrolled study; no blinding	Yip et al., 2007

Herbs	Effect on immune system	No. of subjects	Duration	Study Design	Source
Chinese mixed formula: Shin-yi-san, Xiao-qing-long-tang and Xiang-sha-liu-jun-zi-tang	Enhanced IL-10 and decreased IFN-gamma, IL-5 production and COX-2 mRNA expression.	17 females and 33 males suffering from perennial allergic rhinitis	3 months (once daily)	Uncontrolled	Yang et al., 2001
Camellia sinensis(green tea)	Enhance gamma delta T cell function. Prevented cold and flu illness and symptoms	52 healthy men and 72 healthy women 18-70 years old	3 months (twice daily)	Randomized, double-blind, placebo-controlled study	Rowe et al., 2007
Prebiotic antioxidant bread, which contained green tea powder, herbs and tomato paste	No change was found in the 23 immunological parameters measured (including CD3, CD4, CD8, CD4:CD8, NK, CD57, CD8+, CD57+, CD25, CD4+CD25+, CD122, CD4+CD54+, CD19, CD3+HLA-DR, ICAM-1). CD19 percentage increased after intervention with the bread (P=0.023).	20 male smokers and 18 male non-smokers	Standardised diet with bread provided for 5 wks	Randomized, parallel, double-blind (n=38)	Seidel et al, 2007
Black tea	The peripheral blood mononuclear cells collected produced 2-3 fold more interferon gamma.	11 healthy non-tea-drinking individuals	5 to 6 cups/ day for either 2-4 wks	Controlled trial	Kamath et al., 2003
	Significantly reduced platelet activation and plasma C- reactive protein	37 healthy non-smoking men aged 18-55 years	1050 mg tea extract dissolved in 250 ml water taken each day for 6 wks	Double-blinded placebo-controlled trial (n=37)	Steptoe et al., 2007

The use of herbs and 'traditional medicines' for controlling infectious diseases is essential and wide-spread. Antibiotics can produce serious side-effects, their efficacy is waning as drug-resistant bacterial strains emerge, and in many parts of the world antibiotic therapy is unavailable due to poverty and inadequate healthcare provision. In addition, those who are more vulnerable to infection and often rely on antibiotic therapy for their survival, e.g. HIV patients, cancer patients, children and elderly, have higher risk of developing resistant bacterial infections and thus a higher chance of developing complications and fatal outcome [Cars and Nordberg, 2004; Dronda and Justribo, 2007].

Plants synthesize chemicals as part of their defense against pathogens. For instance, flavones and hydroxylated phenols are naturally synthesized by plants in response to infection [Tan and Vanitha, 2004]. The antimicrobial activity of herbs and plants has been associated with their chemical constituents, such as phenols, phenolic acids, coumarins, terpenoids and alkaloids [Kaefer and Milner, 2008]. Many herbs and natural agents have been reported to have an effect on controlling infectious diseases such as influenza and HIV infection by having antibacterial and antiviral activity, and numerous studies have been published on the effect of different herbs against a variety of microbes [Yoon et al., 1994; Kee, 1999; Lam and Ng, 2002; Tan and Vanitha, 2004; Lee et al., 2006; Shan et al., 2007] (Table 1.9).

Furthermore, effects of herbs against resistant pathogens have been reported. The ethanolic extracts of *Dendrobenthamia capitata, Elsholtzia rugulosa, Elsholtzia blanda, Geranium strictipes and Polygonum multiflorum* showed anti-MRSA activity with an MIC≤1.43 mg/ml. It was reported that besides inhibition against β-lactamase, the mechanism of action was also attributed to inhibition against bacterial topoisomerase and efflux pump [Zuo et al., 2008].

Table 1.9

Antimicrobial, Antiviral and Antifungal Effects of Some Herbs

Herb common name and/or Latin name	Effect	Associated effect/Mechanism	Source
Chrysin, Baicalin, Swertifrancheside	Anti-HIV	Inhibits HIV replication, HIV-1RT and stimulates proliferation of T cells and NK cells repectevely.	Kee, 1999
Betelnut, Cassia, Juhua, Guanzhong, cow-itch plant, box myrthe, Huzhong, Shiliupi, Diyu, Huangqin, Belliric myrobalan	Antibacterial	Antibacterial effect against food borne pathogens (*Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus, Escherichia coli,* and *Salmonella Anatum*)	Shan et al., 2007
Ganoderma lucidum (lingzhi)	Antibacterial	Antimicrobial activity against *Micrococcus luteus*	Yoon et al., 1994
Shizandrae Fructus	Antibacterial	Antibacterial activity against different species of Salmonella	Lee et al., 2006
Panax notoginseng (American ginseng)	Antifungal Antibacterial	A protein from this plant, pananotin inhibited mycelial growth in various fungus and inhibited HIV-1 RT by 35.8%. Panaxagin, a peptide, inactivates fungal ribosome. Toxic to *Coprinus comatus, Physalospora piricola, Botrytis cinerea, Fusarium oxysporum* by pananotin.	Lam and Ng, 2002; Tan and Vanitha, 2004
Zingiber officinale	Antibacterial Antifungal	Toxic to *Rhizoctonia solani* and *Pyricularia oryzae*	Tan and Vanitha, 2004
Angelica dahurica	Antibacterial	Toxic to *Aspergillus candidus*	Tan and Vanitha, 2004
Scutellaria barbata	Antibacterial	Aspigenin in the plant was toxic to MRSA	Tan and Vanitha, 2004
Scutellaria albida	Antibacterial	Linalool, a flavonoid was toxic to *Staphylococcus aureus, Bacillus subtilis, Escherichia coli and Pseudomonas aeruginosa*	Tan and Vanitha, 2004

Direct or complementary/synergistic antimicrobial effects of herbs could offer useful adjunct therapies for infectious disease and combating multi-drug resistant bacteria. The possible use of natural products or herbs together with known antibiotics could be of great significance as there is evidence that some herbs can increase the potency of antibiotics, allowing lower doses to be used and thus avoiding undesirable side effects. For instance, it has been reported that galanin, which is the active constituent of *Alpinia officinarum*, showed synergism with gentamicin against MRSA [Lee et al., 2008]. In another study, synergism between baicalein, a bioactive flavone of *Scutellariae radix* (a Chinese herb), and gentamicin was demonstrated against four clinical isolates of VRE (VRE-70, VRE-940, VRE-096 and VRE-721) [Chang et al., 2007]. In addition, baicalin was shown to restore the effectiveness of β-lactam antibiotics against MRSA and other strains of β-lactam-resistant *Staphylococcus aureus*. In the study, 25 μg/ml of baicalin increased the killing effect on MRSA and β-lactam-resistant *Staphylococcus aureus* with 10-50 μg/ml ampicillin, amoxicillin, benzylpenicillin, methicillin and cefotaxime [Liu et al., 2000]. In another study, epigallocatechin gallate (EGCG) in green tea was found to have a synergistic effect with β-lactams. The MIC of β-lactams (100 μg/ml) against MRSA was lowered fourfold by the inclusion of EGCG (i.e. to 25 μg/ml), reversing the high level resistance of 25 isolates of MRSA to benzylpenicillin, ampicillin, oxacillin, methicillin and cephalexin [Zhao et al., 2001].

In summary, many natural products and herbs are believed to have beneficial effects on health, and there is evidence that some may have immunomodulatory and direct or indirect antibacterial effects. The focus in the rest of this review will be on three such agents: *Ganoderma lucidum* (lingzhi), *Camellia sinensis* (green tea) and *Vaccinium myrtillus* (bilberry). These have been selected because of their high content of antioxidants and also due to a growing body of scientific evidence showing these agents to have some effects associated with the immune system and health enhancement [Wachtel-Galor et al., 2004; Cabrera et al., 2006; Zafra-Stone et al., 2007].

***Focus on Ganoderma lucidum* (lingzhi)**

Ganoderma lucidum or 'lingzhi' in Chinese, and known as 'reishi' in Japan and Korea, is a woody mushroom that has been widely consumed for thousands of years in Asia for its medicinal, rather than nutritional, value. Many commercial lingzhi products are available, and this mushroom is one of the most highly regarded traditional medicines in Asian history [Zhou et al., 2007]. The active components of lingzhi include polysaccharides, triterpenes and elemental components such as germanium [Wachtel-Galor et al., 2004; Zhou et al., 2007].

There are a number of *in vitro* studies showing the influence of *G. lucidum* on the immune system. Treatment with *G. lucidum* mycelia was shown to induce moderate release of TNF-α, IL-6 and TNF-γ in human whole blood culture and also to induce nuclear factor-κB (NF- κB) activation in a murine macrophage cell line. NF- κB is an important signal agent for the release of proinflammatory cytokines [Kuo et al., 2006]. Moreover, *G. lucidum* mycelium and spore extracts were found to have immunomodulating effects by stimulating Th1 and Th2 cytokine mRNA expression as well as inducing lymphocyte proliferation [Chan et al., 2005]. In addition, the polysaccharides derived from *G. lucidum* have been shown to induce dendritic cell maturation [Lin et al., 2006; Chan et al., 2007].

Clinical trials to evaluate the effects of G. *lucidum* have suggested potential immunomodulating, anti-cancer and antioxidant effects of this herbal medicine [Wachtel-Galor et al., 2004; Yuen and Gohel, 2005; Chen et al., 2006]. Increase in mitogenic reactivity to phytohaemagglutinin and lymphocyte subsets as well as cytokines concentration in plasma on patients (n=47) with advanced colorectal cancer after supplementation with *G. lucidum* polysaccharides has been reported, although with no statistical significance [Chen et al., 2006]. Moreover, increased antioxidant power in plasma following ingestion of lingzhi was also published in a controlled supplementation study in which 18 healthy adults received 1.44g of lingzhi capsules [Wachtel-Galor et al., 2004]. In another study, a herbal mixture containing *G. lucidum* was shown to relieve symptoms and improve the recovery

time of 28 patients suffering from *Herpes genitalis* and *Herpes labialis*, and the authors suggested that the herbal mixture had immunoadjuvant effect and the observed results might be explained by the effect of this herbal mixture on increasing natural-killer (NK) cell activity [Hijikata et al., 2007]. However, the suggested conclusions based on these studies are yet to be confirmed, and some of the effects were very small.

In addition to reputed immunomodulatory effects, some studies have reported that the water extract of lingzhi has antiviral and antimicrobial effects [Kuo et al., 2006]. Our group found evidence of indirect antimicrobial effects. Although this remains to be confirmed, we found some evidence that lingzhi may act to increase the efficacy of antibiotics such as penicillin and methicillin. The aqueous extract of *G. lucidum* was shown to have antimicrobial activity against *Micrococcus luteus* (MIC, 0.75 mg/ml) and synergistic effects were shown when the extract of *G. lucidum* was combined with cefazolin against *Bacillus subtilis* and *Klebsiella oxytoca* [Yoon et al., 1994]. It has been also reported that three different organic extracts (n-hexane: diethyl ether/chloroform: acetone/methanol) of four species of *G. lucidum* was active against *Pseundomonas syringae* and *Bacillus subtilis* [Ofodile et al., 2005]. Moreover, activities against *Herpes simplex* virus, Hepatitis B virus, HIV, and Epstein-Barr virus by the polysaccharides and triterpenoids from *G. lucidum* showed effects *in vitro* and in animal models. *Ganoderma* species were also found to contain antibacterial constituents inhibiting gram-positive and/or gram-negative bacteria *in vitro* [Gao et al, 2005].

In summary, the potential effects of *G. lucidum* in terms of both immunomodulation and direct or indirect antimicrobial effects should not be ignored [Lin and Zhang, 2004], and further studies in well-designed experimental studies are warranted.

***Focus on Vaccinium myrtillus* (Bilberry)**

Bilberry is a dark blue/purple fruit, and is a popularly consumed type of berry. Bilberry contains a high amount of a type of antioxidant called anthocyanin, a phenolic compound [Zafra-Stone et al., 2007]. Anthocyanins are powerful antioxidants and are reported to have anti-inflammatory effects [Prior and Wu, 2006], as well as hypoglycaemic effects [Matsui et al., 2006; Zafra-Stone et al, 2007]. Besides its use as a delicacy, bilberry is also widely used for its ability to improve night vision and for decreasing vascular permeability and capillary fragility [Upton, 2001]. Anthocyanins found in bilberry have been reported to have effects in relation to oxidative stress, inflammatory response and age-related diseases [Zafra-Stone et al., 2007]. It has been shown that bilberry extract protected primary cultures of rat hepatocytes against oxidative damage, and the cytoprotective properties was suggested to be due to anthocyanin [Valentova et al., 2006]. Several studies have also demonstrated improved plasma antioxidant status after consumption of berries [Cao et al., 1998; Mazza et al., 2002; Netzel et al., 2002; our unpublished data]. This indicates that berry components with antioxidant activity are bioavailable, and dietary intervention with berries or berry extracts may be one of the possible preventive strategies of immune decline. As mentioned, oxidative stress plays a role in suppressing the immune system, and as we age oxidative stress increases [Finkel and Holbrook, 2000]. Therefore, increasing antioxidant defense by increased intake of antioxidant-rich foods may prevent ageing-related immune decline. Moreover, anthocyanins were suggested to have anti-inflammatory effects in a murine asthma model due to down regulation of proinflammatory cytokines and COX-2 [Park et al., 2007].

In addition, bilberry and other berry fruits have been reported to have antimicrobial effects against human pathogens including *Salmonella* and *Staphylococcus aureus* [Puupponen-Pimia et al, 2005]. Other workers have reported inhibitory effects on *Helicobacter pylori* [Chatterjee et al, 2004]. In addition, Chatterjee et al. [2004] found that exposure to berry extracts increased the antimicrobial effects of the drug clarithromycin against *Helicobacter pylori*.

There is an increasing range of bilberry products on the market, and it is becoming a popular health supplement. However, there are very few scientific studies evaluating bilberry's effects in the immune system in relation to inflammation, or in regard to antimicrobial effects. Therefore, further study of bilberry in relation to inflammation and immune status is warranted.

***Focus on Camellia sinensis* (green tea)**

Tea is made from the infused leaves of the plant *Camellia sinensis*. Different types of tea (green, oolong, black) are found, the difference being in the amount of 'fermentation' of the leaves after they are picked [Shukla, 2007]. Green tea in particular is an antioxidant-rich beverage that is commonly consumed in China and other parts of the world [Benzie et al., 1999; Khan and Muktar, 2007]. Apart from being a pleasant and refreshing drink, green tea is reported to improve blood flow, eliminate toxins and promote health [Cabrera et al., 2006]. The major type of antioxidant in tea, the catechins, can bind transition metal ions (iron and copper) and this can have both antioxidant and anti-inflammatory effects [Benzie and Szeto, 1999].

There is accumulating evidence showing that green tea, which is particularly rich in the flavonoid epigallocatechingallate (EGCG), has immunomodulatory effects [Hamer, 2007; Tipoe et al., 2007]. In a randomized, double-blind, placebo-controlled study it was suggested that a *Camellia sinensis* formulation enhanced gamma delta T cell function and prevented cold and flu illness and symptoms [Rowe et al., 2007]. Also green tea extracts are reported to cause a significant concentration-dependent decrease of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNFα and PGE_2) from activated human monocytes and thus justifying the positive effect of topically-applied green tea extracts on radiation-induced skin reactions [Pajonk et al., 2007]. Moreover, the antioxidant effect of green tea has been observed in various studies as well as its anticancer effect [Packer et al., 2004; Khan and Muktar, 2007].

Green tea catechin has also been reported to improve survival in infected animals [Wheeler et al, 2007] and to reduce *Helicobacter pylori* -induced gastritis [Ruggerio et al, 2007]. Two polyphenolic compounds, catechin and epicatechin gallate (ECG), which are constituents of green tea, have been reported to have weak antibacterial properties. In addition, ECG is able to reduce oxacillin resistance in *Staphylococcus aureus* [Yam et al, 1998; Hamilton-Miller and Shah, 2000; Zhao et al., 2001; Stapleton et al., 2004]. This effect can render oxacillin-resistant isolates, such as EMRSA-16, oxacillin sensitive [Stapleton et al., 2004]. However, there is not much evidence regarding the specific effect of green tea on immune parameters and function, although epidemiological studies have shown that green tea has an effect in longevity indicating that daily consumption may help to maintain health and prolong life [Nakachi et al., 2003]. Further research regarding the effect of green tea in the immune function is an interesting area of research to further validate and possibly identify underlying mechanism of its health promoting effects.

Summary

With advancing age there is a progressive decline in the human immune system, and this is responsible for at least part of the many deleterious changes that occur with ageing, such as the increased incidence of cancer, chronic inflammatory disorders, autoimmunity and increased susceptibility to and poorer recovery from infection. Indeed, the impairment in immunocompetence is reported to be apparent as early as 35 years of age, making this a problem that is not confined to the elderly. Due to the ageing of the population, which brings increased susceptibility to infectious disease because of age-related decline in the immune system, there is an increasing priority for prevention, early intervention and improvement in management for immune decline and infectious disease.

Natural products, from herbs, teas and other plant-based foods, offer high potential for immunomodulation and some are reported to have effects in improving or restoring the immune response. There is growing awareness of the role of diet and traditional herbal medicines on health promotion, including immunomodulatory

effects. Various herbs and dietary agents are commonly used for boosting the immune system, although scientific evidence of efficacy is lacking. To address the problems of the advancing age of our population it is of utmost importance to generate valid science-based evidence in regard to immunomodulatory effects of dietary agents in order to devise diet-based strategies that can prevent or slow age-related decline in the immune system. Such evidence requires results of placebo controlled human intervention trials that employ a biomarker approach to sensitively and specifically detect evidence of health-related effects, such as changes in immune status, inflammatory status and oxidative stress. In addition, experimental study of direct and/or indirect antimicrobial effects of herbs and other natural products is urgently needed due to the emergence of antibiotic resistant strains and new microbes, and the lack of new antibiotics to treat these. There is some evidence that lingzhi, green tea and bilberry have health-promoting effects through anti-inflammatory, immunomodulatory, anti-oxidant and antimicrobial properties, but further study is needed for each of these to bridge the knowledge gap. If these dietary agents are confirmed to have immunomodulatory or anti-inflammatory effects, these natural products would be powerful agents for promoting health in our ageing population, and if direct or indirect antimicrobial action is confirmed then a new generation of antibiotic treatment could result. *This is the focus of this study*.

Chapter 2

AIMS AND SPECIFIC OBJECTIVES OF THE STUDY AND ORGANIZATION OF THESIS

Aims of the Study

The study had two main aims and was organized in two main parts: *in vivo* supplementation studies and *in vitro* experimental study.

Part 1: Immunomodulatory effect of dietary agents

In vivo immunomodulatory, anti-inflammatory and antioxidant effects of selected dietary agents (green tea, bilberry and lingzhi) in controlled human intervention trials

Part 2: Antimicrobial effect of dietary agents

In vitro antimicrobial effect of selected dietary agents (green tea, bilberry and lingzhi) in the presence (indirect effects) and absence (direct effects) of known antibiotics (vancomycin and oxacillin)

Main objectives of the in vivo supplementation trials were as follows:

- To investigate the effects of two types of green tea, bilberry and lingzhi on selected biomarkers of immune status (e.g. cellular immunity and lymphocyte subsets) in target subjects.
- To determine if such changes are interrelated with antioxidant balance and oxidative stress

Main objectives of the in vitro experiments were as follows:

- To investigate the antimicrobial effects of two types of green tea, bilberry and lingzhi extracts against isolates of MRSA, VISA and susceptible *Staphylococcus aureus*
- To determine if any such effects were due to direct effects or due to a potentiating interaction with vancomycin and oxacillin

Organisation of the thesis

Chapter 1 provides a comprehensive literature review which presents the context and rationale for the study overall.

Chapters 3-6 are the experimental chapters, and are each written in the form of a scientific paper. Each chapter has sections on introduction, study design, materials and methods, statistical analysis, results, discussion and conclusion. Duplication has been minimized, although there is some overlap between chapter 1 and the introductory section of each experimental chapter. To avoid too much duplication in terms of methods used in the supplementation studies (Chapters 3, 4, 5), full details are given only in chapter 3, and thereafter, in subsequent chapter, the reader is referred to chapter 3 where appropriate.

Chapter 7 is an overall integration of findings, discussion of results, their implications and limitations, with main conclusions and suggestions for future work.

Unless otherwise stated, all laboratory analyses, data collection and data analysis were performed by the author. For those parts of the study that involved human subjects, all procedures had approval of Ethics by the Human Ethics Subcommittee of The Hong Kong Polytechnic University.

A note on presentation of results on lymphocytes total and subsets

In the results, both the absolute number and the percentage of lymphocytes total and subsets are presented. This is to show that both absolute number and percentage were affected. However, it is important to note that results could be misleading when looking at the percentage only, as the effects on the different sub-sets were different, with some being affected more dramatically than other in relative terms. Showing the percentage alongside the absolute number reveals this. However, it is noted also that the absolute numbers of white cells and white cell subsets is the more meaningful type of results.

Chapter 3

A STUDY OF IMMUNOMODULATORY EFFECT OF TWO TYPES OF GREEN TEA IN MIDDLE-AGED SUBJECTS: RESULTS OF A CONTROLLED HUMAN INTERVENTION STUDY

Introduction

A functional food may promote health beyond that of the traditional nutrients it contains [Milner, 2000] One such functional food is tea, a commonly consumed beverage throughout the world [Benzie et al., 1999; Khan and Muktar, 2007]. Tea is made from the infused leaves of the plant *Camellia sinensis*. There are different types of tea (green, oolong, black) and this is due to the amount of 'fermentation' of the leaves after they are picked [Shukla, 2007]. This fermentation involves the oxidation and polymerization of flavanoid antioxidants in the tea leaves [Khan and Muktar, 2007]. Green tea ('unfermented') has higher antioxidant content than semi-fermented oolong teas and the fermented black teas, while still high in antioxidants, have the lowest total antioxidant content [Benzie et al., 1999]. The major type of antioxidant in tea, the catechins, can bind transition metal ions (iron and copper), and these polyphenolic compounds have potent *in vitro* antioxidant properties and are reported also to have anti-inflammatory, lipid lowering and other health related effects [Benzie and Szeto, 1999; Hamer, 2007; Tipoe et al., 2007; Butt and Sultan, 2009].

Tea is an important dietary source of antioxidant power which can be ingested frequently and in relatively large volumes without any known harmful effects [Yang et al, 2009]. Epidemiological data suggest that green tea consumption may reduce the risk for certain cancers and cardiovascular diseases [Kuriyama, 2008; Cabrera et al., 2006]. Both these disorders are strongly linked to advancing age and to inflammation and oxidative stress

[Franceschi, 2007]. The commonly seen age-related decline in the immune system is also a factor. The immune system is affected by various factors, such as malnutrition, excessive exercise, stress and advancing age. It is well-documented that ageing is associated with specific changes in peripheral blood lymphocytes, and absolute numbers of different immune cells (including T lymphocyte subsets and B lymphocytes) have been reported to decrease with age [Ferguson et al., 1995]. Counting these cells in venous blood is a common approach in assessment of immune status and in the study of immunomodulation by disease, drugs, age, exercise, malnutrition and functional foods.

Cancer risk is greatly increased when immune surveillance is decreased [Prehn and Prehn, 2008]. In addition, immune decline predisposes to infection and poor response to vaccination, and also limits the protective response to infection. All these effects of age-related immune decline mean that from middle age onwards, risk of infectious diseases increases. In addition, loss of immune surveillance leads to increased risk of cancer with age. Therefore, a functional food, such as green tea, which could slow or help prevent age-related immune decline would be a valuable aid to healthy ageing. It is unlikely that tea could be used as a therapy for those with established disease, but its putative antioxidant, anti-inflammatory and immunomodulatory effects in combination may have significant long-term health benefits when taken regularly.

In various studies it has been observed that green tea, which is particularly rich in the flavonoid epigallocatechingallate (EGCG), has antioxidant as well as anti-cancer effects, which include skin, liver and lung cancer [Packer et al., 2004; Cabrera et al., 2006; Khan and Muktar, 2007]. Green tea catechin has also been reported to improve survival in infected animals [Wheeler et al, 2007]. However, while there are many epidemiological studies of tea, especially in relation to cardiovascular disease and inflammation, there have been only a few human trials investigating the effects of tea and tea extracts on immune status [Reviewed by Hamer, 2007]. A study of black tea showed an increase in the *in vitro* response of gamma delta T cells to nonpeptide antigens, whole bacteria, and lipopolysaccharides in 11 healthy subjects after 4 weeks supplementation [Kamath et al., 2003]. In relation to green tea, *in vitro* study reported

that green tea extracts caused a significant concentration-dependent decrease of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNFα and PGE_2) from activated human monocytes [Pajonk et al, 2007], and the authors suggested a role for topically-applied green tea extracts as means to ameliorate radiotherapy-induced skin reactions [Pajonk et al., 2007]. There is only one published study of green tea (which used an extract, not the beverage) on immune function/status. In a randomized, double-blinded, placebo-controlled study of parallel design, 124 healthy adults aged 18-70 years took 2 capsules of green tea extract or placebo for 3m [Rowe et al., 2007]. The green tea extract was reported to prevent cold and flu illness and symptoms and increased gamma delta T cell function when challenged *in vitro* with ethylamine (a molecule that specifically activates human gamma delta T cells) [Rowe et al., 2007]. No white cell numbers or lymphocyte sub-sets were looked at, but in a sister study [Nantz et al., 2009] they reported that serum amyloid-alpha (described as a marker of inflammation) was decreased in the green tea extract group. It is important to note that the green tea formulation dosage was equivalent to ingestion of ~10 cups of green tea per day, and also that cold and flu symptoms were self-reported by a questionnaire.

There is clear need to find agents and therapies that can prevent age-related immune decline or boost the immune system. To date there has been very limited study of effects of green tea on immune function, and there are no published human studies to date on the potential immunomodulatory effects of green tea. This was the focus of this part of the current study in which a placebo-controlled, human intervention study of cross-over design was conducted using a biomarker approach to investigate effects of green tea on antioxidant, inflammatory and immune biomarkers. Two different types of green tea were tested in this study in order to be able to generalize results more confidently to green tea. The teas used are both well-regarded and commonly consumed green teas in Hong Kong, and are called ‘Loonjin’ and ‘Screw-shaped’ teas (Fig 3.1).

The specific objectives of this part of the study were:

1. To investigate changes in selected biomarkers of immune status (lymphocyte subsets) after supplementation with green tea

2. To investigate difference in response to supplementation with two types of green tea (Loonjin and Screw-shaped)
3. To determine changes in a inflammatory marker (hsCRP) after green tea supplementation
4. To investigate the inter-relationships of response to treatment between measured biomarkers of immune status along with biomarkers of oxidative stress and antioxidant defense.

Figure 3.1

Loonjin and Screw-shaped Green Tea

Materials and Methods

Subjects

Eighteen apparently healthy Chinese subjects of age 35 to 50 years were recruited. Criteria of exclusion included: subjects with medically prescribed diet; under slimming regime; with chronic diseases; smokers; overweight; on regular medication including traditional Chinese medicine. Pregnant/ lactating women or subjects who had been hospitalized in the previous 12 months were also excluded. A briefing session was held and written informed consent was obtained before the subjects participated in the study.

The study was approved by the Human Subjects Ethics Sub-committee of The Hong Kong Polytechnic University

Methodology

The trial was conducted over a period of 20 weeks. This cross-over, single-blinded study consisted of three experimental 'treatments'. Upon entry into the study, subjects were divided on a non-selective basis but stratified for number (n=6 per treatment) into three treatment groups. Treatments were water (as placebo); Loongjin green tea (LGT); Screw-shaped green tea (SSGT). Both Loonjin and Screw-shaped green tea used in this study were kindly supplied by Ying Kee Tea House (Address: 8/F, Wah Shing Centre, 5 Fung Yip Street, Siu Sai Wan, Hong Kong). Treatments were coded. Subjects in each group took 150 ml of either 1.0% w/v tea or water twice a day for 4 weeks. Tea bags, brewing instructions and a mug of standard size were supplied to the volunteers. During their participation in the study, the subjects were asked not to change their regular diet and lifestyle habits apart from taking the supplement. The intervention periods were separated by a washout period of 6 weeks. Each subject took all three treatments in a random order. Compliance of treatment was checked by regular contact with the subjects and by counting the tea bags returned from the subjects at the end of each intervention period. For each 4 weeks' intervention period, 60 tea bags were provided to each subject and 56 tea bags were expected to be used after the intervention period. Eighty- five percent compliance (return of <14 tea bags was considered to be satisfactory).

Specimen Collection

Before and after each intervention period, urine and a fasting venous blood sample was collected into EDTA and heparin commercial blood collection tubes for biochemical tests. Totally, 6 blood samples (pre- and post- each of 3 treatments) were collected from each subject. After collection the samples were kept chilled and processed within 6h.

Reagents, cell preparation and staining for immunophenotyping

A standardized method using a recommended panel of two-colour combinations of fluorescein isothio-cyanate (FITC) and phycoerythin (PE)-conjugated monoclonal antibody reagents obtained from a single manufacturer (Beckman Coulter Ltd, CA, USA) was used to determine the expression of each antigen or antigen combination for identification of the major white cell subsets (Table 3.1).

Table 3.1

Panel of Monoclonal Antibodies Used and Lymphocytes Identified

FITC-tagged Antibody to	PE-tagged Antibody to	Lymphocyte Subset(s) Identified
CD45	CD14	Lymphocytes
IgG1	IgG1	Negative Control
CD3	CD4	CD4+ T-cells
CD3	CD8	CD8+ T-cells
CD3	CD19	T-cells, B-cells
CD3	CD56	T-cells, NK-cells

The immunophenotyping was performed on a Cytomics FC 500 (Beckman Coulter) flow cytometer. Gated lymphocyte populations were verified by labelling with antibody to CD45 and dual-labelled samples were run and analysed using appropriate negative, isotype and lymphocyte controls. All samples were processed within 6 hours of blood collection. For each sample, 10 µl of each monoclonal antibody was added to 50 µl of well-mixed anticoagulated whole blood in test tubes. The mixture was gently mixed and incubated for 20 minutes at room temperature in the dark. After incubation, a lyse/no wash method (Immunoprep for Q-prep, Beckman Coulter) was used for processing each specimen. Afterwards, 50 µl of flow-check fluorospheres (Beckman Coulter Ltd) were added for determining the absolute number of each parameter being measured. Immediately after processing, the samples were taken for flow cytometric analysis, in which a minimum of 5,000 lymphocytes were analysed. A single-platform technology was used, in which both absolute number and percentage of lymphocyte subsets were determined by the flow cytometer.

Measurement of hsCRP by commercial kit

Plasma hsCRP level (between-run CV is 3.5%) was measured using the Tina-quant CRP (latex) high sensitive immunoturbidimetric assay (Roche/Hitachi, USA) on the Hitachi (Roche) 902 automatic analyzer (This biomarker was measured by another member of our team and the results supplied to the author).

Assessment of antioxidant status/oxidative stress

Plasma total antioxidant status and ascorbic acid was measured using the FRAP and FRASC assay [Benzie and Strain, 1996, 1999; Choy and Benzie, 2000], alpha tocopherol (by HPLC), uric acid (by commercial kit), malondialdehyde (MDA; by HPLC) and allantoin (by HPLC) were measured (according to our usual protocols). Urine was measured for the oxidation products 8oxodG and F2 isoprostanes, both by LCMS/MS methods [Halliwell & Gutteridge, 2007; Lee et al., 2010] (These biomarkers were measured by another member of our team and the results supplied to the author).

Statistical analysis

The statistical analysis was carried out using GraphPad Prism (version 4.00 for Windows, GraphPad Software, San Diego California USA). In this cross-over study, 16 subjects is estimated be able to detect a true difference of 0.4 in the mean CD4+/CD8+ ratio (a key variable) with 80% power and at the 5% significance level. Recruiting 18 subjects allows for some drop out or loss of data through technical problems.

In statistical analysis, responses to the different teas were compared to response to placebo using one-way Repeated Measures ANOVA with Tukey's Multiple Comparison Test, with log transformation of skewed data. Codes were broken after data analysis. Statistical significance is defined at P value <0.05. Inter-relationships between biomarkers of interest were explored using Pearson's r or Spearman rho, as appropriate. Statistical analysis, using ANOVA for repeated measures, was carried out among the three pre-treatment results (n=18) to investigate baseline differences throughout the study.

Results

Participants were aged 42.6(3.6) years on average and all were of Chinese ethnicity (9 men, 9 women). One of the subjects did not comply adequately, and so data of only 17 subjects were analysed. None of the subjects reported any adverse side effects. The characteristics of 18 subjects for each treatment at entry (i.e. pre-each treatment period) are shown in Table 3.2. It is noted that a significant difference was found in baseline results ($P < 0.05$) in the total lymphocyte percentage in the third treatment period. Also a significant increase of CD3+/CD4+ T helper cells absolute number ($P<0.05$) and an increase of borderline significance in CD19+ was observed in the third treatment period.

A summary of the results (n=17) of the different immune biomarkers measured to assess the response to the three different treatments is shown in Table 3.3. Responses for the different lymphocyte subsets absolute number measured are shown in Fig 3.2-3.7. In addition, responses for the hsCRP are shown in Fig 3.8. Negative values indicate a decrease during the treatment period. In Figures, mean values are shown with standard errors of the mean presented by vertical bars.

According to Table 3.3, it can be seen that there was a slight but non-significant increase in lymphocyte number and cytotoxic T cell number (CD8+) in response to both green teas when compared with water response. An increase of borderline significance in lymphocyte number was observed in response to both green teas when compared with water response. An overall increase in helper T cell number (CD4+), B cell number and CD4/CD8 ratio was observed after all three treatments. On the other hand, an overall decrease in cytotoxic T cell percentage (CD8+) and monocytes number and percentage was observed after all three treatments. There was an increase in response in NK cell number and percentage after Loonjin green tea, while a decrease in response was observed after Screw-shaped green tea and water. In addition, wide variations in the number of granulocytes were observed after all three treatments. However, the responses did not show statistically significant difference among the treatments except for a significant decrease of response in helper T cell (CD4+) percentage after treatment with Loonjin green tea compared with water treatment.

Table 3.2

Baseline (Pre- treatment) Mean (SD) Values of White Blood Cells and Lymphocyte Subsets Before Each of the Three Intervention Periods (T1, T2, T3); n=18

	Cells per µl blood Mean (SD)			Percentage (%) Mean (SD)			P	
	T1	**T2**	**T3**	**T1**	**T2**	**T3**	**Cells/µl**	**%**
Lymphocytes	1369 (459)	1341 (320)	1596 (273)	20.5 (6.4)	20.4 (3.7)	24.5* (4.8)	P=0.082	*P=0.047
Monocytes	251 (43)	260 (61)	252 (75)	4.1 (0.7)	4.0 (0.6)	3.9 (0.9)	P=0.92	P=0.83
Granulocytes	2340 (588)	2805 (909)	2497 (684)	37.4 (5.8)	41.9 (7.0)	41.1 (8.8)	P=0.27	P=0.31
CD3+ CD4+	467 (163)	516 (1256)	597* (105)	34.3 (6.5)	35.6 (4.5)	34.5 (4.9)	*P=0.024	P=0.72
CD3+ CD8+	306 (106)	318 (107)	354 (82)	23.0 (6.2)	22.8 (6.7)	21.1 (4.5)	P=0.3	P=0.58
CD19+	197 (83)	217 (80)	266 (82)	14.4 (4.1)	15.0 (4.3)	15.0 (4.2)	P=0.051	P=0.88
CD56+	228 (142)	248 (121)	285 (150)	15.1 (7.0)	17.7 (7.3)	17.0 (7.3)	P=0.48	P=0.59
CD4+/CD8+ ratio	1.7 (0.7)	1.7 (0.5)	1.8 (0.5)	1.6 (0.7)	1.7 (0.5)	1.7 (0.5)	P=0.86	P=0.85

Table 3.3

Summary of Responses (Post minus Pre Treatment Value) in Immune Biomarkers Across Three Different Treatments

MEAN (SD)	Loonjin response	Screw-shaped response	Water response	ANOVA P value
Lymphocytes%	2.1 (4.7)	0.04 (5.8)	-0.5 (5.9)	P=0.33
Lymphocytes (cells/µl)	150 (359)	103 (339)	-85 (271)	P=0.058
CD4+ T helper %	-0.3* (5.1)	1.2 (4.0)	3.9 (5.2)	*P<0.05
CD4+ T helper (cells/µl)	48 (93)	54 (128)	80 (143)	P=0.74
CD8+ T cytotoxic %	-1.3 (3.8)	-1.1 (6.0)	-3.1 (7.9)	P=0.60
CD8+ T cytotoxic (cells/µl)	33 (98)	13 (130)	-35 (150)	P=0.32
CD19+ B cell %	1.4 (3.3)	-0.8 (4.1)	0.8 (3.9)	P=0.23
CD19+ B cell (cells/µl)	28 (72)	10 (55)	28 (91)	P=0.72
CD56+ NK cell %	1.6 (6.5)	-1.9 (7.0)	-1.3 (3.8)	P=0.22
CD56+ NK cell (cells/µl)	27 (126)	-12 (120)	-16 (114)	P=0.54
CD4/CD8 ratio	0.1 (0.3)	0.2 (0.6)	0.4 (0.7)	P=0.4
Monocytes %	-0.3 (0.8)	-0.1 (0.8)	-0.5 (1.0)	P=0.41
Monocytes (cells/µl)	-23 (65)	-9 (67)	-35 (83)	P=0.48
Granulocytes %	-1.8 (4.5)	3.7 (11.3)	0.6(7.9)	P=0.23
Granulocytes (cells/µl)	36 (641)	-35 (493)	41 (824)	P=0.95

***Statistically significant difference (P<0.05) compared to response to placebo in post test analysis.

Figure 3.2 shows that the lymphocyte absolute number was increased after the treatment with the two types of tea but a decrease was found after treatment with water. However, the response did not show statistically significant difference among the treatments. Figure 3.3 shows that there was an overall increase of absolute number of T helper cells after all three treatments, but the increase did not reach statistical significance among the treatments as the variation in results was rather wide.

Figure 3.2

Lymphocytes (cells/µl) response to Loonjin, Screw-shaped and Water After 4 weeks Supplementation (n=17)

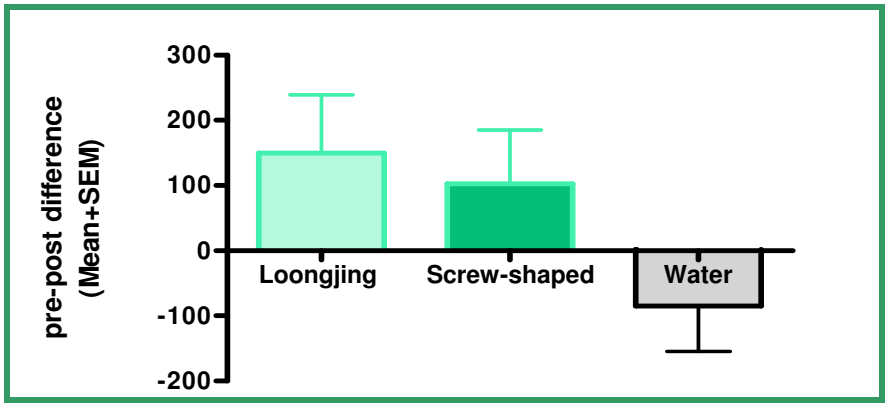


Figure 3.3

T helper cells CD4+ (cells/µl) response to Loonjin, Screw-shaped and Water After 4 weeks Supplementation (n=17)

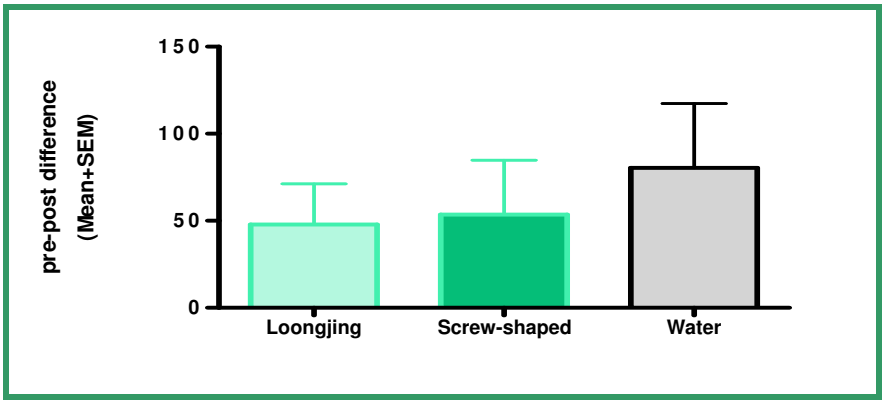


Figure 3.4 shows that there was a slight increase in CD8+ cytotoxic absolute number after treatment with Loonjin and a marginal increase after treatment with Screw-shaped green tea and a decrease after water. However responses did not reach statistical significance.

Figure 3.4

T cytotoxic cells CD8+ (cells/µl) response to Loonjin, Screw-shaped and Water After 4 weeks Supplementation (n=17)

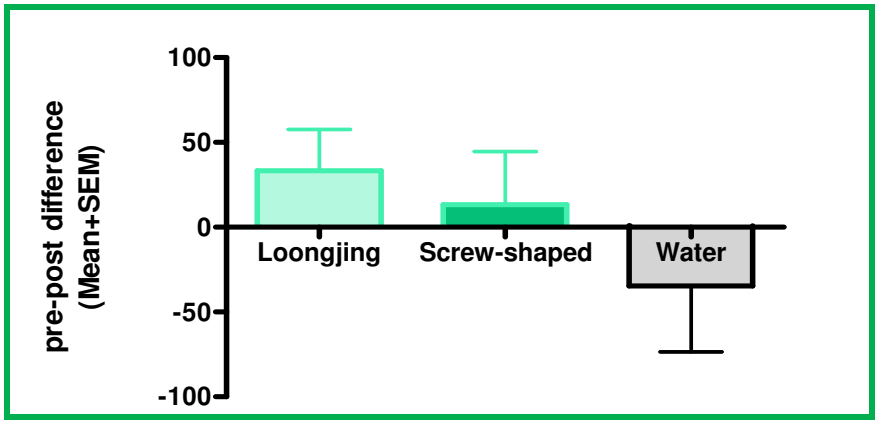


Figure 3.5 shows that there was an overall increase in the absolute number of B cells after all treatments. However, responses did not reach statistical significance when compared to response to water.

Figure 3.5
B cells CD19+ (cells/µl) response to Loonjin, Screw-shaped and Water After 4 weeks Supplementation (n=17)

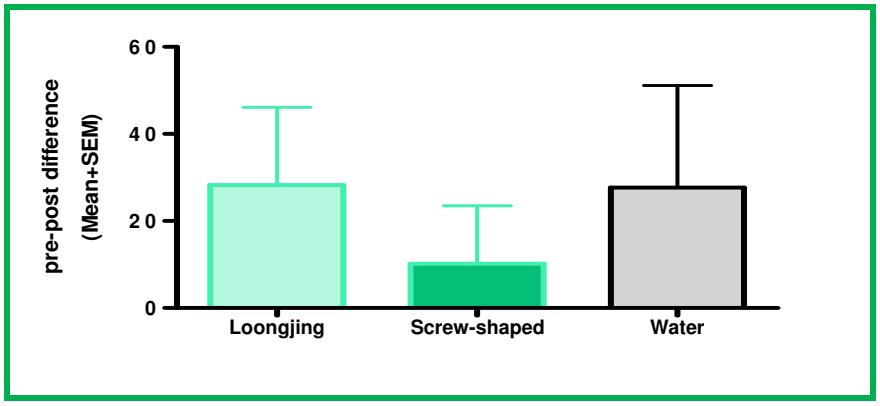


Figure 3.6 shows that there was an increase of NK cells absolute number after treatment with Loonjin and slight decreases were seen after treatment with Screw-shaped green tea and water. The responses did not show any statistically significant difference among the treatments.

Figure 3.6
NK cells CD56+ (cells/µl) response to Loonjin, Screw-shaped and Water After 4 weeks Supplementation (n=17)

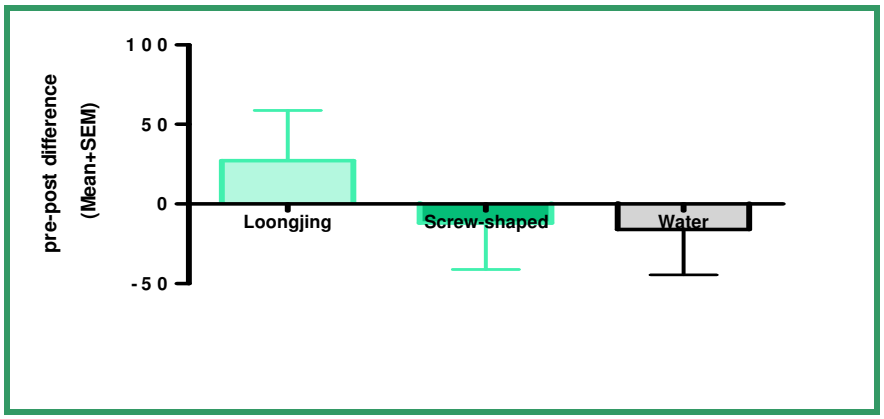


Figure 3.7 shows that there was an increase of CD4/CD8 ratio after treatment with water and Loonjin and a slight decrease was observed after treatment with Screw-shaped. However, all responses did not reach statistical significance. Figure 3.8 shows that that there was a decrease (> 20% decrease) in hsCRP after treatment of both Loonjin and Screw-shaped green tea, but increase (> 20% increase) in hsCRP after water treatment. However, variation was wide and the response did not show a statistically significant difference among the treatments.

Figure 3.7

CD4/CD8 ratio response to Loonjin, Screw-shaped and Water After 4 weeks Supplementation (n=17)

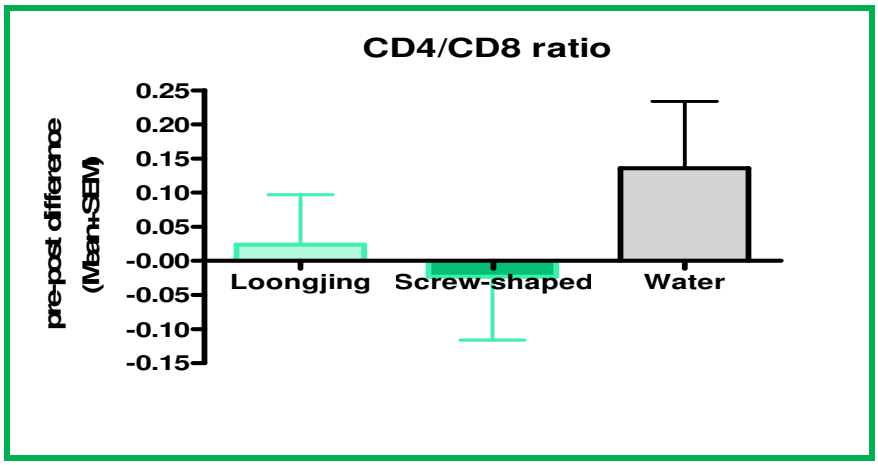
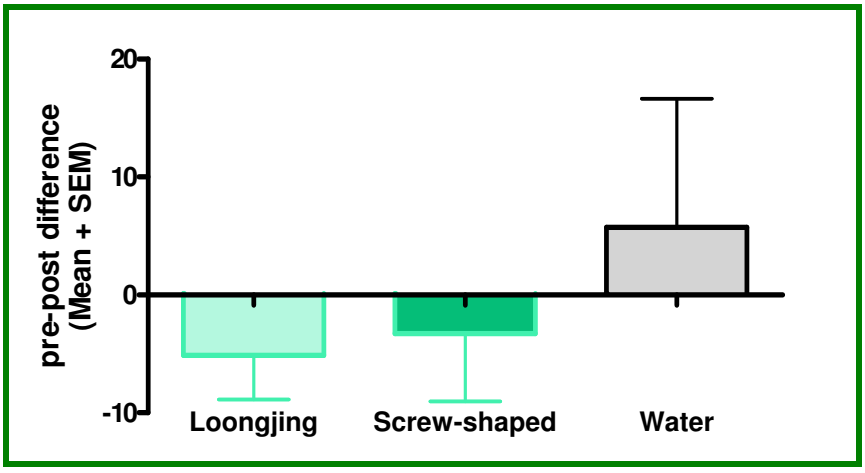


Figure 3.8

hsCRP response to Loonjin, Screw-shaped and Water After 4 weeks Supplementation (n=17)



Inter-relationships in response to treatment in terms of lymphocyte subsets and markers of oxidative stress and antioxidant status were investigated (Tables 3.4 and 3.5). Some potentially interesting significant correlations were seen. In Figure 3.9 it can be seen that there was a significant direct correlation ($r = 0.56$; $P<0.05$) between the plasma total antioxidant content (as the FRAP value) and the number of CD4+ cells (T Helper cells) after Screw shaped tea. However, this relationship was not seen after Loongjin tea. In Figure 3.10 it can be seen that there was a fairly strong significant direct correlation ($r = 0.75$; $P<0.001$) between the plasma ascorbic acid concentration and % CD19+ cells (B cells) after Loongjin tea. However, this relationship was not seen after Screw shaped tea.

In relation to other significant correlations, it was noted that these were driven largely by one data point in each case (Figures 3.11-3.15) and that when these points were not included no significant correlation were seen between these variables.

Correlations were also investigated between baseline (pre-supplementation) results for lymphocyte subsets and markers of oxidative stress and antioxidant status. No significant correlations were seen (results not shown).

Table 3.4

Inter-relationships in Response to Screw-Shaped Green Tea Treatment in Terms of Changes in Lymphocyte Subsets and Changes in Markers of Oxidative Stress and Antioxidant Status (Figures in table as Pearson r values) (n=17)

SSGT response	Lympho %	Lympho Abs no	CD4%	CD4 Abs no	CD8%	CD8 Abs no	CD19%	CD19 Abs no	CD56%	CD56 Abs no	CD4/CD8
FRAP	0.34	0.34	0.30	0.56*	-0.32	-0.22	-0.39	-0.12	0.61#	0.41	0.38
Asc Acid	0.06	0.33	0.0000	0.16	-0.33	-0.27	0.27	0.33	0.28	0.21	0.27
Vit E	-0.09	-0.10	-0.33	-0.31	-0.05	-0.13	-0.009	-0.41	0.33	0.28	-0.07
MDA	-0.44	-0.48#	-0.04	-0.42	-0.005	-0.27	0.17	-0.50#	0.007	-0.06	-0.05
SOD	-0.11	-0.07	0.41	0.27	-0.16	-0.18	-0.16	-0.11	0.14	0.01	0.30
GPx	0.06	0.22	-0.15	0.20	-0.21	-0.14	0.24	0.13	0.15	0.26	0.10
VCAM	-0.36	-0.34	0.43	-0.22	0.006	-0.09	-0.24	-0.28	-0.09	-0.30	0.13

*P<0.05

#P<0.05 but significance removed with exclusion of one data point (see Figures 3.11-3.13)

SSGT= Screw-shaped green tea

Table 3.5

Inter-relationships in Response to Loonjin Green Tea Treatment in Terms of Changes in Lymphocyte Subsets and Changes in Markers of Oxidative Stress and Antioxidant Status (Figures in table as Pearson r values) (n=17)

LGT response	Lympho %	Lympho Abs no	CD4%	CD4 Abs no	CD8%	CD8 Abs no	CD19%	CD19 Abs no	CD56%	CD56 Abs no	CD4/CD8
FRAP	-0.27	-0.28	-0.15	-0.25	-0.06	-0.35	0.035	-0.23	0.20	0.16	0.09
Asc Acid	0.06	-0.20	0.22	-0.24	-0.06	-0.29	0.75**	0.07	-0.27	-0.28	0.03
Vit E	0.223	0.27	-0.093	0.38	-0.11	0.29	-0.60#	0.29	-0.05	0.16	0.10
MDA	-0.30	-0.048	-0.26	-0.18	-0.27	-0.18	-0.32	-0.16	0.71#	0.11	0.24
SOD	-0.19	-0.32	0.27	-0.01	0.28	-0.004	0.24	-0.06	-0.30	-0.26	-0.15
GPx	-0.048	-0.090	0.20	-0.083	-0.16	-0.21	0.15	0.12	0.17	-0.32	0.13
VCAM	-0.24	-0.25	0.32	*0.11*	0.06	-0.16	0.12	0.07	-0.04	-0.006	0.25

**P<0.001

#P<0.05 but significance removed with exclusion of one data point (see Figures 3.14 and 3.15)

LGT= Loonjin green tea

Figure 3.9

Correlation Between Response in Total Plasma FRAP and Response in T helper cells Absolute Number: Screw-shaped Green Tea Treatment (r = 0.56; P=0.02; n=17)

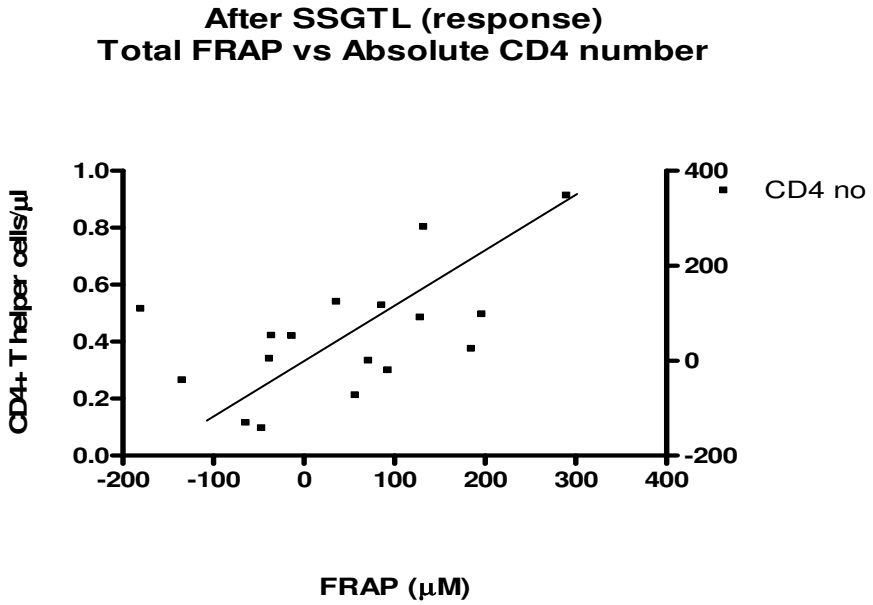
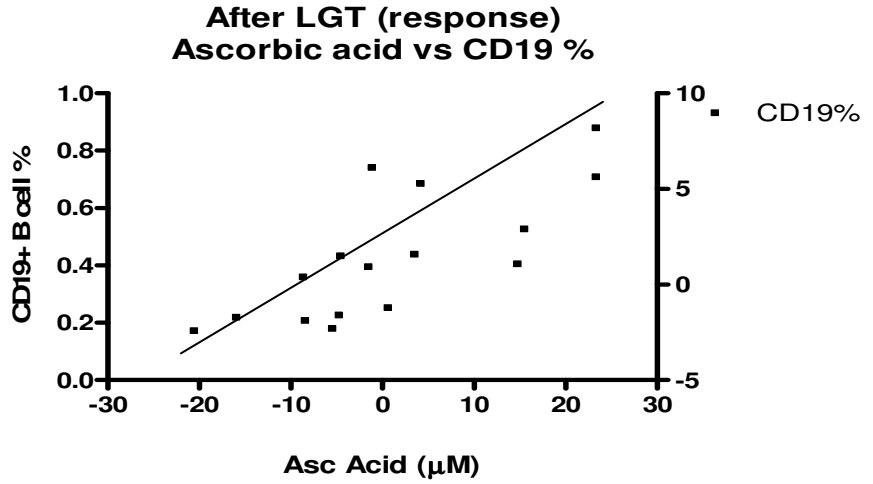


Figure 3.10

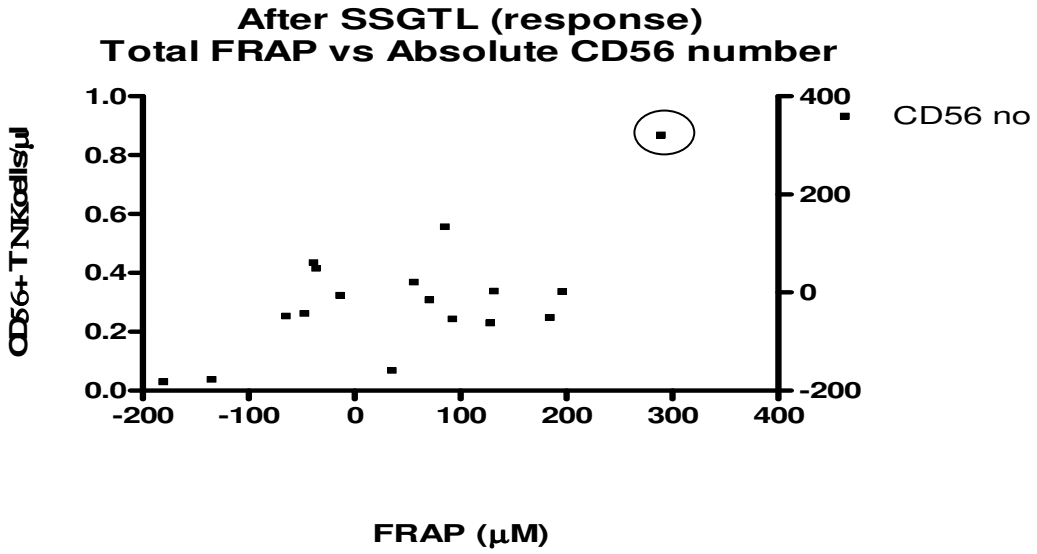
Correlation Between Response in Plasma Ascorbic Acid and Response in the B cells: Loonjin Green Tea Treatment (r = 0.75; P=0.0008; n=17)



Spurious correlations, which are lost after the removal of one data point, are shown in Figures 3.11-3.15. In Figure 3.11, it can be seen that there was a significant direct correlation ($r = 0.61$; $P<0.01$) between the plasma total antioxidant content and the number of NK cells after Screw shaped tea. This was driven by one data point (circled).

Figure 3.11

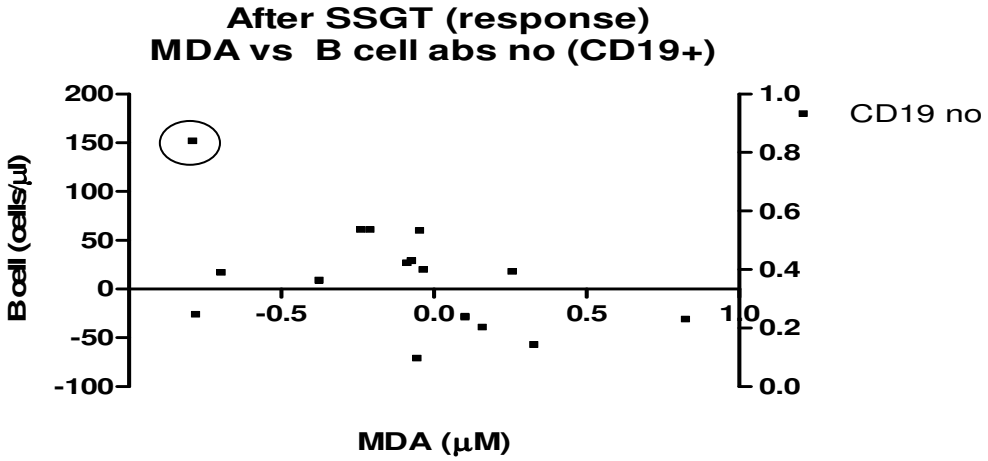
Correlation Between Response in Plasma FRAP and Response in the NK cells Absolute Number: Screw-shaped Green Tea Treatment (r = 0.61; P=0.009; n=17)



In Figure 3.12, it can be seen that there was a significant inverse correlation ($r=-0.50$; $P<0.05$) between plasma MDA (a marker of oxidative stress) and the number of B cells after Screw-shaped green tea. However, there is no significant correlation after the removal of the circled data point.

Figure 3.12

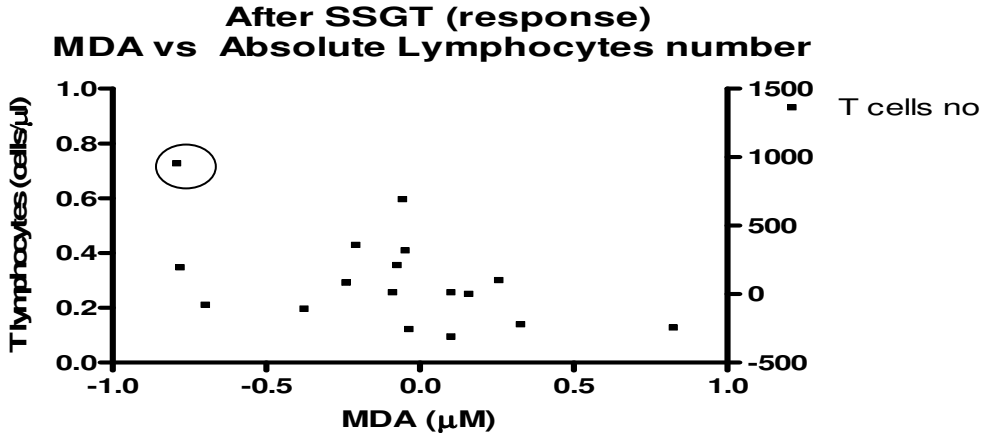
Correlation Between response in Plasma MDA and response in the B cells Absolute Number: Screw-shaped Green Tea Treatment (r =- 0.50; P=0.04; n=17)



In Figure 3.13, it can be seen that there was a significant inverse correlation between MDA and the total number of T cells. However, this correlation is lost after the removal of one data point (circled).

Figure 3.13

Correlation Between response in Plasma MDA and response in Total Lymphocyte Absolute Number: Screw-shaped Green Tea Treatment (r = -0.48; P=0.05; n=17)



In Figure 3.14, it can be seen that there was a significant inverse correlation between lipid standardized Vitamin E and B cells % (r=-0.60; P<0.05) after Loongjin tea. However, this correlation is not significant after the removal of the outlier data point. Moreover, it can be seen that this correlation is largely driven by one data point and no correlation trend is seen at all. Similarly in Figure 3.15, there was a significant inverse correlation between MDA and NK cells after Loongjin tea. But no correlation is seen after excluding one data point (circled).

Figure 3.14

Correlation Between response in Plasma Vitamin E and response in the B cells %: Loonjin Green Tea Treatment (r = -0.60; P=0.01; n=17)

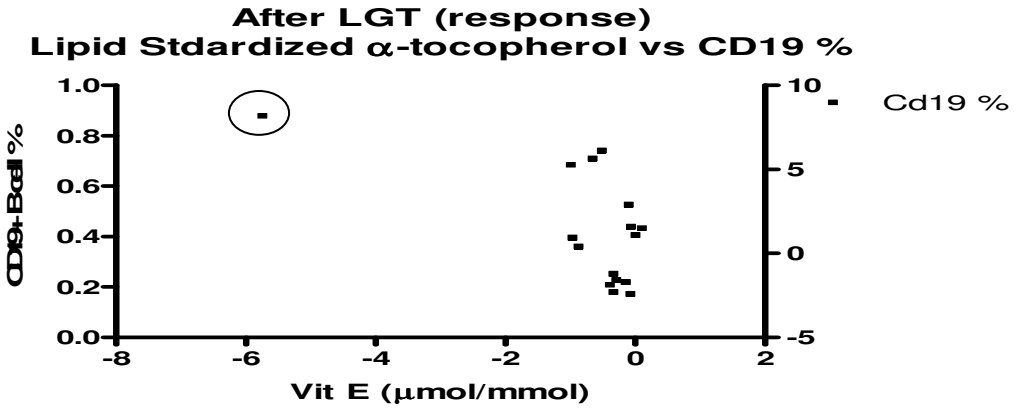
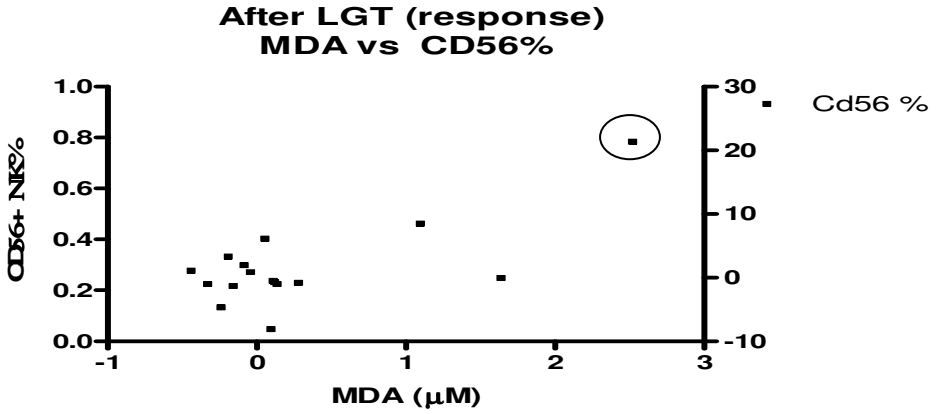


Figure 3.15

Correlation Between response in Plasma MDA and response in the NK cells %: Loonjin Green Tea Treatment (r = 0.71; P=0.002; n=17)



Discussion

Green tea is a potential immunomodulator because of its high antioxidant content. There are some data from animal and *in vitro* studies to support this view [Hamer, 2007; Pajonk et al., 2007; Wheeler et al, 2007], but there is only one human trial to date. This used high dose green tea extract for 3 months and focused mainly on clinical outcome of cold and flu symptoms, but included measurement of cytokine release from activated gamma delta T cells [Rowe et al., 2007]. The authors reported improvement in both types of outcome. In a subsequent paper from the same group and from (we believe) the same study, an inflammatory biomarker, serum amyloid alpha, was also reported to be improved after the tea intervention [Nantz et al., 2009]. This current study is the first human controlled intervention trial to investigate the effect of green tea on biomarkers of cellular immunity and inflammation. White cell numbers and the major lymphocyte subsets were studied, along with hsCRP (a sensitive biomarker of inflammation), before and after 4 weeks' supplementation with two types of green tea. Overall, very little if any effect of green tea was detected, although there was some evidence of increased total lymphocytes after each tea, and decreased hsCRP. Decreases in hsCRP averaged 30% after Loonjin and 23% after Screw-shaped green tea, in comparison to an average increase in hsCRP of 33% after water. However, variation was wide, and tea responses results did not reach statistical significance. Nonetheless, results may indicate some anti-inflammatory effect of green tea. Previous studies of green tea focusing on cardiovascular risk and inflammation show conflicting results of effects of tea or green tea extract on hsCRP. In a study of 55 type 2 diabetes patients (31 men and 24 women) in a randomized, controlled trial of cross-over design, consumption of 9g/d of green tea (900 ml of 1% w/v tea, the same concentration but three times greater amount per day than in this current study) for four weeks did not have any effect on hsCRP [Ryu et al., 2006]. However, a study where 30 healthy male smokers consumed a high dose of green tea catechins (580mg/day) showed a significant decrease of hsCRP after 2 weeks' supplementation [Oyama et al., 2010]. As noted above, supplementation with high dose extract of green tea was associated with a 42% decrease in another biomarker of inflammation, serum amyloid alpha [Nantz et al., 2009]. Overall, previous and current results are supportive of an anti-inflammatory effect of green tea. Such effect, if

confirmed, would bring long term health benefits in terms of inflammation-related disease. A longer supplementation period than the four weeks used here and/or a higher dosage of green tea may be required for confirmation.

In this study, there was also a small but statistically significant decrease in CD4+ T Helper cells seen after one of the teas (Loonjin). While increased total lymphocytes and decreased hsCRP could be regarded as beneficial, decreased T helpers cells is not desirable. With ageing, CD4+ cells decrease [Haynes and Maue, 2009; Gruver et al., 2007], and so a desirable immunomodulatory effect would be to increase CD4+ cells. There are no published reports of effects of green tea supplementation on CD4+ cells, or indeed on any white cell numbers or subsets. However, there are some reports of positive effects of increased antioxidant intake on cellular immunity. Vitamin E and β-carotene, both dietary derived antioxidants, were found to have immunoenhancing effects and to boost the immune response in aged persons [reviewed by Meydani et al., 1995]. It was also reported in a review that antioxidant supplementation with zinc, selenium, vitamin E and carotenoids essentially reverses several age-associated immune deficiencies by increasing total lymphocytes and T-cell subsets and increasing killer T cell activity [Knight, 2000b]. For example, vitamin C supplementation (500mg/day for 1 month) was shown to enhance the proliferative response of T lymphocytes in older people of >70 years [Kennes et al., 1983]. In another study, with 40 healthy volunteers (22-55 years), it was reported that 1g/day of ascorbic acid for 28 days stimulated the immune system through enhancing T cell proliferation in response to infection [Jeng et al., 1996]. In addition, in a randomised parallel double-blinded study, it was reported that 5 weeks' supplementation with a prebiotic antioxidant bread, which contained green tea powder, significantly increased the percentage of CD19+ cells in smokers (n=20) [Seidel et al., 2007].

While this current study did not reveal any significant improvement in cellular immunity in the healthy middle aged adults studied, the previous reports of antioxidant-related immunomodulation suggest that there may nonetheless be some immune function benefit from increased intake of antioxidants. If this is true, then it might be

expected that there would be some relationship between antioxidant status and immune function or in changes in immune function with changes in antioxidant status occurring as a result of supplementation. There are no previously published reports on such relationships. In this study we found no significant correlations between baseline levels of cell-based immunity biomarkers and any of the antioxidant and oxidative stress biomarkers measured. However, there were two potentially interesting, significant, direct correlations of responses seen. One was between the supplementation-related change in plasma FRAP and the change in CD4+ cell number, although it is noted that this was only seen with Screw-shaped tea. The other was between the change in plasma ascorbic acid and the change in % of CD19+ cells, although it is noted that this was only seen with Loongjin tea. It must be acknowledged that these apparent relationships might be due to Type I error, as many inter-relationships were investigated. In addition, it is unlikely that the green tea supplementation affected plasma ascorbic acid directly. We did not measure vitamin C level of the tea, but dried tea leaves in hot water are unlikely to supply a significant amount of vitamin C. It is possible that green tea polyphenols could 'conserve' or recycle ascorbic acid in the gut or in plasma. However, it is noted that there was no evidence of generally increased ascorbic acid status in the volunteers after taking green tea. Therefore, while the significant, positive associations seen between the changes in FRAP and CD4+ T cells with one tea, and between changes in ascorbic acid and CD19+ B cells with the other tea are intriguing, these cannot at the moment be explained and require confirmation in further study. Still, they are supportive of the reported positive effects of increased antioxidant intake on immune function.

The limitations of this study should be noted. It was carried out with a relatively small volunteer group in good general health. Variation in the immunity biomarkers measured is wide, and small changes are difficult to detect. It remains to be seen whether these results can be generalized to people who already show a marked immune decline. Moreover, the intervention was for only four weeks, so longer term effects could not be evaluated. There was no measurement of biomarkers of humoral immunity function, which could indicate wider changes in immune status, so it is not clear whether there

were other effects on other immune components. We did not measure tea polyphenols in plasma or urine and so the amount absorbed is unknown. The strengths of the study include the design (placebo controlled cross-over trial), a relatively homogenous group (all ethnic Chinese, aged 35-50 years, non-smokers, in good health), the dietary-relevant dose of tea was used, a wide range of biomarkers were measured, and two green teas were given.

In conclusion, this controlled, human intervention study is the first (to our knowledge) on effects of antioxidant-rich green tea on biomarkers of cellular immunity and inflammation. No convincing evidence of positive effects on white cell numbers or sub-sets was seen. There was some indication of an anti-inflammatory effect, but variation in the response of the inflammation biomarker (hsCRP) was wide, and changes did not reach statistical significance. Some potential interesting results were observed in terms of inter-relationships between changes in antioxidant status and changes in some white cell subsets. Further studies are suggested in order to clarify the effect of regular consumption of green tea on immune status.

Some of the results in this chapter have been presented as follows:

ARW Lau, SW Choi, WM Lo, KC Wong, IFF Benzie. The immunomodulatory & anti-inflammatory effects of green tea: results from a controlled human intervention study. Hong Kong-Macau Symposium on Chinese Medicine (August 2009)

ARW Lau, SW Choi, WM Lo, KC Wong, IFF Benzie. A study of immunomodulatory & anti-inflammatory effects of green tea: results of a controlled human intervention trial-oral presentation. International Scientific Conference on Nutraceuticals and Functional Foods (June 2009)

Chapter 4

A STUDY OF POTENTIAL IMMUNOMODULATORY EFFECT OF BILBERRY IN TYPE 2 DIABETIC PATIENTS: RESULTS OF A CONTROLLED HUMAN INTERVENTION STUDY

Introduction

Bilberry is a purple-colored berry rich in anthocyanins with powerful *in vitro* antioxidant properties [Kemper, 1999]. This fruit is usually promoted as beneficial for vision, and has been used traditionally for treatment of ocular disorders [Parker and Parker, 2003]. However it has been used also for treatment of diabetes and promotion of cardiovascular health [Upton 2001; Canter and Ernst 2004; Mauray et al., 2009]. Bilberry anthocyanins have been widely investigated *in vitro* and several studies have demonstrated anthocyanins to have a variety of potential biological effects, including anti-inflammatory, antioxidant, inducing apoptosis in cancer cells, and lowering blood glucose in diabetic mice [Zafra-Stone, 2007; Dai et al., 2009; Grace et al., 2009; Willlis et al., 2009]. If confirmed, these effects would contribute to promotion of healthy ageing and lowering risk of cancer, cardiovascular disease, cognitive dysfunction, and various diseases associated with increased oxidative stress, such as diabetes [Zafra-Stone, 2007; Willlis et al., 2009].

Bilberry has been reported to have some immunomodulatory effects. For instance, a commercial anthocyanin-rich extract from bilberry was shown to increase the number of neutrophils and monocytes in peripheral blood of mice treated with 5-fluoroucil, a chemotherapeutic agent which induces myelotoxicity [Choi et al., 2007]. The drug-induced depletion of myeloid cells in the spleen and bone marrow was also shown to be less after treatment of 500mg/kg with the bilberry extract for 10 days [Choi et al., 2007]. In another study in which mice were treated with selected antibiotics (cefuroxime, cefoperazone and doxycycline), an extract from bilberry fruit (undiluted and diluted at

1:10) was demonstrated to positively affect the survival of mouse thymocytes, increase the count of splenocytes and increase the agglutination titre of serum. Antibiotics have been reported to affect phagocytosis, macrophage function and T and B cell proliferation [Drozd and Anuszewka, 2009]. The authors suggested bilberry stimulated the immune system and thus justified its use as a measure to improve the immune status of patients undergoing antibiotic therapy [Drozd and Anuszewska, 2009]. In addition, anthocyanins have been reported to have anti-inflammatory effects. In a murine asthma model, anthocyanins were shown to decrease air-way inflammation induced by ovalbumin by downregulating Th2 cytokines, proinflammatory cytokines and COX-2 [Park et al., 2007].

In addition to potential immunomodulatory effects, increased intake of antioxidant-rich bilberry may benefit health through opposing oxidative stress. In conditions of increased oxidative stress, deleterious oxidation-induced changes to lipids, protein and DNA are found [Halliwell, 2007]. Ageing is associated with increased oxidative stress, increased risk of various diseases and a decline in immune function [De la Fuente and Miquel, 2009]. The age-related decline in immune status begins in the 4^{th} decade of life, and affects mainly cell-mediate immune response [Sansoni et al., 1993; Chandra, 2004]. For example, 19 older subjects (66-82 years) were reported to have decreased helper T-cell (CD4+) response to influenza vaccine and a very poor cytotoxic T lymphocyte (CD8+) response compared to those aged < 50 years [McElhaney et al., 1998]. The causes of the age-related immune decline are not completely known, but malnutrition, thymic involution, UV exposure, psychological stress and oxidative stress are all involved [Neill et al., 1998; Kiecolt-Glaser, 1999; Liu and Mori, 1999; Gleeson, 2007; Gruver et al., 2007]. As noted in earlier chapters, the consequences of this age-related decline in cell mediated immune status include lower immune surveillance, higher risk of infection, lower immune response to infection, and poor response to vaccination [Perskin and Cornstein, 1992; Frasca et al., 2005; Karasek, 2006]. Therefore, strategies that could prevent age-related decline or improve cell-mediated immune function associated with ageing, antibiotic use, and increased oxidative stress would have high impact in the ageing population.

Type 2 diabetes mellitus (DM) is considered as a state of increased oxidative stress and accelerated ageing [Choi et al., 2008]. Diabetes is a group of metabolic diseases characterized by hyperglycaemia [Choi et al., 2005: American Diabetes Association website, 2010]. The long-term manifestations of DM often result in serious metabolic complications such as cardiovascular disease, nephropathy and retinopathy. The development and progression of complications of diabetes are also associated with increased oxidative stress [Choi et al., 2005]. Type 2 DM is an age-related disease and the prevalence is very high and increasing in Hong Kong [Lam, 2009]. A few studies have shown that diabetic patients have impaired innate and adaptive immune responses, and alterations in immunocompetent cells have also been recorded [Chang and Shaio, 1995; Fontana et al., 1999].

There is limited clinical evidence to provide adequate support for the use of antioxidants in diabetes, and no studies have been performed to date to consider immunologic and oxidative stress markers in relation to antioxidant supplementation in Type 2 DM. The aim of this part of the study was to explore whether supplementation with an antioxidant rich food (bilberry) benefits immune status (in terms of white cell number and selected white cell subsets) in Type 2 DM patients and whether these effects are associated with changes in antioxidant status or oxidative stress. Also in this part of the study, the comparison between certain immune parameters between healthy subjects and Type 2 DM patients was investigated to confirm differences found in earlier studies [Chang and Shaio, 1995; Fontana et al., 1999].

The specific objectives of this part of the study were:

1. To investigate changes in immune markers (white blood cell count and selected lymphocyte subsets) in Type 2 DM subjects taking a bilberry supplement.
2. To determine the inter-relationships between supplementation-related changes in the selected immune markers and those of biomarkers of antioxidant defense and oxidative stress.

3. To compare the markers of immune status of people under increased oxidative stress (Type 2 DM patients) with a group of apparently healthy subjects of similar age and sex and the same ethnicity (Chinese).

Materials and Methods

Subjects

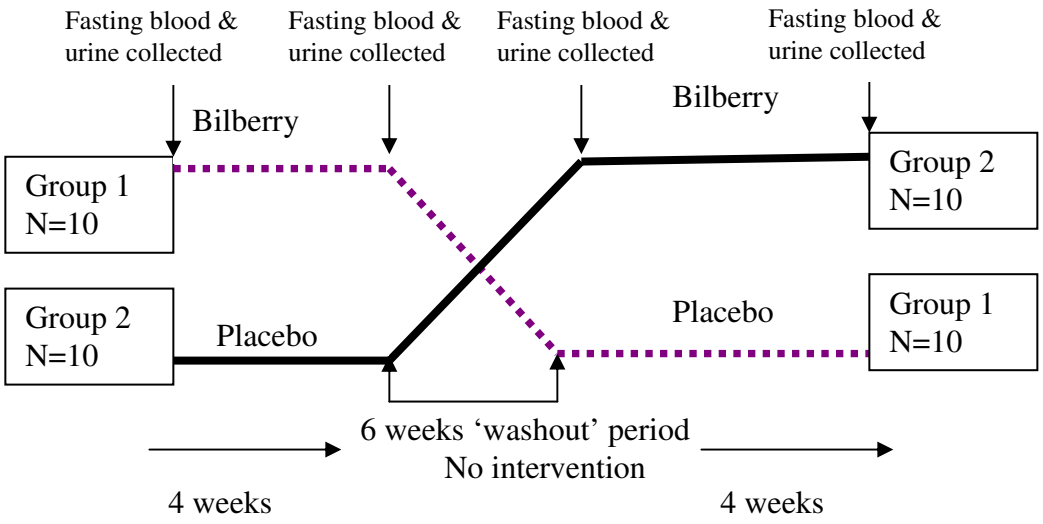
Twenty Type 2 DM consenting subjects, aged 30-70 years were recruited from the Diabetic Clinic in the Prince of Wales Hospital, Shatin, Hong Kong. Ethical approval was granted by the Ethics Subcommittees of The Hong Kong Polytechnic University and the Prince of Wales Hospital. Subjects did not have record of serious illness other than their diabetes, and their entry body mass index was >23 kg/m^2. Subjects with other chronic diseases, diabetic complications, or taking regular antioxidant or vitamin supplementation were excluded from this study. Subjects were requested not to change their regular diet and lifestyle habits during the 14 weeks of participation in the trial, apart from taking the supplement. No treatment changes were recorded during the study.

Methodology

This study was a double-blinded placebo-controlled cross-over intervention study Subjects were assigned to one of two treatments (bilberry or placebo capsules) in alternate order. The commercial capsules were sponsored and provided by PolyU PTec Co. Ltd (and obtained from Guilin Layn Natural Ingredients Corp, Guilin, PRC) (Appendix I). Each capsule contained 200mg bilberry extract or (for placebo) starch. Each subject took four capsules per day (two in morning and two in evening). 10 subjects took 800mg/day (supplying a total of 204.8mg anthocyanin/day) of bilberry extract and 10 took placebo for four weeks, after which there was a six week washout period. Subjects were then crossed-over onto the other treatment for four weeks. Compliance was checked by regular contact with the subjects and by counting the number of capsules returned from the subjects at the end of each intervention period (80% compliance was considered to be satisfactory). Before and after each 'treatment' fasting venous blood was collected into EDTA and heparin blood tubes, and urine was collected into plain containers. The study outline is given in Fig 4.1.

Figure 4.1

Overview of the Study



Laboratory testing

Within 6 hours of sample collection, the total white blood cell count was measured (Cell Dynn 3200 Haematology analyzer, Abbot, USA). Measurement of white blood cell subsets by flow cytometry used the same procedures as described in detail in Chapter 3 under the *"Reagents, cell preparation and staining for immunophenotyping"* subheading. Results are presented as mean±SEM for each type of lymphocyte subset. For biomarkers of antioxidant status/oxidative stress the same procedures as described briefly in Chapter 3 under the "*Assessment of antioxidant status/oxidative stress"* subheading are presented. For the comparison with non-diabetic subjects, 18 apparently healthy Chinese subjects of similar age and sex were recruited with informed consent, and the white cell subsets were measured in fasting samples by the same methods as for the Type 2 DM subjects.

Statistical Analysis

The statistical analysis was carried out using GraphPad Prism (version 4.00 for Windows, GraphPad Software, San Diego California USA). In this cross-over study, 20 subjects are estimated to be sufficient to detect a true difference of 0.4 in the mean

CD4/CD8 ratio (a key variable) with 85% power and at the 5% significance level. In statistical analysis, response to treatment was compared to response to placebo by using paired t-test. Comparison of variables at baseline (pre-supplementation) between Type 2 diabetic patients and healthy subjects were made by Mann Witney test. Pearson's or Spearman's correlation analysis, as appropriate, was used to explore inter-relationships between biomarkers of interest. Significance was set at $P<0.05$.

Results

In the supplementation trial, three of the subjects did not comply adequately, and so the data of only 17 subjects were analysed. None of the subjects reported any adverse side effects. The characteristics of 20 subjects for each treatment at entry (i.e. pre-each treatment period) are shown in Table 4.1. It is noted that no significant difference was found between the two sets of pre-treatment results ($P >0.05$).

Table 4.1

Mean, Standard Deviation of White Blood Cells and Subsets Before Each of the Two treatments Periods; n=20

Parameter	Cells/µl Mean(SD)		Percentage (%) Mean(SD)	
	Pre-1st Treatment	Pre-2nd Treatment	Pre-1st Treatment	Pre-2nd Treatment
Lymphocytes	1919 (349.8)	1976 (399.5)	29.4 (6.2)	29.4 (5.2)
Total WBC	6797 (1451)	6816 (1346)	-	-
Monocytes	483 (156.5)	481 (147.8)	7.1 (1.4)	7.0 (1.5)
Neutrophils	4089 (1246)	4205 (1081)	59.9 (6.9)	60.4 (6)
Eosinophils	177 (94.2)	166 (107.2)	2.7 (1.6)	2.4 (1.3)
Basophils	63 (14.3)	67 (22.8)	1.0 (0.3)	1.0 (0.4)
CD3/CD4	783 (218.8)	815 (228.3)	40.8 (8.1)	41.2 (7.9)
CD3/CD8	429 (227.7)	414 (180)	21.3 (8.9)	20.7 (7.8)
CD19	239 (119.5)	264 (145.8)	12.4 (5.8)	13.0 (6.5)
CD56	245 (120.6)	268 (125.6)	12.7 (6.5)	14.0 (7.2)
CD4/CD8 ratio	2.1 (1.2)	2.1 (1.5)	2.2 (1.2)	2.2 (1.2)

A summary of the results (n=17) of the different immune biomarkers measured to assess the response to treatments are shown in Table 4.2. No significance difference was found in response to treatment when compared with placebo ($p>0.05$). Responses for the different lymphocyte subsets percentage measured are shown in Figures 4.2-4.7. Negative values indicate a decrease during the treatment period. Mean values are shown with standard errors of the mean presented by vertical bars. In addition, inter-relationships found between white blood cell count and markers of antioxidant status are shown in Figures 4.8 and 4.9.

According to Table 4.2 and Figures 4.2-4.5, it can be seen that there was a slight but non-significant increase in the lymphocytes % helper T cells % (CD4+), neutrophils % and CD4/CD8 ratio in response to bilberry when compared with placebo response. An increase of borderline significance in the cytotoxic T cells (CD8+) % was observed in response to bilberry when compared with placebo response. Slight but non-significant decrease with bilberry were seen in lymphocyte cell number, helper T cell (CD4+) number, cytotoxic T cell (CD8+) number, B cells number, NK cells number, NK cell %, neutrophils number and basophils number when compared with placebo response.

Table 4.2

Summary of Response (post- minus pre-treatment value) of Immune Biomarkers across Treatments

Parameter	Response to Bilberry Treatment Mean (SD)	Response to Placebo Treatment Mean (SD)	P value
Lymphocytes%	0.4 (4.8)	-0.1 (3.9)	P=0.67
Lymphocytes (cells/µl)	-57 (230.2)	-20 (267.1)	P=0.66
CD4+ T helper %	0.5 (2.8)	-0.7 (4.3)	P=0.86
CD4+ T helper (cells/µl)	-14 (92.3)	-22 (137.0)	P=0.43
CD8+ T cytotoxic %	0.7 (3.3)	-1.4 (3.9)	P=0.06
CD8+ T cytotoxic (cells/µl)	-38 (141.5)	-13 (88.8)	P=0.89
CD19+ B cell %	0.0 (3.6)	-1.4 (3.0)	P=0.71
CD19+ B cell (cells/µl)	-3 (78.2)	-34 (100.8)	P=0.98
CD56+ NK cell %	-2.1 (3.8)	-0.5 (2.0)	P=0.14
CD56+ NK cell (cells/µl)	-45 (76.5)	-0.6 (31.2)	P=0.08
CD4/CD8 ratio	0.2 (0.6)	-0.2 (1.9)	P=0.56
Total WBC (cells/µl)	-63 (1513.4)	149 (1160.8)	P=0.72
Monocytes %	-0.1 (1.2)	0.1 (1.7)	P=0.59
Monocytes (cells/µl)	-14 (75.4)	4 (134.3)	P=0.95
Neutrophils %	0.2 (6.1)	-0.5 (5.1)	P=0.9
Neturophils (cells/µl)	-32 (1397.7)	-7 (937.9)	P=0.61
Eosinophils %	0.3 (0.7)	0.4 (1.6)	P=0.97
Eosinophils (cells/µl)	8 (32.2)	20 (106.6)	P=0.81
Basophils %	0.02 (0.3)	-0.02 (0.3)	P=0.96
Basophils (cells/µl)	-0.3 (16.0)	-2.6 (24.8)	P=0.92

Figure 4.2

Lymphocytes % response to Bilberry and Placebo After 4 weeks Supplementation (n=17)

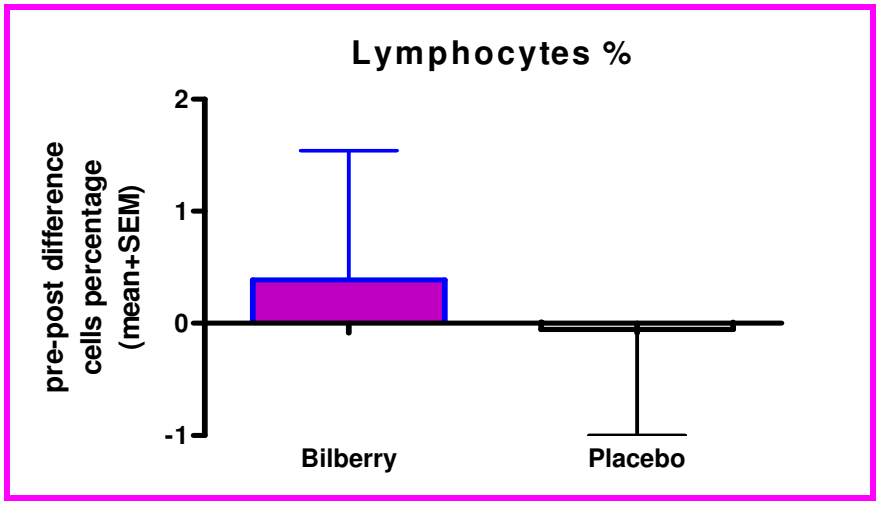


Figure 4.3

CD4+ T helper % response to Bilberry and Placebo After 4 weeks Supplementation (n=17)

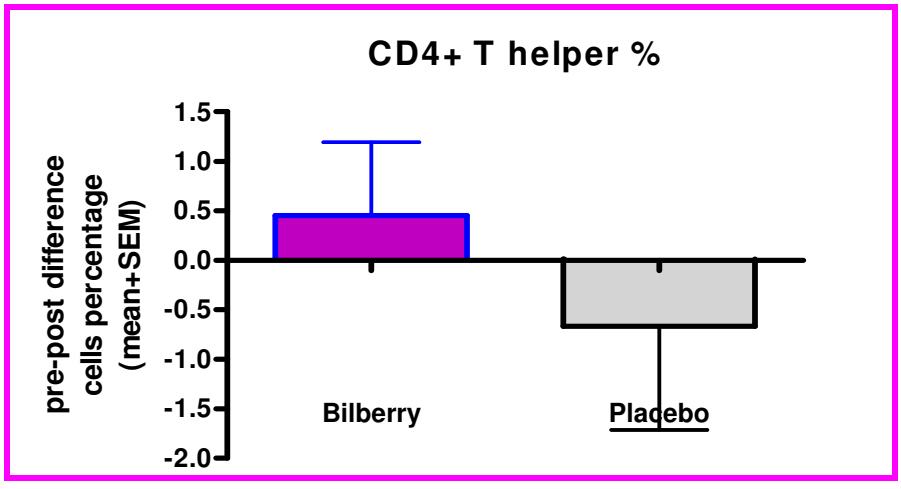


Figure 4.4

CD8+ T cytotoxic % response to Bilberry and Placebo After 4 weeks Supplementation (n=17)

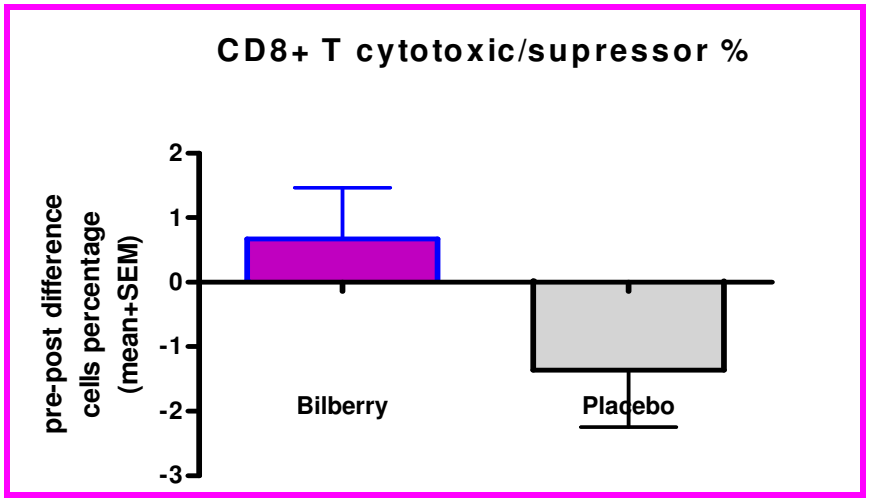


Figure 4.5

CD4/CD8 ratio response to Bilberry and Placebo After 4 weeks Supplementation (n=17)

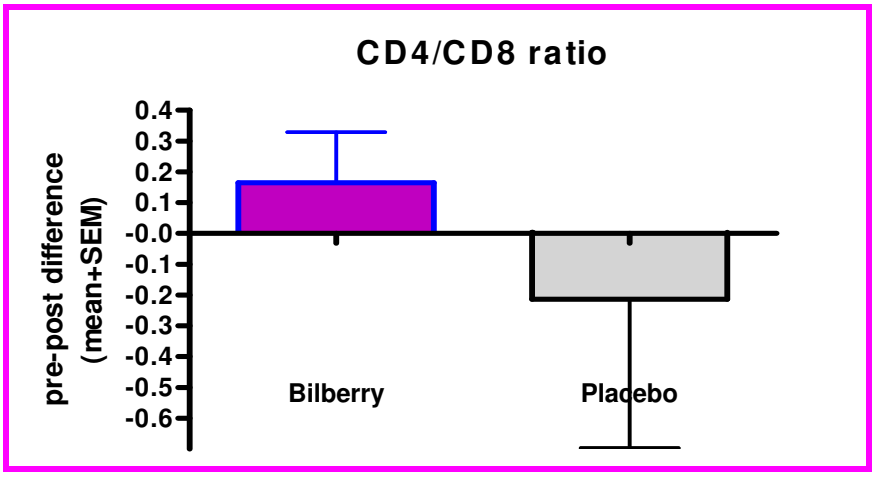


Figure 4.6 shows that there was no statistically significant change in CD19+ (B cell) number or % after bilberry compared to after placebo, although there was around a 13% decrease in number with placebo. Figure 4.7 shows responses in NK cells % after bilberry and placebo treatment. The NK number decreased by ~16%, and % decrease was ~2%. However these changes were not significant when compared to placebo.

Figure 4.6

CD19% B cell response to Bilberry and Placebo After 4 weeks Supplementation (n=17)

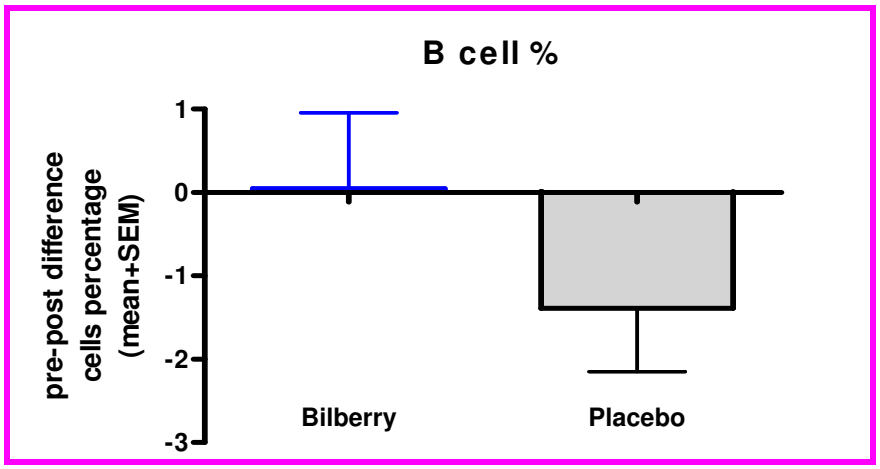
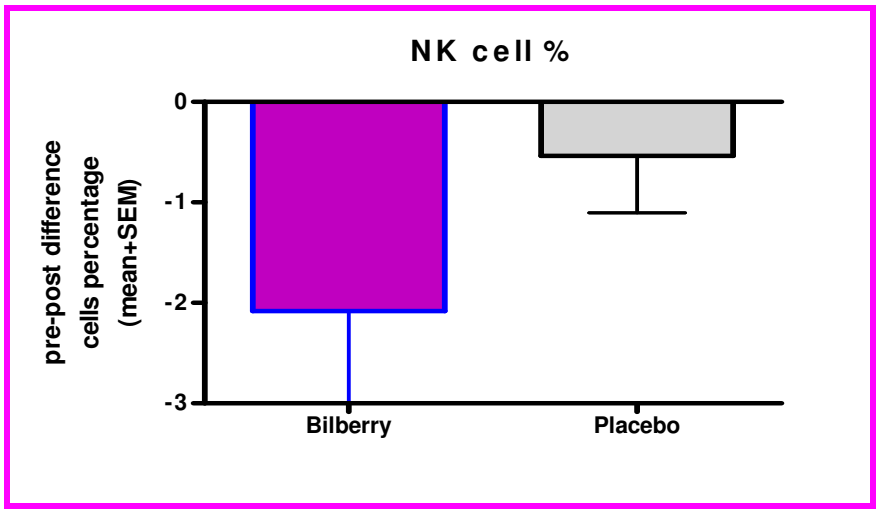


Figure 4.7

CD56+ NK cell % response to Bilberry and Placebo After 4 weeks Supplementation (n=17)



Inter-relationships at baseline and in response to treatment in terms of lymphocyte subsets and markers of oxidative stress and antioxidant status were investigated (Tables 4.3 and 4.4). Some potentially interesting significant correlations were seen at baseline (Figures 4.8 and 4.9). In addition, some significant correlations were observed in response to bilberry treatment in terms of some lymphocyte subsets, differential WBC count and markers of oxidative stress and antioxidant status, shown in Figures 4.10-4.13.

In relation to other significant correlations, it was noted that these were driven largely by one data point in each case (Figures 4.14 and 4.15) and that when these points were not included no significant correlation were seen between these variables.

Table 4.3

Inter-relationships at Baseline in Terms of Lymphocyte Subsets, WBC Count and Biomarkers of Oxidative Stress and Antioxidant Status in 20 Type 2 DM Subjects (Pearson r value; n=20)

	FRAP	Asc Acid	Vit E (Lipid Standardised)	Allantoin	Urinary 8-oxo-G
Lympho%	-0.41	0.09	-0.31	-0.22	0.49*
Lympho (cells/µl)	-0.44*	0.13	0.08	0.28	0.23
CD4 %	0.04	-0.30	0.05	0.13	0.08
CD4+ (cells/µl)	-0.35	-0.27	-0.14	0.18	0.33
CD8+ %	0.00	0.19	-0.32	0.33	0.01
CD8+ (cells/µl)	-0.35	0.14	-0.32	0.38	0.23
CD19+ %	-0.14	0.36	0.05	-0.29	0.21
CD19+(cells/µl)	-0.33	0.34	0.02	-0.17	0.29
CD56+ %	0.42	0.19	-0.32	-0.26	0.20
CD56+(cells/µl)	0.33	0.26	-0.31	-0.13	0.29
CD4/CD8 ratio	0.47	-0.07	-0.10	-0.14	0.48
Total WBC (cells/µl)	-0.05	0.06	0.34	0.40	-0.17
Monocytes %	0.36	-0.34	0.23	0.16	-0.37
Monocytes (cells/µl)	0.15	-0.13	0.40	0.38	-0.35
Neutrophils %	0.33	-0.11	0.12	0.31	-0.35
Neutrophils (cells/µl)	0.06	0.01	0.32	0.41	-0.27
Eosinophils %	-0.08	0.37	0.21	0.07	0.16
Eosinophils (cells/µl)	-0.12	0.41	0.29	0.26	0.02
Basophils %	-0.16	0.28	-0.20	-0.04	0.27
Basophils (cells/µl)	-0.29	0.38	0.10	0.24	0.08

*P<0.05

Table 4.4

Inter-Relationships Between Pre/Post Differences After Bilberry in Terms of Lymphocyte Subsets, WBC Count and Markers of Oxidative Stress and Antioxidant Status (Pearson r Value; n=17)

Changes in:	FRAP	Asc Acid	Vit E (Lipid Standardised)	Allantoin	Urinary 8-oxo-G
Lympho%	-0.20	-0.38	0.42	-0.04	-0.32
Lympho (cells/µl)	0.31	0.10	0.31	0.38	-0.01
CD4 %	-0.32	-0.41	-0.29	-0.25	-0.21
CD4+ (cells/µl)	0.13	-0.19	0.24	0.29	0.04
CD8+ %	0.00	0.24	0.27	-0.09	0.00
CD8+ (cells/µl)	0.31	0.32	0.35	0.16	0.03
CD19+ %	0.37	0.10	-0.33	-0.22	-0.05
CD19+(cells/µl)	0.42	0.08	-0.14	-0.03	0.01
CD56+ %	-0.20	0.18	0.15	0.38	0.25
CD56+(cells/µl)	-0.04	0.26	0.31	0.45	0.18
CD4/CD8 ratio	-0.35	-0.45	-0.03	0.13	-0.03
Total WBC (cells/µl)	0.34	0.54#	-0.65#	0.13	-0.15
Monocytes %	-0.56*	-0.79*	0.36	-0.23	-0.29
Monocytes (cells/µl)	-0.02	-0.05	0.09	-0.06	0.09
Neutrophils %	0.44	0.61*	-0.39	0.04	0.34
Neutrophils (cells/µl)	0.48	0.64*	-0.30	0.09	0.24
Eosinophils %	-0.45	-0.34	0.18	0.01	-0.09
Eosinophils (cells/µl)	-0.04	0.19	0.15	0.33	0.21
Basophils %	-0.31	-0.01	0.15	-0.12	0.35
Basophils (cells/µl)	0.04	0.50	0.11	0.15	0.54

*P<0.05

#P<0.05 but significance removed with exclusion of one data point (see Figures 4.13 and 4.14)

At baseline two significant inter-relationships were observed. In Figure 4.8 it can be seen that there was a significant negative correlation (r = -0.44; P<0.05; n=20) between the plasma total antioxidant content (as the FRAP value) and the number of total lymphocytes. In Figure 4.9 it can be seen that there was a significant direct correlation (r=0.49; P<0.05; n=20) between urinary 8-oxoG and the total lymphocyte percentage.

Figure 4.8

Correlation of Total Lymphocytes Absolute Number vs. Total Plasma FRAP at Baseline (r = -0.44; P<0.05; n=20)

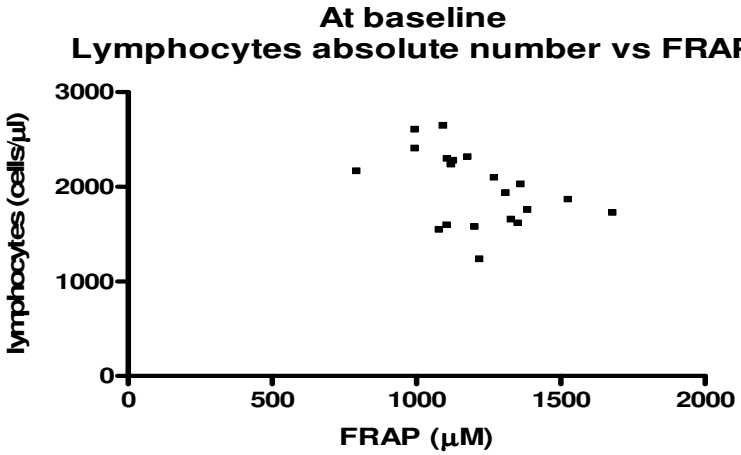
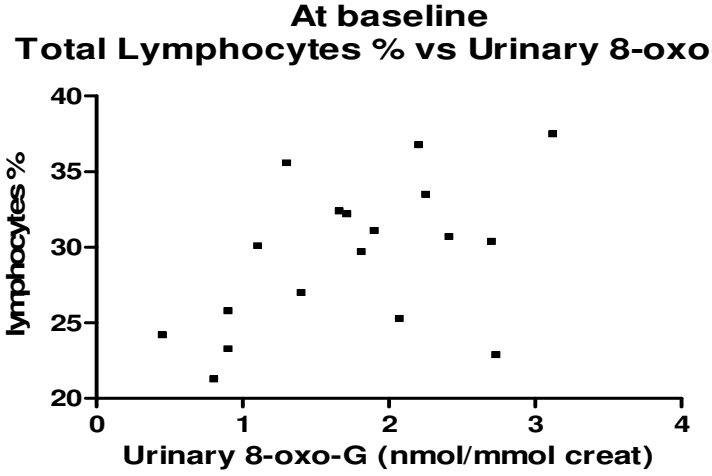


Figure 4.9

Correlation of Total Lymphocytes % vs. Urinary 8-oxodG (r = 0.49; P<0.05; n=20)



Inter-relationships were also observed in some cellular immune biomarkers and markers of antioxidant status. In Figure 4.10 it can be seen that in response to bilberry treatment, there was a negative correlation (r=-0.56; P<0.05) between monocytes % and the total plasma FRAP. In Figure 4.11 a negative correlation (r=-0.79; P<0.001) between monocytes % and ascorbic acid was also observed in response to bilberry treatment.

Figure 4.10

Correlation of Pre/Post Difference After Bilberry Treatment of Monocytes % vs. Total Plasma FRAP (r=-0.56; P<0.05; n=17)

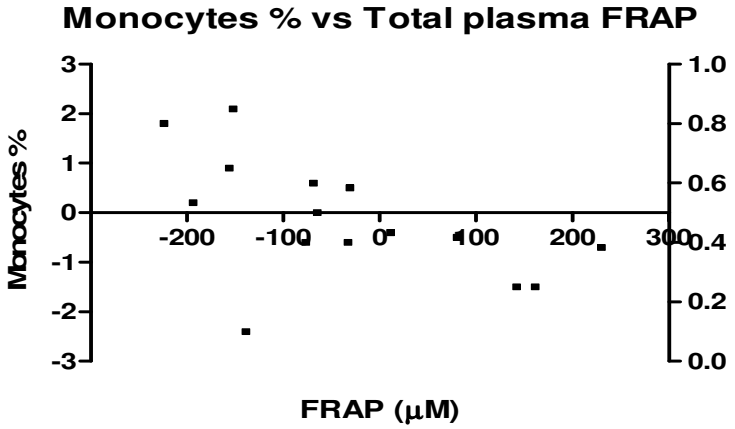
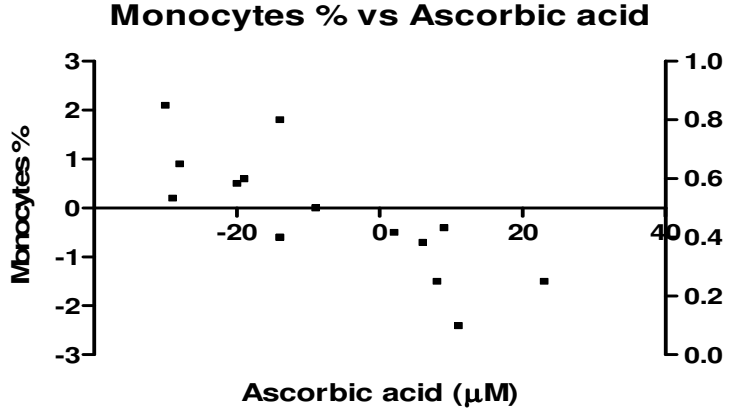


Figure 4.11

Correlation of Pre/Post Difference After Bilberry Treatment of Monocytes % vs. Ascorbic Acid (r=-0.79; P<0.001; n=17)



In Figure 4.12 it can be seen that in response to bilberry treatment there was a direct correlation (r=0.64; P<0.01) between neutrophils cell number and the plasma ascorbic acid. In Figure 4.13 it can be seen that a direct correlation (r=0.61; P<0.05) between neutrophils % and the plasma ascorbic acid was observed in response to bilberry treatment.

Figure 4.12

Correlation of Pre/Post Difference After Bilberry Treatment of Neutrophils Cell Number vs. Plasma Ascorbic Acid (r=0.64; P<0.01; n=17)

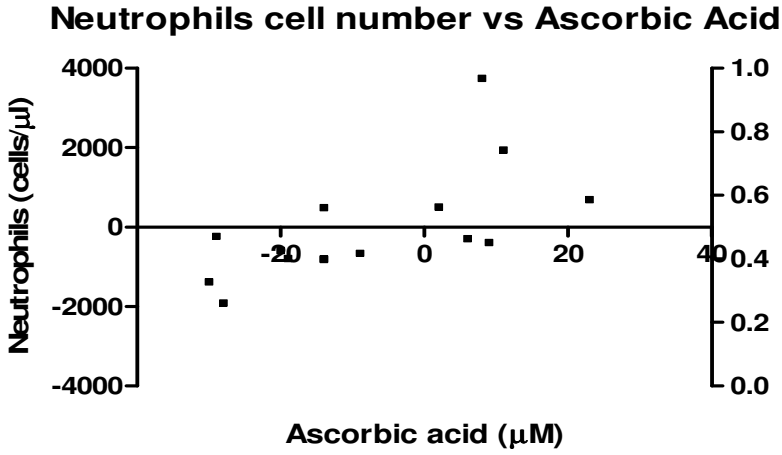
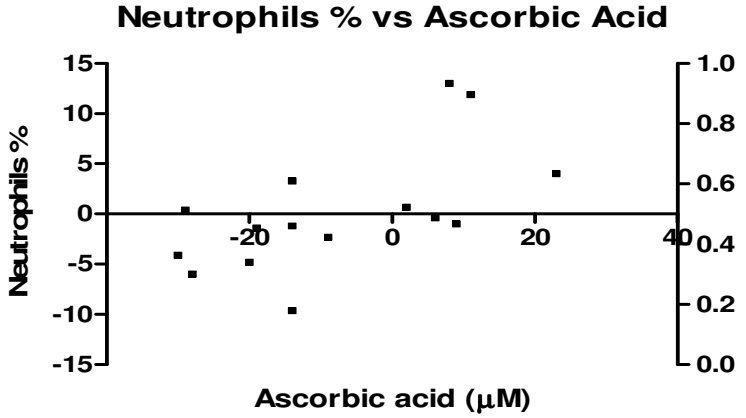


Figure 4.13

Correlation of Pre/Post Difference After Bilberry Treatment of Neutrophils Cell % vs. Plasma Ascorbic Acid (r=0.61; P<0.05; n=17)



Spurious correlations, which are lost after the removal of one data point, are shown in the following figures. Figure 4.14 shows that there was an apparent correlation, in the response to bilberry treatment, between the total WBC count and the ascorbic acid value ($r=0.54$; $P<0.05$). Figure 4.15 shows that there was an apparent inverse correlation, in the response to bilberry treatment, between the Total WBC count and Vitamin E ($r=-0.65$; $P<0.01$). However, these correlations were lost after the removal of the circled data point.

Figure 4.14

Correlation of Pre/Post Difference After Bilberry Treatment of Total WBC Number vs. Total Ascorbic Acid ($r=0.54$; $P<0.05$; $n=17$)

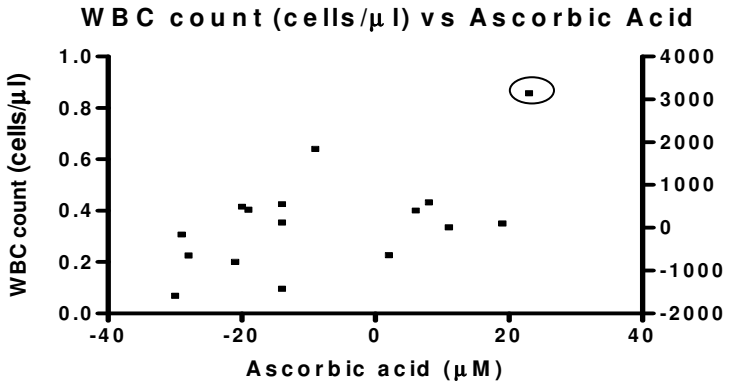
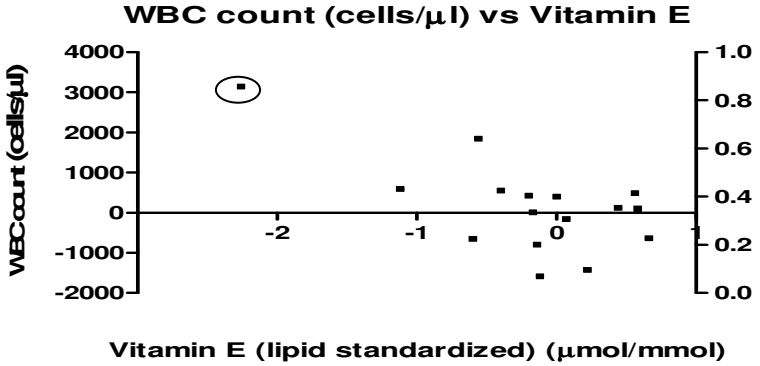


Figure 4.15

Correlation of Pre/Post Difference After Bilberry Treatment of Total WBC Number vs. Vitamin E ($r=-0.65$; $P<0.01$; $n=17$)



The characteristics of 20 Type 2 DM and 18 apparently healthy subjects are shown in Table 4.5.

Table 4.5

Characteristics of 20 Type 2DM and 18 Healthy Subjects

Type 2 DM subjects n=20	Healthy subjects n=18
Age (years): Mean (SD) 55.8 (9.5)	Age (years) Mean SD 42.6 (3.6)
Median 55	Median 42.5
Range 31-70	Range 35-50
Sex distribution (female/male): 11/9	Sex distribution (female/male): 9/9

Comparison of lymphocyte subsets at baseline (pre-supplementation) between Type 2 diabetic patients (n=20) and a group of normal subjects of similar age and sex (n=18) are shown in Figures 4.16 and 4.17. Total lymphocyte% and number in Type 2 DM subjects were significantly higher ($P<0.05$) compared with healthy subjects. T Helper (CD4+) cells number was also higher ($P<0.05$) in the diabetic subjects, but the CD4+ % difference did not reach statistical significance. Type 2 DM subjects showed significantly lower NK cell% ($P<0.05$) but no difference in NK cell number. No significant difference was found in the other lymphocyte subsets measured.

Figure 4.16

Comparison of Percentage of Lymphocyte Subsets between 20 Type 2 DM Subjects and 18 Healthy Subjects

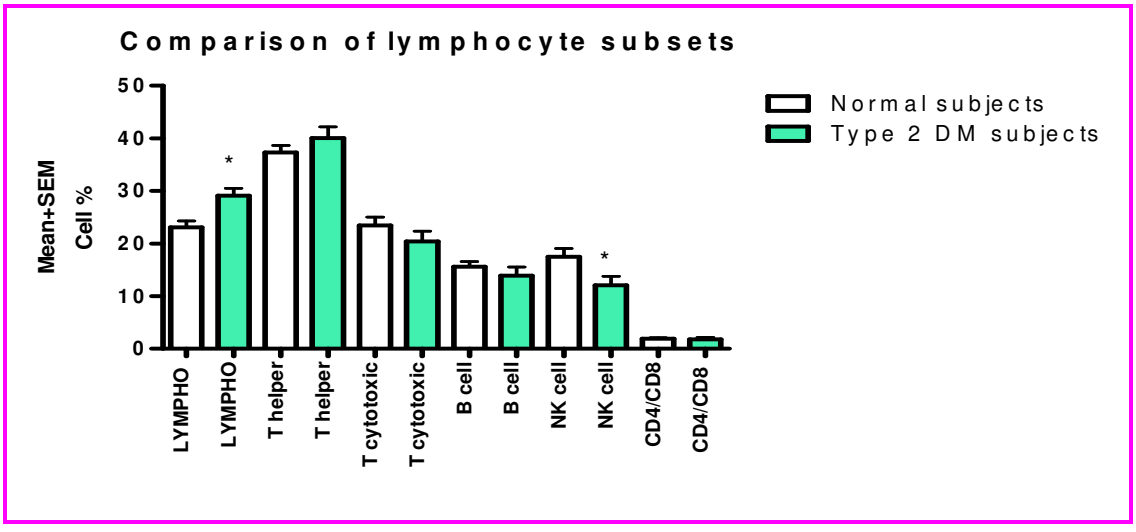
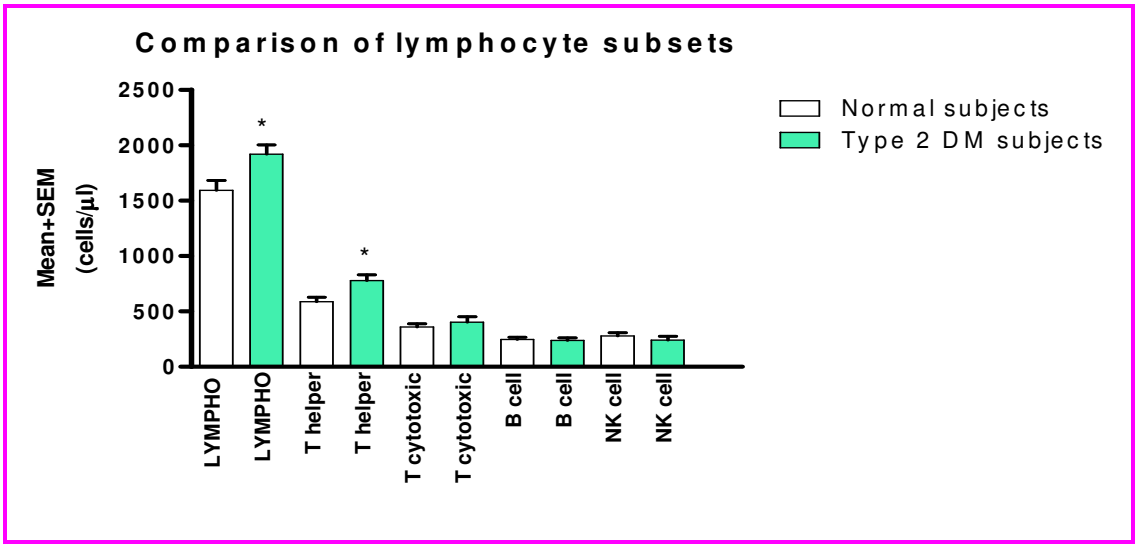


Figure 4.17

Comparison of Total Number of Lymphocyte Subsets between 20 Type 2 DM Subjects and 18 Healthy Subjects



Discussion

Anthocyanins are flavonoid compounds, which are potent antioxidants. Anthocyanin concentration in the fresh bilberry is approximately 0.1-0.25% and concentrated commercial extracts are standardized to contain 25% anthocyanins [Chandra et al., 2001]. Anthocyanins have been shown to have anti-inflammatory effects, however there is limited evidence showing the effect of this antioxidant agent on cellular immunity. Dietary antioxidants have been reported to increase certain immune response [Meydani et al., 1995; Butt and Sultan, 2009; Salman et al., 2009]. Although, the mechanism of how antioxidants can enhance immune response is still unclear, it is hypothesized that the antioxidant effect of antioxidant-rich agents can oppose the deleterious effects of oxidative stress on the immune cells [Ames et al., 1993; Knight, 2000b]. Moreover, diabetes is a disease associated with high oxidative stress [Choi et al., 2005, 2008]. Also, it has been reported that diabetic subjects are prone to infections as they often show decreased innate and adaptive immune responses [Okano et al., 2008]. As a result, it might be expected that dietary agents rich in antioxidant would enhance the immune

status in people under high oxidative stress, such as diabetic patients. Many studies exist on the evaluation of anti-inflammatory effects of different types of natural products on Type 2 DM. However, there are no clinical studies available investigating the effect of bilberry on the cellular immunity in Type 2 DM. Therefore, this double-blinded placebo controlled intervention trial was performed to study the effect of 4 weeks' bilberry supplementation on certain immune parameters in Type 2 DM subjects. We also compared baseline values in Type 2 DM subjects with a group of healthy subjects of similar age and sex. Results showed no significant effects of bilberry supplementation. However, there was some evidence of increased % of CD8+ T cytotoxic cells after bilberry. Such an effect could indicate improved immune surveillance. This possible beneficial effect observed in this study is of potential importance. This is because we found that our Type 2 DM subjects had slightly lower % CD8+ T cytotoxic cells when compared with healthy subjects, although the difference did not reach statistical significance. In addition, our Type 2 DM subjects showed significantly lower % NK cells when compared with healthy subjects studied ($P<0.05$). As mentioned earlier, NK cells play an important role in removing 'changed' cells, a key function of immune surveillance. It has been suggested in many studies that DM increases the risk of developing a variety of cancers which include cancer of colon, pancreas, breast and liver [reviewed by Coughlin et al., 2004]. In addition, in a study with 1,353 Type 2 DM subjects followed up for 9.8 years, it was reported that the cancer mortality rate was higher compared to non-diabetic subject: the standardized cancer mortality Risk Ratio of the Type 2 DM subjects was 1.47 (95% CIs 1.22 to 1.76; $P<0.05$) [Landman et al., 2010]. Therefore, further study to investigate possible effects of antioxidant supplementation on CD8+ T cytotoxic cells and NK cells would be useful because of the vital role of these cells on immune surveillance and protection against cancer.

The pathogenesis of insulin resistance in Type 2 DM is closely associated with chronic low-grade inflammation and it has been reported that worsening of insulin sensitivity and complications in Type 2 DM is related to high WBC count [Vozarova et al., 2002]. A study of 1,480 Type 2 DM subjects found that advancement of diabetic nephropathy was associated with increased counts of total WBC, monocytes, and neutrophils [Chung

et al., 2005]. Also, increased numbers of activated CD4+ and CD8+ T cells were recorded in patients with diabetic ketoacidosis and hyperglycaemia. The authors stated that high concentration of glucose and free fatty acids provide an environment of oxidative stress and activation of the inflammatory pathways [Stentz and Kitabchi, 2003]. The higher lymphocytes and T helper cells (CD4+) in the diabetic subjects studied here is consistent with a chronic inflammatory state. Interestingly, at baseline (pre-treatment data), there was significant correlation seen between a biomarker of oxidative stress (urine 8-oxodG) and lymphocytes%, and a significant inverse relationship between a biomarker of antioxidant status (plasma FRAP) and lymphocytes number. Together, results are consistent with increased oxidative stress with inflammation, and a possible role for antioxidants in modulating inflammation.

In the present study, Type 2 DM subjects (n=20) were found to have lower NK cell% than healthy subjects ($P<0.05$; n=18)). Total lymphocyte% and number were significantly higher and T helper (CD4+) cells number was also higher ($P<0.05$) when compared with healthy subjects. In contrast to the findings presented here, in previous study the number of T cells (CD3+) from Type 2 DM subject (n=34) was not significantly different from healthy subjects (n=22) [Chang and Shaio, 1995]. Also, 142 Type 2 DM patients did not show any significant differences in lymphocyte subsets, such as CD4+ , CD8+ and CD56+ when compared with 34 healthy individuals [Okano, 2008]. However, as reviewed by Stentz and Kitabchi [2003], reduction in the total number of T-lymphocytes is a more consistent finding in DM, but T-lymphocytes abnormalities were reported to be greatest in those with poor glycaemic control [Stentz and Kitabchi, 2003]. The difference observed here in Type 2 DM when compared with healthy subjects could be due to variable or poorly controlled diabetes in these subjects. To explore this, HbA1c data was retrieved from patient records (data not shown) and lymphocytes % and number at baseline were compared in those with HbA1c above or below 7.5%. This cut-off of 7.5% was used here a crude indicator of ‘acceptable’ or ‘poor’ glycaemic control. No significant differences were seen in lymphocytes % and number between the two groups, and on further exploration no correlation was seen between HbA1c and lymphocytes % or number (results not shown). However, it should

be noted that the number of subjects was small and there was not much difference in the mean between both groups (with HbA1c above or below 7.5%).

In this study, there were fairly strong, inverse and statistically significant correlations between the change in monocytes % and the changes in fasting plasma FRAP and changes in plasma ascorbic acid after bilberry treatment. Moreover, direct significant correlations between changes in ascorbic acid and changes in neutrophils % and number after bilberry treatment were found. This supports the possible modulation of chronic inflammation by antioxidants as suggested by the relationship seen in the baseline data. However, the direct relationship seen between supplementation-associated changes in plasma ascorbic acid and changes in both % and number of neutrophils is interesting. Bilberry is rich in anthocyanins, but low in ascorbic acid (3mg/100g) [Upton, 2001]. Therefore, there is unlikely to be a direct effect of bilberry supplementation on plasma ascorbic acid, although an indirect, or 'conserving', effect by bilberry antioxidants is possible. However, on inspection of the data, we observed that over half the subjects showed a decrease in ascorbic acid after bilberry. We do not suggest that the bilberry caused this decrease and it is noted that pre-post differences in ascorbic acid were not significantly different after bilberry compared to after placebo. This indicates that the difference in ascorbic acid was not caused by the supplementation but most probably due to some dietary changes, affecting both groups, during the course of the study. The volunteers were asked to keep to their usual diet during the course of the study (14 weeks per subject, over several months in total), but their diet was not 'controlled' except for the supplementation part, and dietary intake of ascorbic acid could well have varied over the winter to summer period of the study overall. The reason for the significant correlation seen between the changes in neutrophil % (and number) and the changes in plasma ascorbic acid after bilberry treatment is not clear. It is noted that this relationship was not seen at baseline. We have been unable to find previously published data on neutrophil numbers in Type 2 DM compared to normal subjects, and unfortunately the healthy subjects studied here did not have complete blood counts (which furnish the % and numbers of monocytes, neutrophils, etc) due to resource limitations. Therefore, at this time we do not know if Type 2DM patients have different

numbers of neutrophils than healthy subjects, although it has been reported that there are defects of neutrophil chemotactic, phagocytic and microbicidal functions in diabetic patients and experimental animal studies, and the dysfunction was related to the degree of hyperglycaemia and oxidative stress [reviewed by Alba-Loureiro et al., 2007]. It is possible to speculate that diabetic patients may also have lower neutrophil numbers, and that ascorbic acid might help restore number and function. This remains to be confirmed. Nonetheless, these results support published studies in which vitamin C (ascorbic acid) has been reported as a stimulant of leukocyte function, especially of neutrophil and monocyte movement [Deruelle and Baron, 2008]. Vitamin C has also been reported to protect neutrophils from reactive oxygen species generated during phagocytosis, and in a supplementation study, vitamin C (1-3 g/day) given to healthy adults was shown to enhance neutrophil chemotaxis *in vivo* [Wintergerst et al., 2006]. However, no studies of effect of vitamin C or other antioxidants on neutrophil numbers have been reported.

The limitations of this study should not be ignored. Firstly, some subjects did not comply with the study, and the limited sample size might have contributed to the lack of statistical significance observed. Also, there was a marked variability in the immune variables measured, making it difficult to detect small changes. However, studying a larger group within the time and resources constraints of this study was not possible. Another limitation is that plasma anthocyanins were not measured, and thus their bioavailability or plasma response could not be evaluated. The dose used was 'dietary-relevant' and was for only 4 weeks. A higher dose or a longer duration of supplementation may have been useful. Moreover, functions of immune cells and levels of inflammatory cytokines were not investigated as these tests are resource intensive, and so a wider range of effects on immune response could not be studied.
In conclusion, in this controlled human intervention study, four weeks' supplementation with an extract of anthocyanin-rich bilberry did not have marked effect on selected immune biomarkers in Type 2 DM patients. There was some indication of improvement in cytotoxic T cells (CD8+ %) cells which could have implications for immune surveillance. It was observed that Type 2 DM subjects have lower NK cells%, higher total lymphocytes % and number, and higher T helper (CD4+) cell numbers when

compared with healthy subjects (P<0.05). These findings are consistent with an inflammatory state in Type 2 DM, and inflammation is associated with increased oxidative stress (and lower antioxidants). The significant direct correlation found between baseline urine 8-oxodG and lymphocytes%, and a significant inverse relationship baseline 'total antioxidants' in plasma (as the FRAP value) and lymphocytes number support this scenario. A possible modulatory role for antioxidants in immune function is suggested by the inverse relationship seen between changes in monocyte % and plasma FRAP and an even stronger inverse relationship was seen with ascorbic acid. Further studies are suggested in order to clarify effects of bilberry, and other antioxidant-rich foods, on the immune system in Type 2 DM subjects.

Some of the results in this chapter have been presented as follows:

ARW Lau, SW Choi, T Chu, B Tomlinson, IFF Benzie. Possible immuno-enhancing effects of bilberry in subjects under increased oxidative stress: results of a controlled human intervention study. The 4th International Functional Food Symposium: New Horizons in Chinese Medicine and Health Foods (October 2009)

A.R.W. Lau, S.W. Choi,T. Chu, B.Tomlinson, I.F.F. Benzie. Possible immuno-enhancing effects of bilberry in subjects under increased oxidative stress: results of a controlled human intervention study. Progress in Nutrition volume 12 (Number 1; 2010) Page 80.

Chapter 5

RADIATION-INDUCED EFFECTS ON CELLULAR IMMUNITY IN NASOPHARYNGEAL CANCER PATIENTS AND POTENTIAL IMMUNOMODULATORY EFFECTS OF LINGZHI (*GANODERMA LUCIDUM*): PRELIMINARY RESULTS OF A CONTROLLED HUMAN INTERVENTION STUDY

Introduction

Ganoderma lucidum (commonly known as lingzhi or reishi) is a type of woody mushroom, which is renowned in Chinese culture, with many claimed health enhancing effects, including anti-tumour, immunomodulating and anti-inflammatory effects. Indeed, lingzhi has been very popular among Chinese and cancer patients as a health food supplement and has been claimed also to decrease side effects of chemotherapy such as nausea, vomiting and anorexia [Wachtel-Galor et al., 2004; Wang et al., 2005].

Cancer is the major cause of death in Hong Kong, and nasopharyngeal carcinoma (NPC) ranks as the seventh most common cancer affecting the population (according to the Hong Kong Department of Health Report 2009). Cancer has many causes, but depressed immune surveillance allows changed, pre-cancerous cells to survive and greatly increases risk of cancer [Penn, 2006]. Indeed, it has been reported that the frequency of malignancy is roughly 10,000 times greater in subjects with primary immunodeficiency than in the general age-matched population [Penn, 2006]. In addition, there is evidence showing that patients with head and neck cancers have decreased immune function, in particular cellular immunity [Bokhorst-De Van der Schueren, 1998].

Radiotherapy is the main form of treatment used to treat NPC [Lee et al., 2002]. However, NPC patients receiving radiotherapy experience many side effects, which

include swallowing and eating difficulties, sore throat and mucositis. Moreover, radiotherapy has been reported to have many deleterious effects on immune function. In a study with 70 cancer patients, it was reported [Verastegui et al., 2003] that patients undergoing radiotherapy to the head and neck area had severe lymphopenia, lower counts in B cells and total lymphocyte counts including both CD4+ and CD8+ cell subsets. In addition it was reported that none of the patients had fully recovered their baseline (pre-radiotherapy) levels of lymphocytes (particularly CD4+ cells) by 60 months of follow-up [Verastegui et al., 2003]. Other studies have also shown radiation-induced immunosuppression in cancer patients receiving radiotherapy [Belka et al., 1999; Santin et al., 2000].

There are anecdotal reports of lingzhi being able to decrease radiation-induced side effects. In a clinical supplementation study, with 105 cancer patients receiving chemotherapy and/or radiotherapy, capsules made of a Chinese medicinal herb complex containing a small amount of lingzhi (3mg, with a total dose of 27mg lingzhi/day) were given for 6 weeks [Zhuang et al., 2009]. It was reported that the depletion of leukocytes and neutrophils was significantly less in the treated (n=55) vs. the placebo (n=50) treated subjects [Zhuang et al., 2009]. Mounting supporting evidence has shown lingzhi to have immune-modulating effects [Lin and Zhang, 2004; Chen et al., 2006]. As reviewed by Lin [2005] many animals and *in vitro* studies have shown linghzi polysaccharides to have various effects on immune function including promoting the function of antigen presenting cells, mononuclear phagocyte system, humoral immunity and cellular immunity [Lin, 2005]. In an uncontrolled clinical trial on patients with advanced colorectal cancer, various immune markers were reported to increase, though not significantly, after treatment with *G. lucidum* polysaccharides [Chen et al., 2006]. In addition no toxicity has been reported with lingzhi intake [Wachtel-Galor et al., 2004]. However controlled human studies showing the effect of lingzhi on the immune system are sparse and there is only one published study [Zhuang et al., 2009] that has investigated the effect of linghzi on radiotherapy-induced immune suppression. However, it is noted that within the herbal mixture used, <1% w/w was lingzhi. Furthermore, the patients in the study by Zhuang et al. [2009] included those with

cancer of breast, colon, lung as well as NPC, covering Stages I to IV, and many were receiving chemotherapy as well as radiotherapy.

The aims of this study were to investigate the effect of radiotherapy on selected markers of cellular immunity in NPC patients, and to explore the effect of lingzhi on radiotherapy-induced changes. In addition, the cell based immune markers in the cancer patients at baseline (immediately prior to radiotherapy) were compared to those of similar age- and sex- matched non-cancer patients. The cancer patients studied were part of a relatively homogenous group. All were ethnic Chinese attending the same oncology unit, had been recently diagnosed with NPC of similar staging and were about to begin a standard course of radiotherapy, under the direction of the same oncology team.

This was a double-blinded, placebo-controlled intervention trial of parallel design. The intervention was a single herb (lingzhi). The specific objectives of this part of the study were:

1. To investigate the effect of radiotherapy (RT) on selected biomarkers of cell-based immune status in NPC patients
2. To monitor the recovery of immune biomarkers after completion of radiotherapy
3. To investigate if lingzhi can modulate radiotherapy-induced changes in immune biomarkers
4. To compare the immune biomarkers in NPC patients with those in non-cancer subject matched for age and sex, particularly the cells concerned with immune surveillance (CD8+ cytotoxic T cells and CD56+ NK cells).

Note on NPC patients and RT:

The NPC patients were recruited, treated and samples collected at their treating hospital and by members of their oncology team. Inclusion, exclusion criteria, staging and selection of RT were set by the oncologist in charge. The NPC patients recruited were in the early stage cases (T1, T2, N0 & N1) and who would receive RT. The prescribed dose was 2Gy/fr. at 100%I.L., 5fr/wk, 35 fr. to 70Gy and stop.

Inclusion criteria were:

1. Patients with primary nasopharyngeal carcinoma (NPC) of T1, T2 with or without N1 will be recruited
2. Patients in 1 who will receive Intensity-modulated radiotherapy (IMRT) at standard fractionation to a total dose of 70 Gy.
3. Patients who were able to sign the informed consent prior to study entry.

Exclusion criteria were:

1. Those who have difficulty understanding or complying with instructions
2. Those who receive chemotherapy.

Materials and Methods

Subjects

NPC consenting subjects (early stage cases), aged 39-86 years were recruited from the Oncology Department in the Princess of Margaret Hospital, Kwai Chung, Hong Kong. Ethical approval was granted by the Ethics Subcommittees of The Hong Kong Polytechnic University and the Princess Margaret Hospital Ethical Review Board. The inclusion criteria included patients with NPC of T1, T2 with or without N1; patients about to receive intensity-modulated RT at standard fractionation to a total dose of 70 Gy. Those patients who had difficulty understanding or complying with instructions were excluded from this study. Subjects were requested not to change their regular diet and lifestyle habits during the 11 weeks of participation in the trial, apart from taking the supplement. No treatment changes were recorded during the study for the 15 subjects whose data are presented.

Note on sample size and allocation to treatment:

Initially it was planned to study at least 70 subjects (35 in each arm). However, due to the stringent inclusion and exclusion criteria, and to some drop outs or chemotherapy being added to some subjects' treatment regimen (in which patients were withdrawn from the study), recruitment was very slow. Also, although treatment coded 'A', or 'B'

was done on an alternate basis, more drop-outs (usually before actual start of therapy) or treatment changes were more frequent in one group than the other. Therefore, at the time when the data for this report were required to be analyzed, the numbers of subject who had completed the study was only 15, with 9 in one group and 6 in the other group.

Methodology

This study was a double-blinded placebo-controlled parallel intervention study. Subjects were assigned, by the treating oncologist, to one of two coded treatments (lingzhi or placebo capsules). The commercial lingzhi ('deer horn' lingzhi) and the placebo capsules were kindly provided by Pharmatech Hong Kong Limited (Appendix II). One lingzhi capsule contained 350mg of lingzhi extract. The intervention group received lingzhi (two capsules, twice a day) during the course of 7 weeks of radiotherapy (starting on day 1 of radiotherapy) and continuing for four more weeks after completion of radiotherapy. The total supplementation period was 11 weeks. The control group received visually identical placebo capsules (two capsules, twice a day) during the course of radiotherapy, but a supplementary dose of lingzhi was given for 4 weeks on completion of radiotherapy. Compliance of treatment was checked by regular contact with the subjects and by counting the number of capsules returned from the subjects at the end of each intervention period (85% compliance was considered to be satisfactory). Fasting venous blood was collected immediately before RT started, at mid course (~3.5 weeks) on completion (after 7 weeks' RT) and 4 weeks after completion. Fasting venous blood was collected into EDTA blood tubes at the RT clinic and transported to our laboratory on ice, and immune biomarkers were measured within 6h of blood collection.

For the comparison of immune biomarkers between NPC patients and non cancer subjects, data from other parts of this study were used to create a databank of age- and sex matched immune biomarker results. Of the 38 Chinese subjects of similar age and sex in the non cancer group, 18 were from the green tea study part (Chapter 3) and 20 were from the bilberry study part (Chapter 4). In all cases, baseline (entry) data were used.

Laboratory testing

Within 6 hours of sample collection, the total white blood cell count was measured (Cell Dyne 3200 Haematology analyzer, Abbot, USA). Measurement of white blood cell subsets by flow cytometry used the same procedures as described in detail in Chapter 3 under the *"Reagents, cell preparation and staining for immunophenotyping"* subheading.

Statistical Analysis

The statistical analysis was carried out using GraphPad Prism (version 4.00 for Windows, GraphPad Software, San Diego California USA). In this parallel study, 35 subjects were estimated to be sufficient to detect a true difference of 0.4 in the mean CD4/CD8 ratio (a key variable) with 80% power and at the 5% significance level. However, due to time restriction and slow recruitment of patients, only 15 subjects had completed intervention by the end of 2009, the deadline for inclusion here. Therefore, results must be regarded as only very preliminary. For comparison between placebo and lingzhi groups and across the four timepoints, Two-way ANOVA was used. Comparison of variables at baseline (pre-supplementation) between NPC and non cancer subjects was made by Mann Whitney test. Significance was set at $P<0.05$.

Results

Effect of RT on immune cells

A summary of the results (n=15) of the different immune biomarkers measured to assess the effect of RT on cell-based immune status in NPC patients are shown in Tables 5.1(A) and 5.2(B) and Figures 5.1 to 5.3. According to Table 5.1(A) and Figure 5.1, it can be seen that the % of total lymphocytes and the % of each subset were affected to a different extent by RT. For example, the total lymphocyte % decreased dramatically, by 70%, during the course of RT. The most affected lymphocyte subset in relative (% terms) was CD19+ B cell, with a 62% decrease observed at mid-course (after ~3.5 weeks) of RT, and a further small decrease was seen at completion of RT (after 7 weeks of RT). The %CD4+ helper T cell was significantly lower at mid course and at completion of

RT when compared to baseline ($P<0.001$), but the decrease was not as dramatic as seen in the total lymphocytes% and B cells%. In contrast, the %CD8+ cytotoxic T cell was not affected during the course of RT. The %CD56+ NK cell was ~50% higher at mid course of RT when compared to baseline ($P<0.01$), though the increase was less marked and not statistically significant on completion of RT. In addition, it can be seen that the CD4/CD8 ratio was significantly lower at completion of RT ($P<0.05$) when compared to baseline, and was slightly, but not significantly lower at mid-course.

In relation to the real picture of absolute cell numbers, from Table 5.1(B) it can be seen that the total lymphocytes and subsets number all had significantly and markedly decreased at mid-course and at completion of RT when compared with baseline ($P<0.01$). The most dramatic decreases were seen in CD19+ B cells (>90% decrease), CD4+ T cells (>85% decrease). For CD8+ T cytotoxic cells and CD56+ NK cells there was ~75% decrease during RT. For all subsets, the greatest decreases were seen from baseline to mid course.

Table 5.1

The Effect of RT on Lymphocytes Subsets % (A) and Number (B)

(Summary of values (Mean+SD; n=15) Across Three Different Timepoints)

(A)

MEAN (SD)	Baseline	Mid-course	Completion
Lymphocytes%	25.5 (8.9)	9.8 (4.0)***	7.7 (4.8)***
CD4+ T helper %	32.0 (7.8)	25.2 (8.9)***	23.1 (8.9)***
CD8+ T cytotoxic %	23.2 (11.0)	23.5 (13.3)	26.2 (15.0)
CD19+ B cell %	13.2 (10.6)	5.0 (7.6)***	4.4 (4.1)***
CD56+ NK cell %	19.5 (14.8)	29.5 (17.9)**	23.8 (18.1)
CD4/CD8 ratio	1.6 (0.6)	1.3 (0.8)	1.1 (0.8)*

(B)

MEAN (SD)	Baseline	Mid-course	Completion
Lymphocytes (cells/µl)	1917 (663.8)	568.3 (251.8)**	388.6 (169.9)**
CD4+ T helper (cells/µl)	623.0 (221.5)	143.4 (59.7)***	88.2 (41.3)***
CD8+ T cytotoxic (cells/µl)	414.4 (339.5)	134.9 (80.8)***	108.9 (86.7)***
CD19+ B cell (cells/µl)	211.3 (111.7)	24.7 (31.9)***	14.3 (13.8)***
CD56+ NK cell (cells/µl)	411.8 (387.5)	200.2(184.3)**	113.4 (137.6)***

Statistically significant difference compared to baseline in post test analysis. *P<0.05; **P<0.01; ***P<0.001.

Figure 5.1

The Effect of RT on Lymphocytes Subsets % in Three Different Time Points; results are mean +/- SEM; n=15

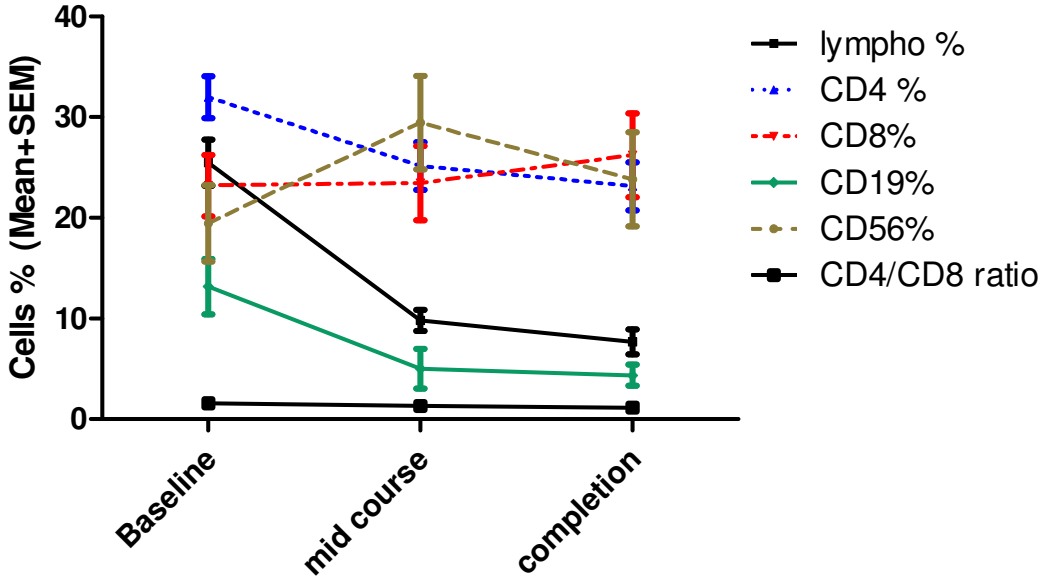
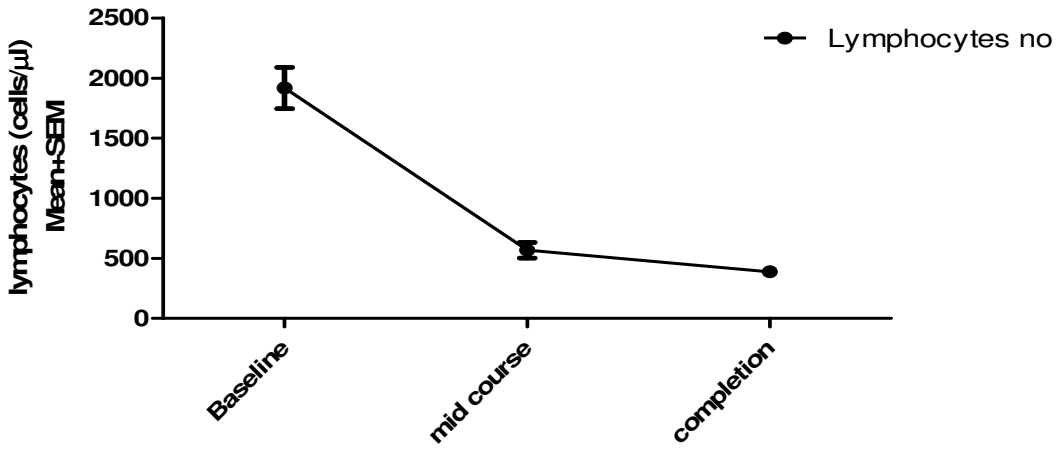


Figure 5.2 shows that there was a dramatic decrease (~70%) in the total lymphocytes number when compared to baseline at mid-course and at completion of RT.

Figure 5.2

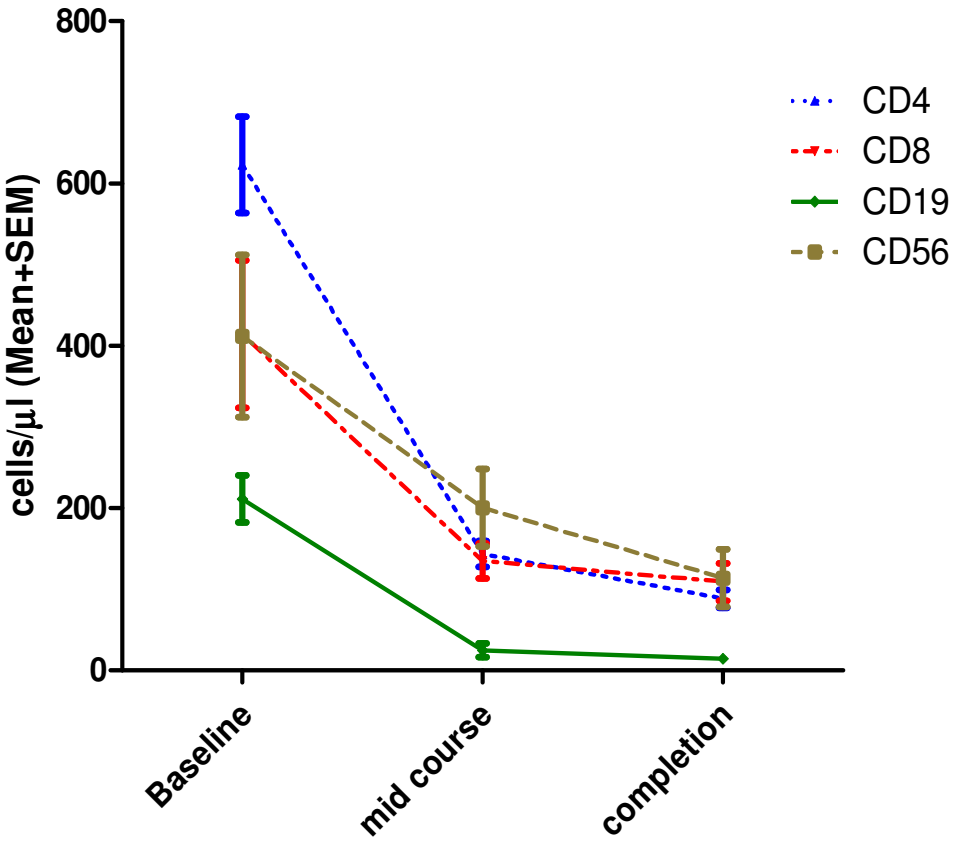
The Effect of RT on Total Lymphocytes Number in Three Different Time Points; results are mean +/- SEM; n=15



In Figure 5.3, it can be seen that CD4+ T helper cell and CD19+ B cell number were the most affected by RT when compared with other lymphocytes subsets, ~80% decrease of these cells was observed at the mid-course of RT. In addition, there was also a significant decrease (>50%) of CD8+ cytotoxic T cells and CD56+ NK cells at mid-course of RT. In addition, after mid-course of RT, further depletion in all the lymphocytes subsets number was seen at completion of RT.

Figure 5.3

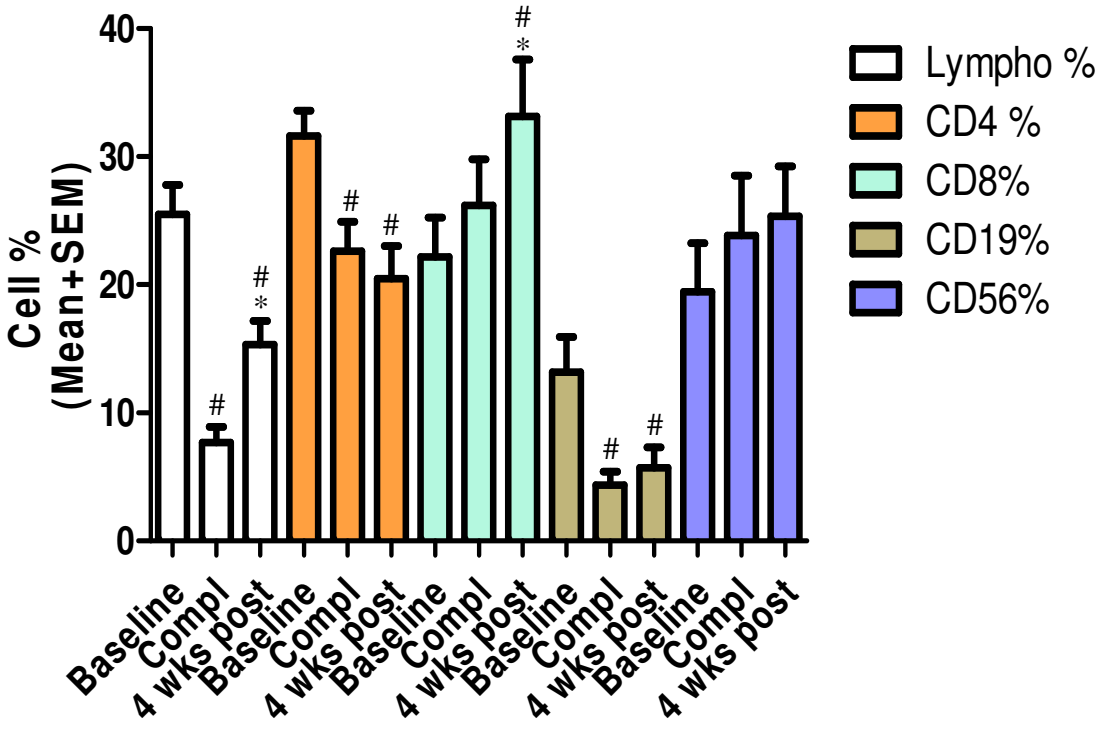
The Effect of RT on Lymphocytes Subsets Number in Three Different Time Points; results are mean +/- SEM; n=15



The post-RT recovery of total lymphocytes and subsets % and number in samples collected at four weeks after completion of RT are shown in Figures 5.4 to 5.6. In Figure 5.4, it can be seen that the total lymphocyte %, which as noted above, had significantly decreased during RT, had significantly rebounded ($P<0.01$) by 4 weeks after RT completion, regaining ~60% of the baseline% (pre-RT). The %CD4+ helper T cells, which had also significantly decreased during RT, showed no signs of rebound by four weeks after completion of RT, with the level even lower than the completion of RT result. The %CD8 (cytotoxic T cells) and %CD56 (NK cells), which were both higher on completion compared to baseline, showed a continued increase at 4 weeks after completion of RT. The %CD8 (cytotoxic T cells) were significantly higher compared to completion and baseline levels, however the %CD56 (NK cells) at 4 week post RT was not statistically significantly different compared to baseline and completion levels. The %CD19 (B cells) had also significantly decreased at completion of RT and remained at depressed levels even at 4 weeks post-completion of RT.

Figure 5.4

The Recovery of Immune Biomarkers (Lymphocyte Subsets %) Before, at Completion and 4 Weeks After Completion of RT; results are mean +/- SEM; n=15



Statistically significant difference compared to baseline in post test analysis; P<0.05

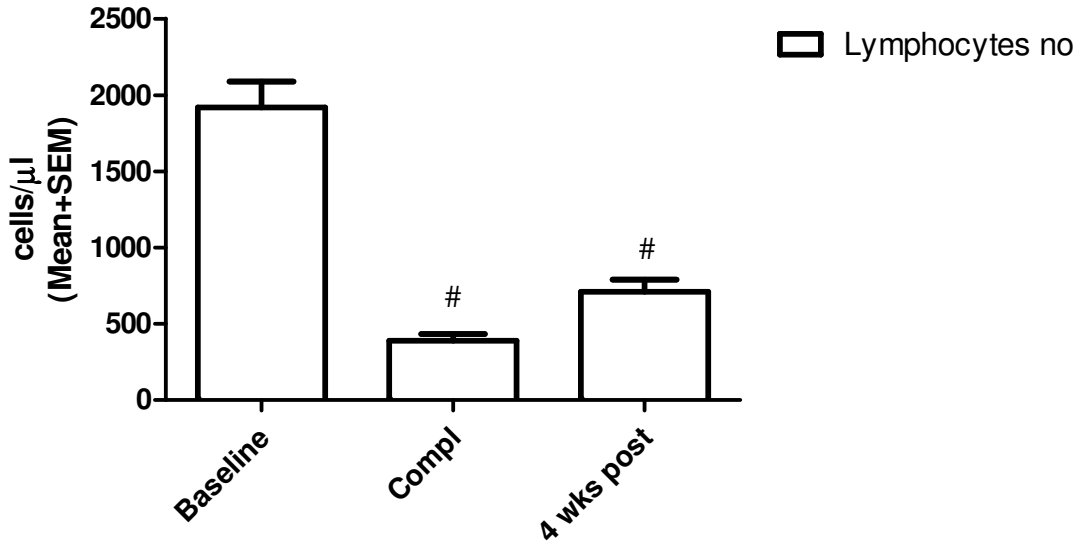
* Statistically significant difference compared to completion in post test analysis; P<0.05

Post-RT recoveries, in absolute cell numbers, of lymphocytes and sub-sets are shown in Figures 5.5 and 5.6 below. In Figure 5.5 it can be seen that the total lymphocyte number, which had significantly and dramatically (by 80%) decreased during RT, had begun to recover at 4 week post-completion of RT, with numbers approaching twice ($P<0.01$) the numbers at completion, although only ~37% of baseline number had been regained.

Figure 5.5

The Recovery of Total Lymphocyte Numbers

Before, at Completion and 4 Weeks After Completion of RT; results are mean +/- SEM; n=15

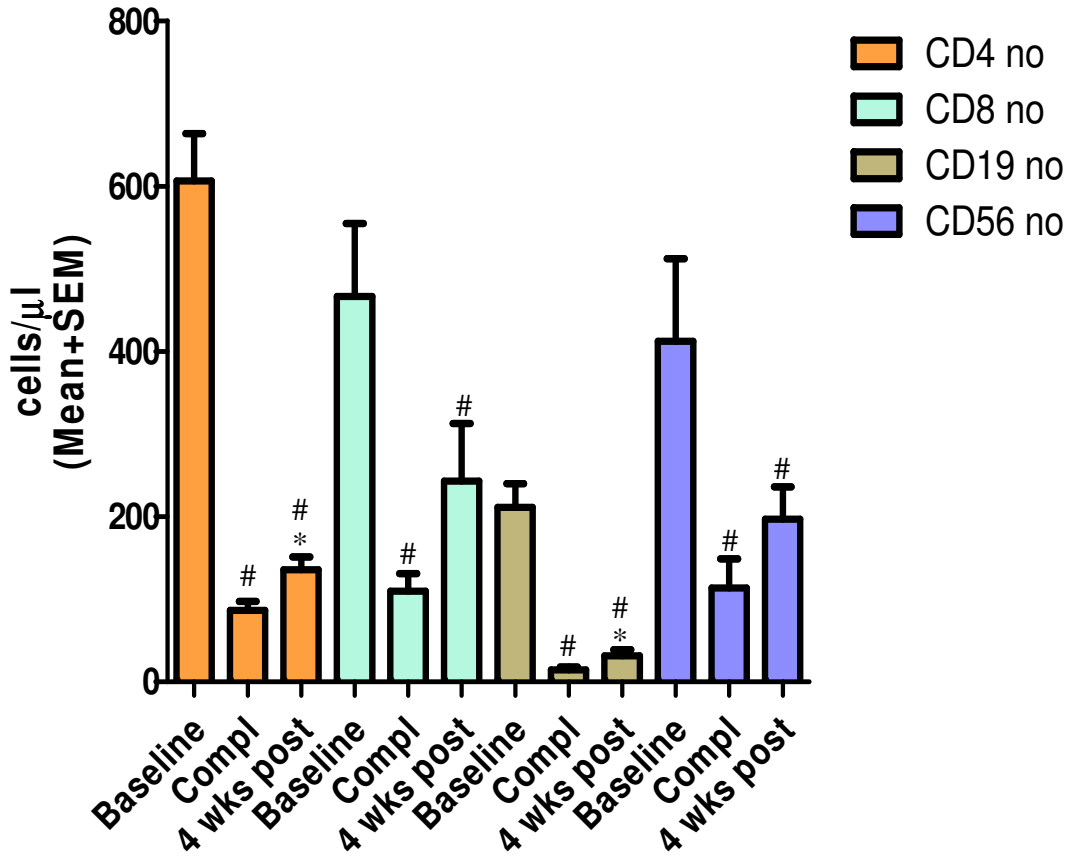


Statistically significant difference compared to baseline in post test analysis; $P<0.05$

In relation to recovery of sub-set cell numbers, from Figure 5.6 it can be seen that the numbers of CD4+ helper T cells, CD8+ cytotoxic T cells, CD19+ B cells and the CD56+ NK cells numbers, all of which had been markedly and significantly decreased at completion of RT when compared to baseline levels, all remained very depressed. CD19+ B cells had been the most dramatically affected by RT (only 7% of baseline number remaining by completion of RT), followed by CD4+ T helper cells (14% baseline number remaining at completion) and CD8+ and CD56+ cells (~25% remaining at RT completion). These subsets had all increased (by 44-65% in terms of cells numbers compared to at RT completion) by 4 weeks after completion. However, only the total lymphocyte number and CD19+ B cells were significantly higher at 4 weeks after completion of RT compared to completion levels. There was no obvious relationship between the magnitude of the RT effect and the rebound. CD4+ cells appeared to rebound fastest, followed by CD56+ and CD8+ and CD19+.

Figure 5.6

The Recovery of Immune Biomarkers (Lymphocyte Subsets Number) Before, at Completion and 4 Weeks After Completion of RT; results are mean +/- SEM; n=15



#Statistically significant difference compared to baseline in post test analysis; P<0.05

* Statistically significant difference compared to completion in post test analysis; P<0.05

Effect of lingzhi on RT-induced changes in immune cells

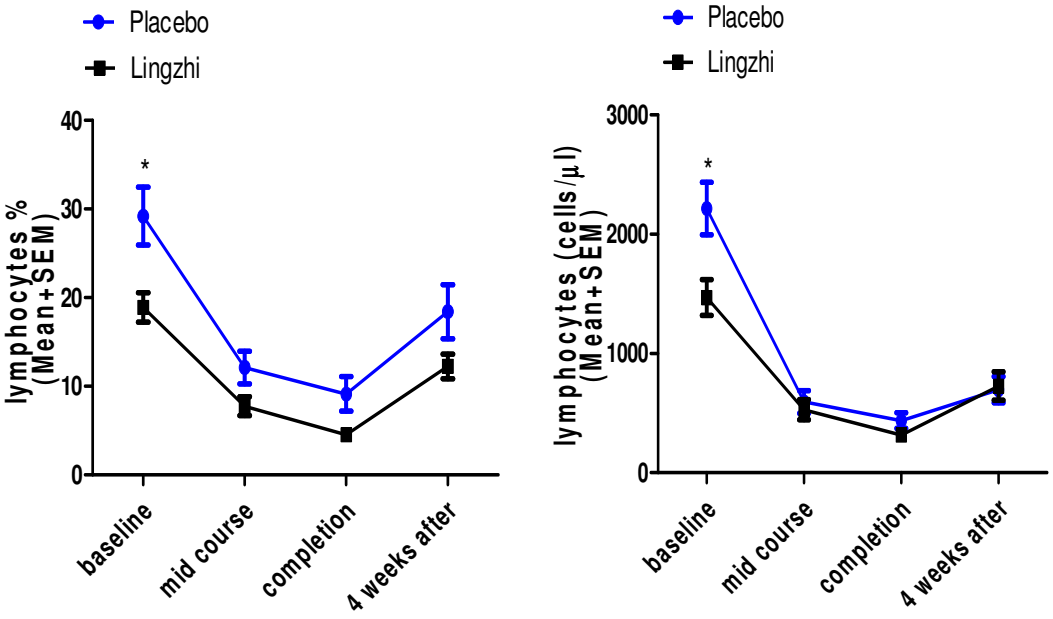
With regard to the effect of lingzhi treatment on RT-induced depression on immune cells results are presented in Figures 5.7-5.12.

In Figure 5.7, it can be seen that at baseline, the total lymphocytes % and number were statistically different (P<0.05) in both groups. It is noted that the groups could not be matched for baseline white cell data because their RT therapy and treatment (A or B (i.e. placebo or lingzhi) was started on the same day as their blood sample was drawn for white cell testing. It is noted also that no statistical difference in lymphocyte number and % between subjects taking placebo or lingzhi was seen at mid-course, at completion or four weeks after completion of RT. By four weeks after completion of RT (during which time both groups were taking lingzhi) the lymphocytes number and % had increased slightly increased in both groups, with both groups showing a similar change (P >0.05).

Figure 5.7

The Effect of Lingzhi on the Total Lymphocytes % and Number;

Placebo: n=9; Lingzhi: n=6; Results are Mean+/- SEM

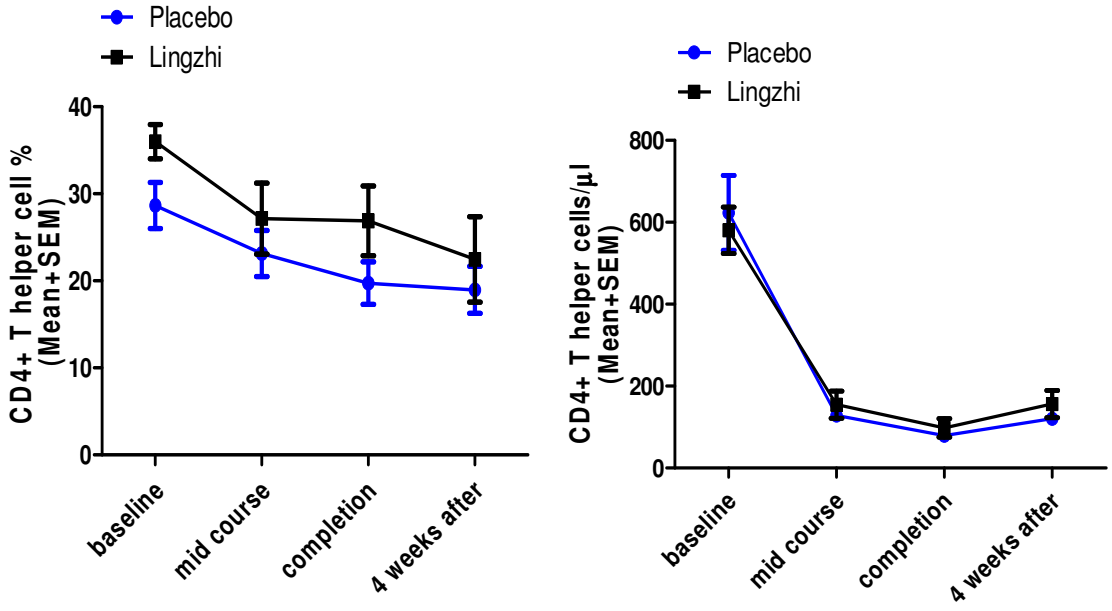


In Figure 5.8, it can be seen that CD4+ helper T cells % was higher (but not significantly so) and the numbers were very similar in subjects taking placebo and subjects taking lingzhi and in both groups a decrease in % and numbers of CD4+ cells was seen, with a similar pattern and magnitude of decrease in both groups. At four weeks after completion of RT, the % of helper T cells continued to decrease in both groups; however the helper T cell number had marginally increased in both groups by four weeks after completion of RT, although results did not reach statistical significance.

Figure 5.8

The Effect of Lingzhi on the CD4+ T Helper Cell % and Number;

Placebo: n=9; Lingzhi: n=6; Results are mean+/- SEM

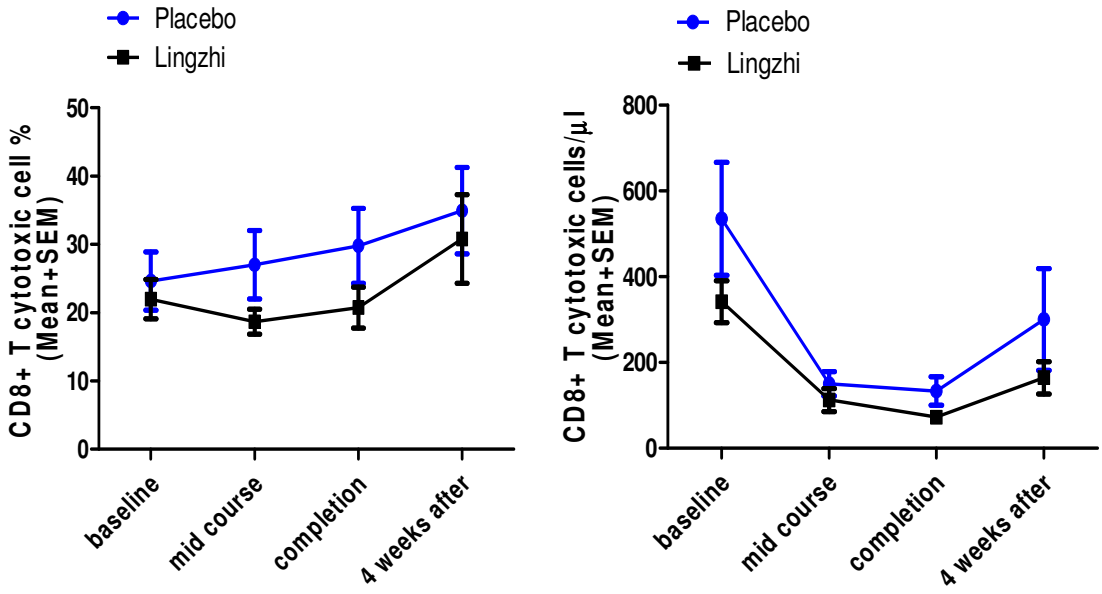


In Figure 5.9 it can be seen that no significant difference in CD8+ cytotoxic T cells % or number was seen between groups at any time point, although the placebo group had higher levels in general (P >0.05). The % and numbers had increased slightly but not significantly increased by four weeks after completion of RT. Increases were similar for both groups.

Figure 5.9

The Effect of Lingzhi on the CD8+ T Cytotoxic Cell % and Number;

Placebo: n=9; Lingzhi: n=6; Results are mean+/- SEM

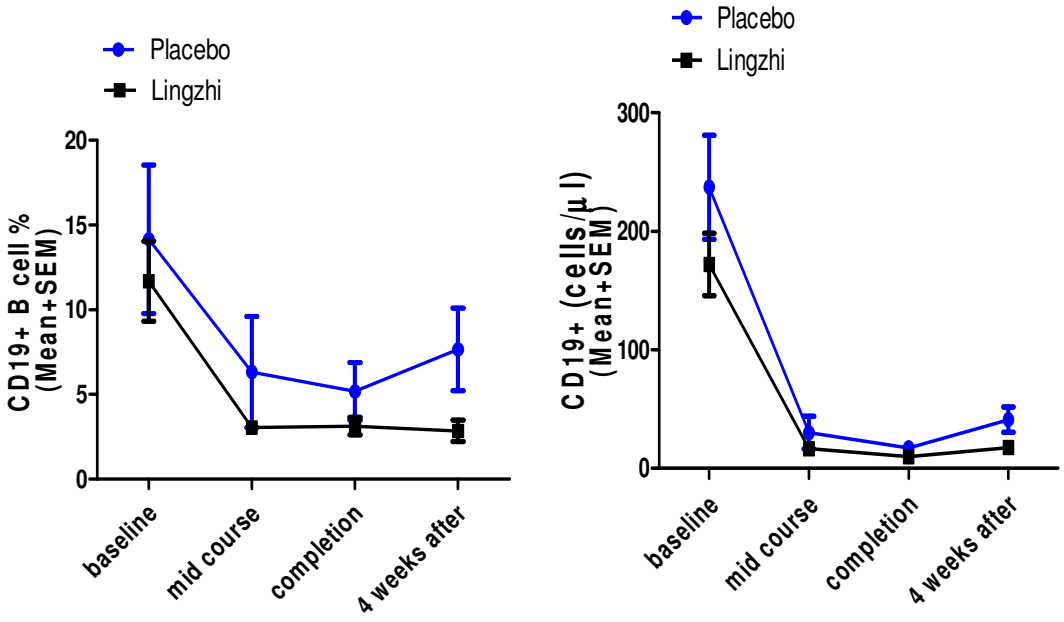


In Figure 5.10 it can be seen that no significance difference was found between placebo and lingzhi groups for CD19+ B cell % or number at any time point, although in general the placebo group had slightly higher levels. At 4 week after completion of RT a slight (but not significant) rebound was seen in placebo group. No evidence of rebound was seen in the lingzhi group.

Figure 5.10

The Effect of Lingzhi on the CD19+ B % and Cell Number;

Placebo: n=9; Lingzhi: n=6; Results are mean+/- SEM

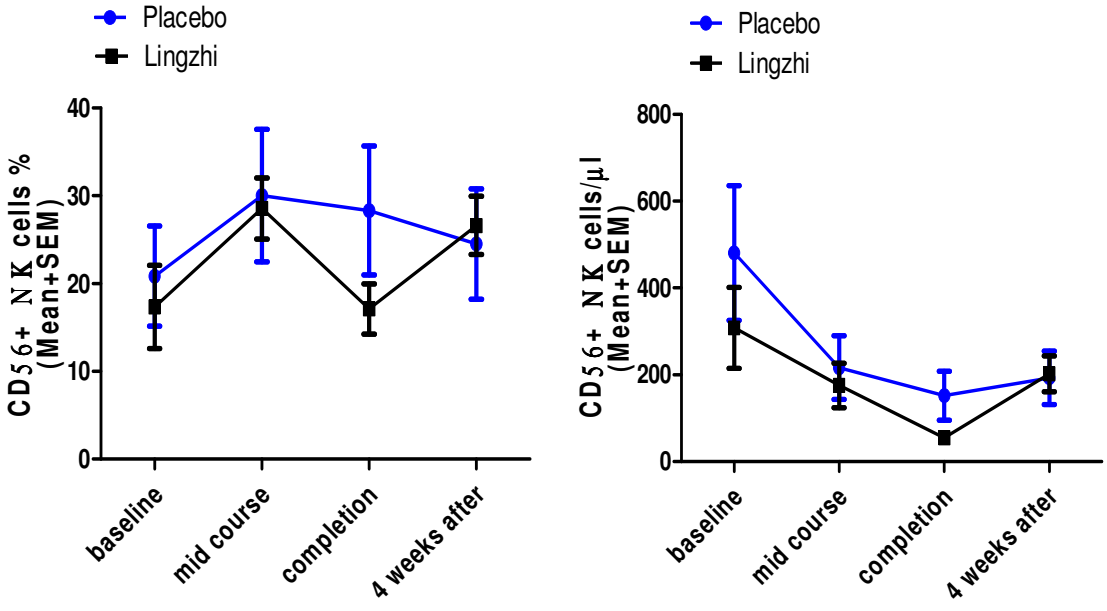


In Figure 5.11 no significance difference was found between placebo and lingzhi groups for CD56+ NK cell % or number at any timepoint. However, the placebo group showed a higher % and number of NK cells at completion of RT when compared with the lingzhi group. Moreover, the number of NK cells had slightly (but not significantly) increased by four weeks after RT in both groups, with both groups now showing very similar % and number of CD56+ cells, indicating that lingzhi group might be rebounding slightly faster than placebo group.

Figure 5.11

The Effect of Lingzhi on the CD56+ NK Cell % and Number:

Placebo: n=9; Lingzhi: n=6; Results are mean+/- SEM

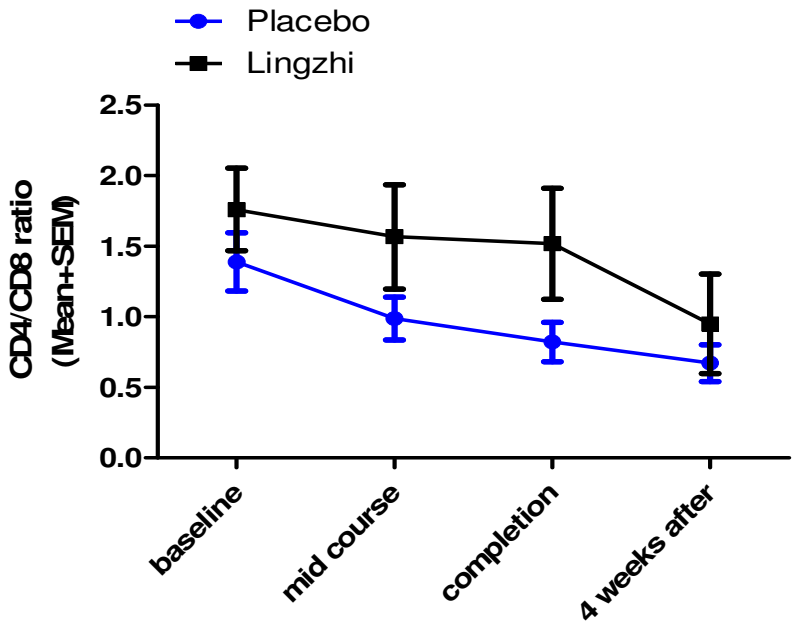


In Figure 5.12, it is seen that no statistically significant difference was found in the CD4+/CD8+ ratio between groups at any time point. Subjects in the placebo group showed slightly lower CD4/CD8 ratios. As with the other results, there was wide variation in results, and the differences between groups were not statistically significant.

Figure 5.12

The Effect of Lingzhi on the CD4/CD8 ratio;

Placebo: n=9; Lingzhi: n=6; Results are mean+/- SEM



Comparison of immune biomarkers in NPC patients and non cancer patients of similar age and sex

The characteristics of Non cancer patients (20 apparently well Type 2 DM and 18 apparently healthy normal subjects) and 15 NPC patients are shown in Table 5.2.

Table 5.2

Characteristics of 53 Subjects for Immune Comparison

Type 2 DM subjects n=20	**Healthy subjects n=18**	**NPC patients n=15**
Age (years): Mean (SD) 55.8 (9.5) Median 55 Range 31-70 Sex distribution (female/male): 11/9	Age (years): Mean SD 42.6 (3.6) Median 42.5 Range 35-50 Sex distribution (female/male): 9/9	Age (years): Mean SD 56.2 (14.5) Median 51 Range 39-86 Sex distribution (female/male): 4/11

The comparison of lymphocytes and subsets % and number are shown in Figures 5.13 and 5.14. In Figure 5.13, it can be seen that non-cancer patients had significantly higher ($P<0.05$) CD4+ helper T cells % when compared to NPC patients. In addition, NPC patients had slightly, but non-significantly, higher CD8+ cytotoxic T cells % and CD56+ NK cells % when compared with non-cancer subjects. In Figure 5.14, it can be seen that there was no significant difference found in the total lymphocytes and subsets number in NPC patients compared to non-cancer subjects.

Figure 5.13

Comparison of the Lymphocyte Subsets % in NPC Patients (n=15) with those in Non-cancer Subjects (n=38) at Baseline; Results are mean+/- SEM

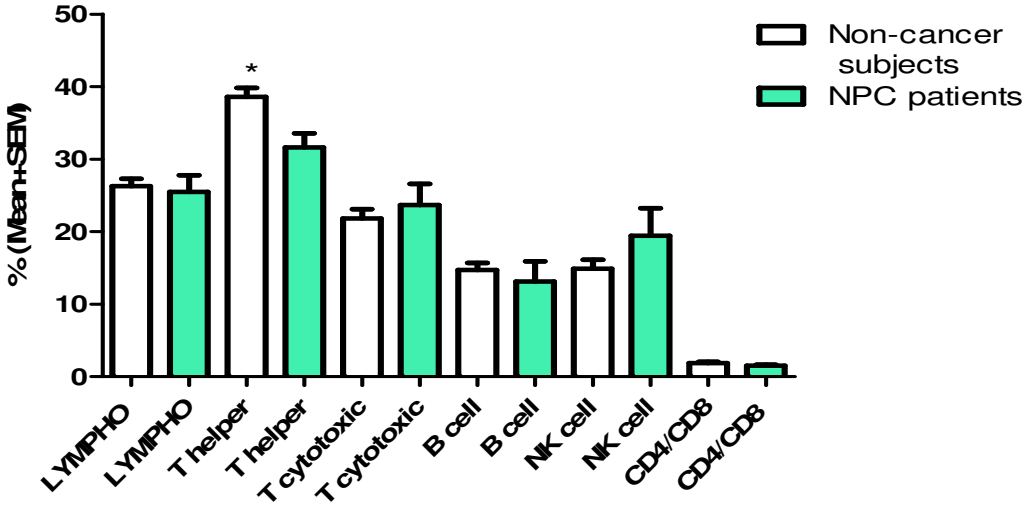
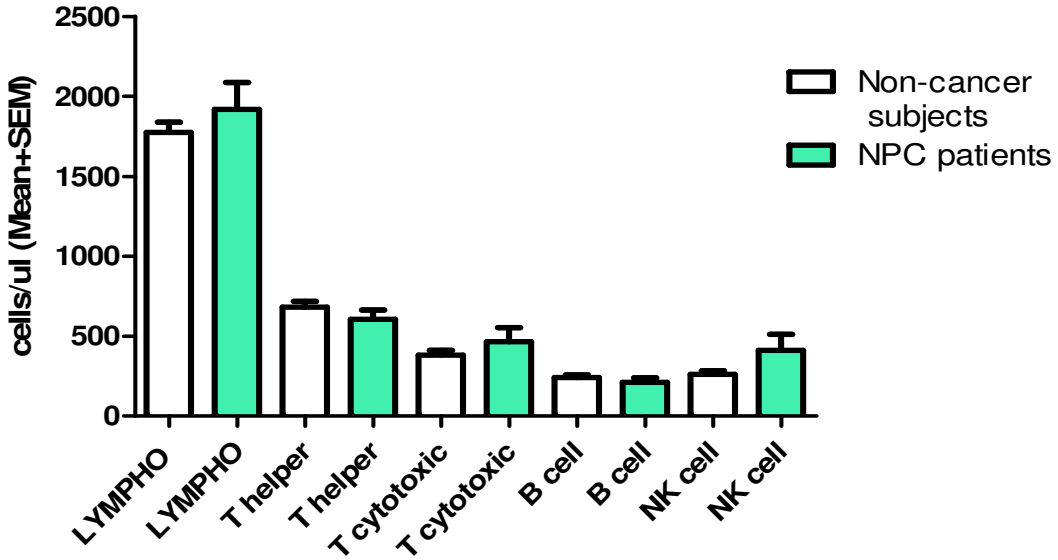


Figure 5.14

Comparison of the Lymphocyte Subsets Absolute Number in NPC Patients (n=15) with those in Non-cancer Subjects (n=38) at Baseline; Results are mean+/- SEM



Discussion

Nasopharyngeal carcinoma (NPC) is rare in Europe and North America. However, it is common in several areas in Southern China, and Hong Kong is one of the regions with a very high incidence of NPC [Spano et al. 2003]. According to the Hong Kong Hospital Authority website, there are about one thousand new cases of NPC per year in Hong Kong [Hospital Authority, 2010]

Radiotherapy (RT) is the primary modality of choice for the treatment of NPC as this method is more successful in improving survival rate than other forms of treatments such as surgery [Lee et al., 2005]. However, continual improvement is needed to further ameliorate the quality of life for NPC patients receiving RT. Indeed, RT side effects include mucositis, partial hearing loss, alterations in taste and salivary dysfunction. Moreover, NPC patients encounter difficulties in chewing and dental complications [Spano et al. 2003; Hospital Authority, 2010].

RT has also been reported to considerably affect lymphocytes, which can lead to clinically manifested infections such as fungal, viral and bacterial infections. In a study in which the effect of RT on cellular immunity in 16 breast cancer patients treated with RT alone (no chemotherapy) was investigated, it was reported that the total T lymphocyte count, CD4+ and CD8+ subpopulations were significantly decreased after 5 weeks of receiving a total RT dose of 50 Gy with daily fraction of 2 Gy for 5 days/week, though some received additional RT [Koukourakis et al., 2009]. The blood samples were taken at four timepoints: before RT, after a cumulative radiation dose of 30 Gy (after 3 weeks' therapy), at completion and at 1 month after the completion of RT. According to the results [Koukourakis et al., 2009], the mean (no SDs were shown in original paper for any sub-set data) total T lymphocytes number (cells/μl) was 1756 at baseline, and this decreased to 1355 after 30 Gy RT dose, to 1233 at RT completion and was 1293 at 1 month post-completion of RT. The mean value of CD4+ number (cells/μl) was 831 at baseline and decreased to 685, 575 and 599 at the other timepoints. Finally, the mean value of CD8+ number (cells/μl) was 446 at baseline, and this decreased to 294, 268 and 306 at the other timepoints respectively. It can be seen that even after 1

month after completion of RT, numbers had not regained pre-RT levels [Koukourakis et al., 2009]. In comparison to the results on 15 NPC patients studied here, the decreases in white blood cell numbers in the breast cancer patients of Koukourakis et al. [2009] showed a similar pattern of decrease, but of considerably smaller magnitude, even though the doses, time points and pre-RT CD4+ and CD8+ numbers were all very similar to those in this current study (Tables 5.3 and 5.4).

In breast cancer patients, radiotherapy treatment is usually done after breast-conserving surgery or mastectomy (removal of breast). Radiotherapy is often used as a preventive measure to destroy any remaining breast cancer cells in the breast, chest wall, or axilla after surgery, thereby reducing the risk of local recurrence of the cancer and improving long-term survival. [Clarke, 2008]. However, in NPC patients, RT without surgery is the standard treatment for NPC, and intensity-modulated radiation therapy (IMRT) is the recent technique used. IMRT modulates the intensity of the radiation beams so that a high dose can be delivered targeted to the tumour while sparing normal tissues. NPC has a tendency to spread to the regional lymph nodes and it has been reported that about 65 to 75 % of cases of NPC involve deep cervical lymph nodes [Jalaludin et al., 1994]. Therefore, for NPC patients IMRT is used to treat the primary tumour along with all the regional lymph nodes, including the supraclavicular nodes [Lee et al., 2002]. The lymph nodes are important in the body's defense against microorganisms and the spread of tumour cells. The heaviest concentration of lymph nodes is located in the cervical spine, the armpit or axillary area and the groin or inguinal region. The most common cells found in the lymph nodes are lymphocytes, macrophages and plasma cells [Sompayrac, 2003]. Therefore, the three to five fold larger effect of RT seen in this current study of NPC patients compared to the effects seen in breast cancer patients could be due to differences in the anatomical region and associated lymph nodes irradiated. Indeed, for the breast cancer patients in the study of Koukourakis et al, [2009], only the breast and chest wall were treated by RT. In other studies, the regions irradiated involved more lymph glands. For example, in the study of Belka et al., [1999], all patients received RT to the paraortal lymph nodes, and all patients with head and neck cancers in the study of Verasteguie et al., [2003] and the NPC patients in this current study, RT was given with

extended fields to the primary and cervical lymph nodes. RT near to large vessels and lymphocyte tissue decreases lymphocytes counts as reported in a long-term study of immune dysfunction after RT in patients with cancers of the head and neck, the pelvis and the central nervous system [Verastegui et al., 2003].

In that study [Verastegui et al., 2003], RT effects on immune cells were investigated in patients with squamous cell carcinoma (SCC) of head and neck (n=50), SCC of uterine cervix (n=10) and tumours of the central nervous system (CNS) (n=10). SCC patients all received 65-70 Gy over 7 weeks, and the CNS patients received 55-65 Gy over ~6 weeks. Blood was collected at baseline, at RT completion and at regular intervals up to 5 years after RT completion. Results showed that CD4+ and CD8+ numbers decreased by, respectively, 73% and 60% in the SCC head and neck cancers [Verasagui et al., 2003]. The total lymphocytes and the B cells had decreased by 70% on completion. These very large decreases on completion of RT were similar to those seen in the NPC patients studied here, and again the RT dose, duration and baseline lymphocytes subsets numbers were similar to those of this current study (Table 5.3 and 5.4) . In the study of Verastegui et al. [2003], the impact of RT was also large in those with SCC uterine cervix, but was much less in the CNS tumour cases, even though the dose and duration of RT was similar to the SCC cases. As with other studies, including this current one, the effect on CD19+ cells was largest, but in the CNS tumour patients the CD19+ cells had decreased only by 32% after RT, compared to 70% in SCC head and neck and 78% in the SCC uterine cervix cases in the same study [Verastegui et al., 2003]. In this current study of NPC patients, CD19+ cells had decreased by 88% by mid-course, and by 93% at completion.

In another study, localized RT of total dose of 26 Gy significantly affected all the 11 different lymphocytes subsets measured, including B cells, T cells subsets and NK cells [Belka et al., 1999]. Ten patients with testicular seminoma were treated with 2 Gy/dose for 13 doses over three weeks. Therefore the dose at completion was slightly less (26 Gy) than the mid-course dose in this current NPC study (35 Gy) and the mid course dose in the breast cancer study (30 Gy) of Koukouris et al. [2009]. In the RT-treated seminoma

patients, total lymphocytes (cells/μl) averaged 1405 at baseline and 462 at completion of 21 days' RT, a decrease of 67%, similar to the change in the NPC patients at mid-course (after 35 Gy). Decreases in CD4+, CD8+, CD19+ and CD56+ cells were also pronounced and were similar to the decreases seen at mid-course in the NPC patient studied here (Table 5.4).

The baseline (pre-RT) cell numbers and relative decreases in immune cells from the studies of Koukourakis et al. [2009], Vesategui et al. [2003], Belka et al. [1999] and this current study are summarized in Tables 5.3 and 5.4.

Table 5.3

Comparison of Baseline Lymphocytes Subsets Cells Number (cells/µl) in Different Studies on Cancer Patients Prior to Radiotherapy (and Not Receiving Chemotherapy)

	This study (2010) 15 NPC Patients	**Koukourakis et al. (2009) 16 Breast Cancer Patients**	**Vesastegui et al. (2003) 50 Head & Neck SCC Cancer Patients**	**Belka et al. (1999) 10 Seminoma Patients**
Total Lymphocytes	1917	1488 (Total T-cells)	1452	1405 (Total T-cells)
CD4+ cells	623	674	777	560
CD8+ cells	414	421	315	421
B cells	211	-	297	153
NK cells	412	-	-	116

Table 5.4

Comparison of Relative Decrease (from pre-RT Level) in Lymphocytes Subsets Cell Numbers in Different Studies by RT Dose Received (Duration of RT Treatment) When Blood Was Collected

	RT Dose 26-30Gy (3-3.5 wks)			RT Dose 60-70 Gy (4-7 wks)			4 wks post RT	1 month post RT	4 months post RT
	This study	Belka et al 1999	Koukourakis et al 2009	This study	Vesastegui et al 2003	Koukourakis et al 2009	This study	Koukourakis et al 2009	Belka et al 1999
Lymphocytes total	70%	67%	17%	79%	70%	20%	63%	15%	39%
CD4+	76%	64%	14%	86%	73%	18%	78%	14%	41%
CD8+	67%	69%	25%	74%	60%	24%	41%	19%	38%
B cell	88%	91%	17%	93%	70%	20%	85%	15%	58%
NK cell	51%	75%	14%	73%	-	18%	52%	14%	12%

This study: 15 NPC patients

Belka et al (1999): 10 seminoma patients

Kourourakis et al (2009): 16 breast cancer patient

Vesastegui et al (2003); 50 SCC of head and neck patients

The results of this current study confirm that RT has a detrimental effect on cellular immunity as seen by the profound depletion in the absolute number of all lymphocytes and subpopulations studied, in particular C19+ cells, which are needed for humoural immunity. This current study has also confirmed that the impact of RT is of prolonged duration. Indeed, the effect may last for many months or even years. Koukourakis et al. [2009] showed that T-lymphocytes, CD4+ and CD8+ cells remained depressed at 1 month after RT completion in 16 breast cancer patients, even though the magnitude of the RT effect was less in these patients. Belka et al. [1999] showed that at 4 months after RT completion, the 10 testicular seminoma patients showed some degree of recovery in all white cell sub-sets measured, but total lymphocytes, CD4+, CD8+ and CD19+ were still only at 40-60% of baseline. However, NK cells had regained about 90% baseline by 4 month post RT (see Table 5.4).

Verastegui et al. [2003] followed RT patients for up to 5 years, with some interesting results. At 12 months post RT the depression in all white cell subsets was still severe in SCC patients. Even at 5 years post RT, total lymphocytes number and number of CD4+ cells remained depressed in the SCC patients with head and neck cancers (the only group followed for 5 years). No data were shown for CD19+ cells post RT, but the authors stated that B cell recovery was complete by 12 months in the SCC patients. The authors stated that the clinical manifestations of the prolonged decrease in white cells were mouth, fungal and viral infection and mild upper respiratory infections, however, no life threatening infections were seen even though the white cells were markedly depressed for a prolonged time [Vesastegui et al, 2003]. Perhaps of more importance, the depressed lymphocyte levels were associated with increased risk of tumour recurrence [Vesastegui et al, 2003]. In this current study we collected post-RT blood only at 4 weeks post RT. As can be seen in Table 5.4, the levels remained very depressed, though some recovery in CD8+ and NK cells was seen at 4 weeks post RT compared to at completion.

In addition, in this current study, the effect of RT on the percentage of the lymphocytes subsets in NPC patients was investigated. It was observed that most of the lymphocyte

subsets % significantly decreased ($P<0.01$) during the course of RT, however CD56+ NK cells % were found to significantly increase at the mid-course of RT, meanwhile CD8+ cytotoxic T cells % were found to significantly increase four weeks after completion of RT. However, it should be noted that although changes in % reflect somewhat different susceptibility of different cells to RT, the results could be misleading as it appears that there was a relative enrichment as shown by the increase of certain lymphocytes subset %, but all were significantly decreased in absolute numbers. Therefore it is important to express the absolute number in order to evaluate the effect of RT and not rely on the %.

In summary, RT has a large, negative and prolonged effect on the immune function. This marked and prolonged immunosuppression effect of RT has important implications. Indeed, it has been reported that there is a strong correlation between the status of cellular immunity and survival of patients with head and neck cancer [Verastegui et al., 2003]. Due to RT induced immune depression, NPC patients may encounter clinical complications such as recurrence of the cancer and development of 'new' cancer, as well as having increased risk of infection. In addition, NPC patients usually need to attend the hospital for treatment, where they can encounter sick people and hence further increase risk of acquiring infectious diseases. Therefore, it is important to try to modulate the drastic effects RT has on cellular immunity, and adjunct therapy with RT could be useful to improve the quality of life of cancer patients going through RT, who are not only severely immunosuppressed but also psychologically depressed [He and Liu, 2005]. For example, zinc supplementation may be helpful in improving cellular immunity in malnourished patients [Verastagui et al, 2003]. Natural products with a reputation for immunomodulation are also worth studying.

Lingzhi (*Ganoderma lucidum*) is a woody mushroom which is the most highly regarded of traditional Chinese medicines. It is widely consumed for its medicinal, rather than nutritional value [Wachtel-Galor et al, 2005, Yuen & Gohel, 2007; Zhou et al., 2007]. Nowadays many commercial lingzhi products are available. Active components of lingzhi include polysaccharides, triterpenes and elemental components such as

Germanium [Wachtel-Galor et al., 2004; Zhou et al., 2007]. In the literature there are a few clinical trials on individuals that suggest potential immunomodulating and antioxidant effects of this herbal medicine [Wachtel-Galor et al., 2004; Yuen and Gohel, 2005; Chen et al., 2006]. For instance, in an uncontrolled study, increase in mitogenic reactivity to phytohemagglutinin and lymphocyte subsets as well as cytokines concentration in plasma of patients with advanced colorectal cancer after supplementation with lingzhi polysaccharides has been reported, although with no statistical significance [Chen et al., 2006]. In a controlled supplementation study following ingestion of lingzhi (1.44g of lingzhi capsules) increased antioxidant power in plasma in 18 healthy adults was also published [Wachtel-Galor et al., 2004]. However, the suggested conclusions based on these studies are yet to be confirmed as some of the effects were small. Nonetheless, the potential effects of lingzhi should not be ignored as there is convincing evidence shown in *in vitro* and animal studies [Lin and Zhang, 2004], thus further studies in well-designed clinical trials to study lingzhi effects on human health are warranted.

This study monitored the effects of lingzhi on cellular immunity in a small group of NPC patients receiving RT. The study is ongoing, but due to very slow recruitment (as a result of stringent entry criteria) and time restriction, data on a very small number of subjects (n=15) are shown here. Results showed that there was no significant difference between the two groups receiving placebo (n=9) and lingzhi (n=6). However, it was observed that for the total lymphocyte number and %, the lingzhi group has a less dramatic initial fall when compared to placebo group. Similarly, subjects taking lingzhi showed a less dramatic initial fall in the CD8+ cytotoxic T cell number when compared to placebo group. In addition, the CD56+ NK cells in the lingzhi group appeared to be rebounding faster 4 weeks after completion of RT when compared to placebo group. Although, these observations did not reach statistical significance they show preliminary evidence of possible effect of lingzhi on counteracting the drastic negative effect of RT on cellular immunity and further studies are warranted.

Finally, it was found that NPC patients pre-RT had a significantly lower helper T cells % (CD4+) when compared with non-cancer patients of similar age and sex. In addition, NPC patients showed a slightly higher cytotoxic T cell (CD8+) and NK cell number and % when compared with non-cancer patients. Helper T cells (CD4+) secrete many different cytokines and therefore are the main players in cell-mediated and humoral immune response [Chaplin, 2006; Zabriskie, 2009]. Moreover, CD4+ helper T cells are required for the priming and maintenance of CD8+ cytotoxic T cells [Wang et al., 2004]. Therefore, NPC patients may be predisposed to higher risk of infection due to lower CD4+ helper T cells, and the slightly higher levels of cytotoxic T cells (CD8+) in the NPC patients may not be reflected in better immune surveillance because of lack of CD4+ priming.

The limitations of this study should be noted. NPC patients receiving RT usually encounter difficulties in eating and swallowing. Thus, it cannot be ignored that other factors such as poor nutrition, zinc deficiency, liver insufficiency and the cancer itself may contribute to low lymphocytes count and impaired cellular immunity. In addition, the recovery of cellular immunity may also depend on age, and nutritional factors. Moreover, this is a very preliminary study in terms of evaluating lingzhi effects. The patients were recruited by the oncology and radiotherapy team of the hospital. Only data from 15 subjects who had completed their intervention by December 2009 are presented here in a preliminary analysis. Of these, nine had taken treatment ‘A’ (placebo), and six had taken treatment ‘B’ (lingzhi) (A or B treatment information was revealed for data analysis after completion of data collection; the A and B codes were broken for this investigator only after completion of data analysis). The trial overall is anticipated to last until 35 subjects have been studied, which is estimated to be late 2010 or early 2011. Therefore, it must be acknowledged that the study as presented has low power due to small numbers and because the markers of interest are also very variable. In addition, because the RT was due to start on day of entry to the study, the method of allocation treatment (lingzhi or placebo as coded treatments) was by alternate allocation at entry. This means that the groups could not be matched for baseline immune status, and due unpredictable drop out, the treatment groups were not matched in number.

Still, the study has strengths and novelty. To our knowledge, this is the first study to investigate RT effects on cell-based immunity on NPC patients, and the first double-blinded, placebo-controlled supplementation trial to investigate possible effects of lingzhi on this. The strengths of this parallel study are: the NPC patients were all diagnosed with similar stage of cancer, going to the same oncology unit and receiving the same RT treatment, and were all ethnic Chinese. The supplementation trial was placebo controlled, with lingzhi extract dosage of 1400mg/day of lingzhi for 11 weeks for some subjects, and only for the final 4 weeks (post RT) for others. The eventual aim is to see if lingzhi during RT or only on completion affects rebound of cell based immunity. In addition, comparison of cell-based immune status in NPC cancer patients with a group on non-cancer subjects of same ethnicity and geographical location (Hong Kong) and of similar age and sex was also performed.

In conclusion, in this study it was shown that RT has a detrimental and dramatic effect on cellular immunity in NPC patients. In addition, it was seen that even after 4 weeks post RT, the total lymphocytes and white cell subpopulations remained depressed and a very slow recovery was seen. There was some evidence that lingzhi might have some potential effect in counteracting the side effects of RT on cellular immunity as seen by the faster rebound and less dramatic fall of certain lymphocytes subsets in lingzhi group when compared to placebo group. However results did not reach statistical difference as the number of subjects was small and variation was very wide. At last, it was observed that NPC patients have significantly lower helper T cells when compared to non-cancer subjects. Thus these patients are at higher risk of infection when compared to the normal population.

Chapter 6

IN VITRO STUDY OF ANTIMICROBIAL EFFECTS OF DIETARY AGENTS: LINGZHI, BILBERRY AND GREEN TEA AGAINST ANTIBIOTIC RESISTANT ORGANISMS (*MRSA* AND *VISA*)

Introduction

Gram positive bacteria are important human pathogens that have gained increasing attention as a result of the use of indwelling medical devices and poor activity of commonly used antibiotics [Wilcox, 2009]. Gram positive bacteria, especially staphylococci mount global challenges as they frequently display resistance to multiple drug classes, including recently introduced agents. Methicillin resistant *Staphylococcus aureus* (MRSA) was first reported in the 1960s and it has become prevalent worldwide [Woodford and Livermore, 2009]. MRSA infections represent a significant clinical burden most frequently causing localized soft-tissue infections but also life-threatening systemic infections. Patients who are elderly, admitted to intensive care units and repeatedly hospitalized are often colonized or infected with resistant bacteria and MRSA isolates are frequently isolated from these patients [Zuo et al., 2008]. Initially, MRSA infections were largely confined to hospitals and health care facilities, however there has now been spread and emergence of community-associated MRSA worldwide [Rice, 2009]. In addition, contact with animals carrying MRSA has been identified as a risk factor for MRSA colonization [Cui et al, 2009]. Antimicrobial resistant organisms, including MRSA have been isolated from both pets and livestock, especially swine [Lloyd, 2007]. Transmission of MRSA between pets, owners and veterinary staff and between pigs and farm workers can occur. It has been demonstrated in a small veterinary hospital that 18% of 78 staff were carriers of MRSA. PCR and PFGE typing confirmed that 82% out of the total MRSA isolates recovered were similar to one of the MRSA strains dominant in UK hospitals (EMRSA-15) [Loeffler et al., 2005]. In another

study where the characterisation of MRSA isolates from dogs and their owners was determined, it was shown that some of the MRSA isolates were similar to highly virulent strains and the authors suggested that rapid changes are occurring in the genes of dog isolates and continued surveillance was essential [Boost et al., 2007]. Moreover, there is a great concern about the emergence of highly toxigenic community MRSA strains as MRSA strains are reported to be greatly clonal [Woodford and Livermore, 2009].

In order to treat MRSA vancomycin, a glycopeptide antibiotic, was introduced as a drug of last resort for the treatment of these resistant organisms. However, in 1995 clinical isolates of glycopeptides intermediately resistant *Staphylococcus aureus* have been reported from several countries [Rybak et al., 2005]. Therefore, the emergence of vancomycin resistant *Staphylococcus aureus* (VRSA) and vancomycin-intermediate *Staphyloccoccus aureus* (VISA) is becoming another concern. The MICs for VISA fall within the range of 2-8 μg/ml, therefore the presence of these organisms increases difficulty in the treatment of infected patients.

The pharmaceutical industry has been passive in developing new agents in response to the threat posed by emerging resistant bacteria. The progress in developing new antibiotics has been very slow and the lack of antibiotics to treat multi-drug resistance bacterial infections is a concern [Fischbach and Walsh, 2009]. Given the increasing spread and problematic consequences of MRSA emergence, new antimicrobial agents and strategies are urgently needed. Different natural products and herbs have been increasingly recognized as possible antimicrobial agents [Yoon et al., 1994; Lee et al., 2006; Shan et al., 2007]. For instance, a green tea compound (epicatechingallate) was reported to reduce oxacillin resistance in *Staphylococcus aureus*, such as EMRSA-16 [Stapleton et al., 2004]. Bilberry and other berry fruits have been reported to have antimicrobial effects against human pathogens including *Salmonella* and *Staphylococcus aureus* [Puupponen-Pimia et al, 2005]. In addition, *Ganoderma* species (lingzhi) were also found to contain antibacterial constituents inhibiting gram-positive and/or gram-negative bacteria *in vitro* [Gao et al, 2005].

Therefore, the specific objectives of this part of the study are:

1. To determine if extracts (hot water) of lingzhi, two types of green tea and bilberry have direct antimicrobial effects on selected microbes [of particular interest is methicillin resistant *Staphylococcus aureus (*MRSA*)* and VISA]
2. To determine if these extracts can act synergistically to lower the MIC of known antibiotics (oxacillin, vancomycin) against the selected microbes.
3. To determine if the MIC of oxacillin and vancomycin can be restored to a therapeutically useful level by co-exposure of MRSA to lingzhi, green tea and bilberry extracts
4. To determine if there is any additive or complementary/synergistic antimicrobial effect against MRSA

Methodology

Bacterial strains, media, growth conditions and chemicals

Clinical strains and standard strains of methicillin resistant and methicillin sensitive *Staphylococcus aureus* (respectively MRSA and MSSA) were cultured following standard microbiological methods in Mueller-Hilton Agar (Oxoid, Hampshire, England). Commercially purchased standard strains, of each of the SCCmec and agr types of MRSA (HIP07930 and COL), VISA (Mu50; ATTC 700699) and *S. aureus* (RN6607) were used as controls. 54 MRSA strains (30 clinical isolates, 10 community colonization isolates, 4 isolates from dogs, 10 isolates from pigs) and 2 MSSA strains were also tested. For testing, bacterial strains were resuspended before use in normal saline to a density equivalent to that of a 0.5 McFarland standard. After incubation the plates were incubated for 24 hours at 37 ^{o}C. The antimicrobial agents (vancomycin and oxacillin) were obtained from Sigma-Aldrich, St. Louis, MO. A stock solution of 550 μg/ml vancomycin and 1200 μg/ml oxacillin were prepared as described by the SGE software [Wexler et al, 1996]. The stock samples were stored at -80 ^{o}C until use.

Preparation of extracts

For the green tea extracts, boiling water (200 ml) was added to 40 g of Screw-shaped or Loongjin green tea to make a 20% w/v 'stock' extract. The liquid obtained was filtered

through Whatman No.2 filter paper and centrifuged at 4000 rpm at 20 ^{o}C for 20 minutes. For the lingzhi and bilberry extracts, ~15 capsules were chosen randomly from the commercially available bottles. Capsules were opened and 12g of powder was mixed with 120 ml of sterile distilled water to give a 10% w/v 'stock' extract. The liquid obtained was centrifuged at 4000 rpm at 20 ^{o}C for 20 minutes. The extracts were adjusted to pH 7 and stock solutions were further diluted to give nominal dilutions from 1% to 0.015% (w/v). The supernatants of the four extracts were filtered using a Millipore filter (Carrigtwohill, Co., Ireland) with a 0.22 μm PVDF membrane under vacuum. The stock samples were stored at -80 ^{o}C until use.

Determination of hydrogen peroxide in extracts

Each extract was tested to determine the concentration of hydrogen peroxide and thus exclude the possibility of the antimicrobial effect of hydrogen peroxide produced by the extracts. This was done by using an in-house protocol (Yuen and Benzie, 2003) as modified from PeroXOquant^{TM} Quantitative Peroxide Assay. Calibration standards of hydrogen peroxide over the range of 0-20 μM were freshly prepared. For working reagent preparation, 1ml of 25mM aqueous solution of ferrous ammonium sulphate (Sigma) in 2.5M sulphuric acid (Merck) was mixed with 100ml aqueous solution of 125μM xylenol orange (Sigma) containing 100mM sorbitol (>99% pure, BDH). In a microtitre plate (Thermo LabSystems, Franklin, MA), 100μl of working standard, controls and extracts preparation were added to the microwell in triplicates. Then, 170μl working reagent was added to the samples in the microwell. The mixture was well mixed and incubated at room temperature for 20 minutes. Absorbance of each microwell was read at 590nm with microplate ELISA reader (Benchmark Plus, USA). Calibrator values were used to plot a calibration line, from which unknowns values were read.

Determination of total phenolics in extracts

The total phenolic content of the extracts was determined by using the Folin-Ciocalteu colometric assay [Vinson and Hontz, 1995]. Working standards of gallic acid (ranging from 0-10 mM) were freshly prepared. In a test tube, 100μl of sample/standard was diluted with 700μl of distilled water. Then, 10% of sodium tungstate and 0.33 mol/l of

sulphur acid were added and allowed to stand for 10 minutes. The mixture was centrifuged at 3000 rpm for 10 min and 200µl of supernatant was collected into new test tubes. Folin-Ciocaltau reagent (100µl) was added, followed by 20% of sodium carbonate. After standing at room temperature for 2 hours, the absorbance was read at 580nm using a spectrophotometer.

Susceptibility testing

To test for direct antimicrobial effect of the extracts, 38 strains (which included selected MRSA from clinical, colonization, dog and pig isolates, and MSSA and VISA standard strains) were tested against extracts of each test agent using dilutions of 10% and 20% w/v stock extracts. This was done by the agar dilution susceptibility method, using a multipoint inoculator (Crane Electronics Ltd., Mast Laboratories Ltd., England). Agar plates contained two-fold serial dilutions of the extracts (in the range of 0.015%-1%) and were inoculated with 10^4 colony forming units (cfu) per spot by the multipoint inoculator. The MIC was defined as the lowest concentration inhibiting growth after incubation at 37 °C for 18 h. [Schawlbe, 2007].

The MICs for oxacillin and vancomycin were determined using a modification of the spiral plater gradient endpoint technique [Wexler et al, 1996], and software specially designed for the purpose (Spiral Biotech, Norwood, MA, USA). The organisms were then tested against a combination of known antibiotic and test agents (extracts) to see if the presence of the test agent had a complementary or synergistic effect on the MIC of the antibiotic. This was performed in a uniform agent versus exponential agent mode, in which one agent is mixed in the agar and the second is added using the spiral plater. The bacterial strains were streaked in radial lines across the gradient with a sterile wool swab. Each bacterial strain was applied in duplicate following the template provided with the SGE software (Appendix III). The distance from the centre of the plate to the point where growth began was measured and the SGE computer software was used to convert these values into MIC values (mg/L). The concentrations of each extract used were selected on the basis of its ability to inhibit growth of the microbes according to the results obtained from the agar dilution method.

Statistical Analysis

The statistical analysis was carried out using GraphPad Prism (version 4.00 for Windows, GraphPad Software, San Diego California USA). ONE-way ANOVA was used to compare groups. Significance was set at $P<0.05$.

Results

Hydrogen peroxide level of extracts

The natural extracts tested showed that at the highest percentage to be used (1% w/v) the level of hydrogen peroxide level was very low. The green tea and lingzhi extracts produced less than 0.03% of hydrogen peroxide. For the bilberry extract, 0.19% of hydrogen peroxide was produced. Four different MRSA strains were challenged with 0.2% freshly prepared hydrogen peroxide and no growth inhibition was observed.

Total phenolics and antioxidant power level of extracts

The total phenolic content and the antioxidant power of the extracts at the highest concentration tested of 1% w/v were determined. Amongst all the extracts, bilberry had the highest phenolic content (10.0mM) and antioxidant power level (31340µM). Lingzhi had a phenolic content of 8.0mM and antioxidant level (1620µM). Loongjin and Screw-shaped green tea had similar phenolic contents and antioxidant power, Loonjing green tea having a phenolic content of 1.18mM and antioxidant power of 5740µM whereas Screw-shaped green tea contained 1.40mM total phenolics and an antioxidant power of 6380µM.

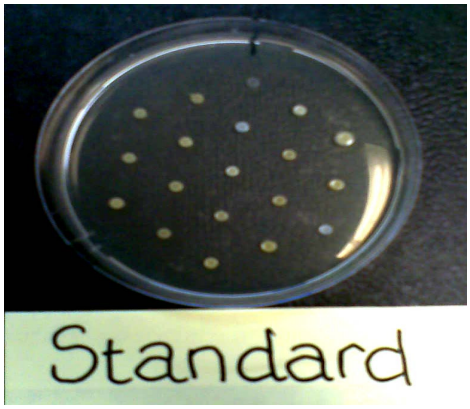
Direct effect of extracts in bacterial growth

The number of MRSA strains inhibited is presented in Table 6.1. The MIC of Screw-shaped green tea for MRSA (n=38) was 0.12%. Similarly, Bilberry MIC was 0.12% while for Loongjin green tea the MIC was 0.25%. For Lingzhi, there was no direct antimicrobial effect observed, even at the highest concentration of 1%.

In Figures 6.2-6.5 pictures showing the effect of different concentrations of the four extracts (loongjin green tea, lingzhi, bilberry and screw-shaped green tea) are presented.

Figure 6.1

Control plate with 18 MRSA Strains and VISA Strain



In Figure 6.2-6.5 it can be seen that bacteria growth was inhibited at certain concentrations (in % w/v) of the extracts except for lingzhi.

Figure 6.2

Effect of Different Concentrations of Loongin Green Tea Extract (LGT) on 18 MRSA Strains and VISA Strain

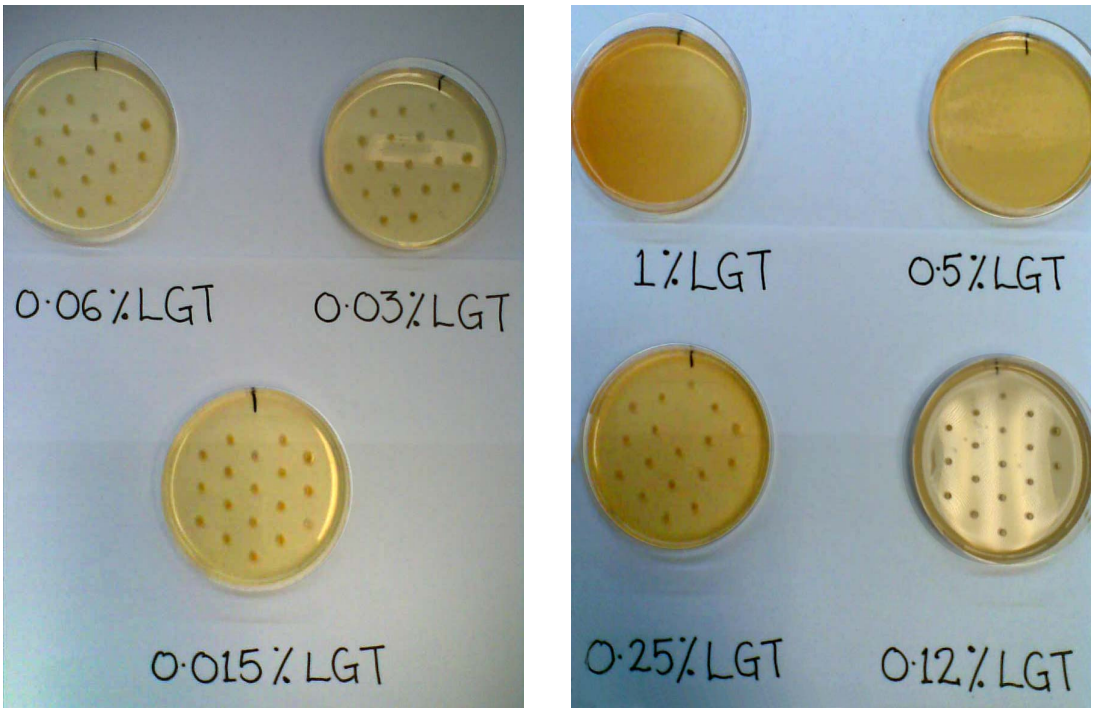


Figure 6.3

Effect of Different Concentrations of Lingzhi Extract (L) on 18 MRSA Strains and VISA Strain

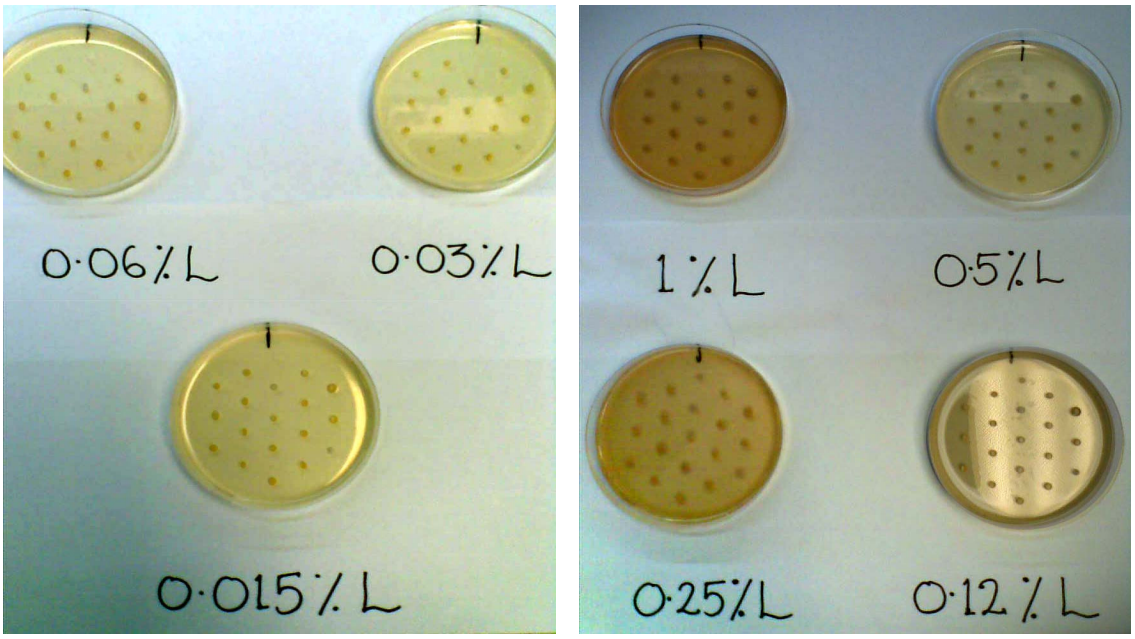


Figure 6.4

Effect of Different Concentrations of Bilberry Extract (BB) on 18 MRSA Strains and VISA Strain

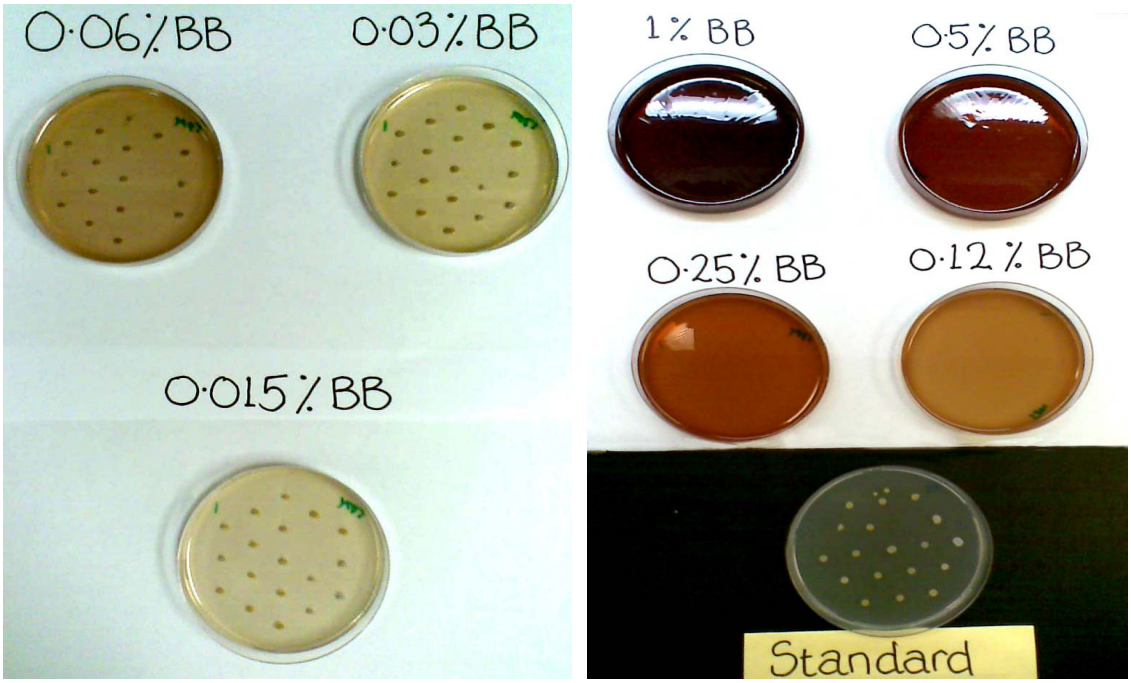


Figure 6.5

Effect of Different Concentrations of Screw Shaped Green Tea (SSGT) on 18 MRSA and VISA Strains

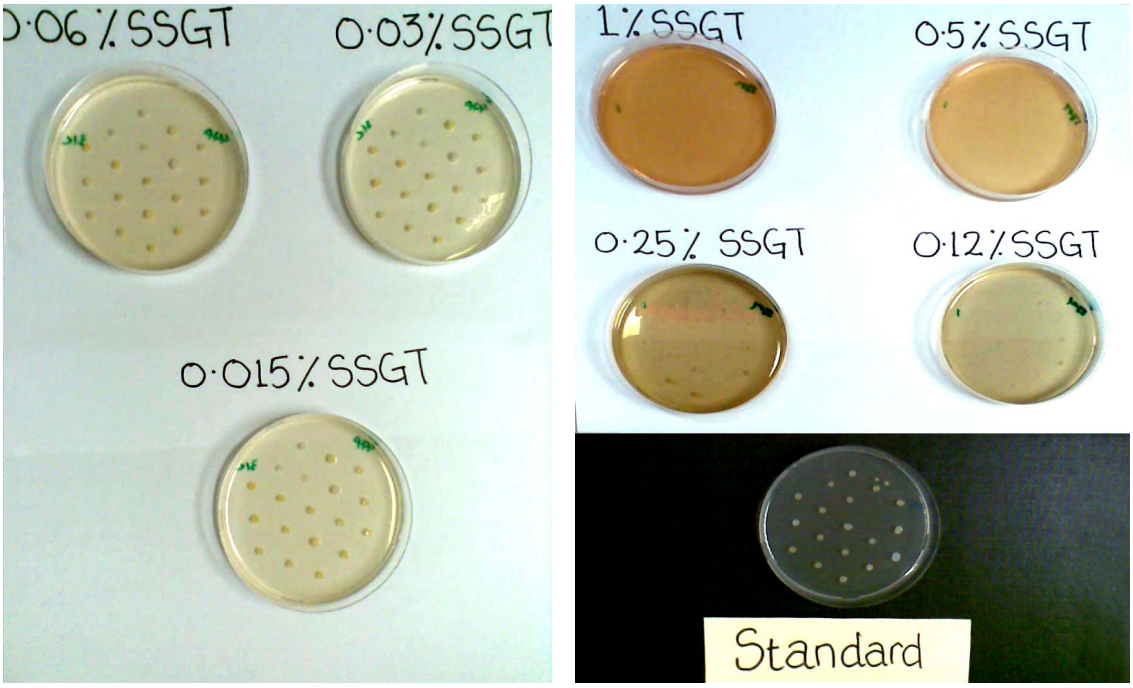


Table 6.1

Summary of MRSA and MSSA Strains Inhibited at Each Concentration % of Different Extracts (out of 38 strains tested)

Extract	0.015% (0.15 mg/ml)	0.03% (0.3 mg/ml)	0.06% (0.6 mg/ml)	0.12% (1.2 mg/ml)	0.25% (2.5 mg/ml)	0.5% (5.0 mg/ml)	1% (10.0 mg/ml)
Screw-shaped green tea	0/38	0/38	0/38	34/38	36/38	38/38	38/38
Loongjin green tea	0/38	0/38	0/38	0/38	35/38	38/38	38/38
Lingzhi	0/38	0/38	0/38	0/38	0/38	0/38	0/38
Bilberry	0/38	0/38	0/38	38/38	38/38	38/38	38/38

In summary, direct antimicrobial effect was seen with bilberry, loonjin and screw-shaped green tea. For the next part of the study to test for synergistic effect,

concentrations, in which there was no obvious direct effect of the extracts, were selected. The concentrations were as follows:
Screw-shaped green tea: 0.06%, 0.03% and 0.015%; Loongjin green tea: 0.12%, 0.06% and 0.03%; Bilberry extract: 0.06%, 0.03% and 0.015%; Lingzhi extract: 1%, 0.5% and 0.25%

Effect of extracts against MRSA in combination with oxacillin and vancomycin
The summary of MIC values of oxacillin and vancomycin (alone and together with different concentrations of extracts) are shown in Table 6.2 and Table 6.3.

In Table 6.2 it can be seen that all the strains were resistant to oxacillin alone with a MIC ≥11.072 µg/ml. The clinical isolates of MRSA were resistant to oxacillin alone and in combination with the extracts (MIC ≥11.072 µg/ml). On the other hand in the colonizing organisms, and the animals MRSA isolates MICs were significantly decreased ($P<0.05$) by oxacillin in combination with the extracts in a concentration dependent manner. Moreover, at 0.06% w/v bilberry together with oxacillin no growth was observed for most of the MRSA isolates. Therefore, the MIC was lower than the recorded one since many of the strains were inhibited. However, the lowest concentration tested for bilberry extract was 0.06% w/v and further studies to find the MIC for this extract is warranted.

In Table 6.3 it can be seen that all MRSA isolates were susceptible to vancomycin with a MIC range of 1.3-1.8 µg/ml. The two standard MRSA had an average MIC of 2.4 µg/ml meanwhile VISA had a MIC of ≥4.4 µg/ml. However, vancomycin together with the extracts decreased the MICs of the standard MRSA and VISA. In addition, vancomycin together with extracts significantly decreased ($P<0.05$) the MICs of the clinical MRSA isolates. The MICs of the colonization MRSA isolates were also decreased by vancomycin together with the extracts; however results did not reach statistical significance. Similarly, the MICs of animal MRSA isolates were decreased by vancomycin together with the extracts. In addition at 0.06% w/v bilberry together with vancomycin a greater impact in reducing the MICs of MRSA and VISA strains was

observed. Growth inhibition for most of the MRSA isolates was seen. Therefore, the MIC was lower than the recorded one since many of the strains were inhibited. However, the lowest concentration tested for bilberry extract was 0.06% w/v and further studies to find the MIC for this extract is warranted.

Table 6.2

Summary of MIC Values of Different MRSA, Standard MRSA and VISA Strains with Oxacillin (Alone and in Combination with Extracts)

	Oxacillin MIC (µg/ml): Mean (SD)				
	Two Standard MRSA Strains	VISA (Mu50) Strain	30 clinical isolates	10 colonization isolates	14 animal isolates
Alone	≥11.072	≥11.072	11.072 (0.00)	11.072 (0.00)	11.072 (0.00)
With 0.25% L	≥11.072	≥11.072	11.072 (0.00)	4.5 (4.3)	8.2 (3.1)
With 0.5%L	≥11.072	≥11.072	11.072 (0.00)	4.9 (3.7)	6.3 (3.8)
With 1% L	≥11.072	≥11.072	10.8 (1.7)	2.7 (2.8)	5.1 (3.2)
With 0.03% LGT	≥11.072	≥11.072	10.8 (1.6)	5.7 (4.6)	6.9 (3.7)
With 0.06% LGT	≥11.072	≥11.072	10.8 (1.7)	6.9 (4.8)	8.7 (3.8)
With 0.12% LGT	≥11.072	≥11.072	10.8 (1.4)	5.9 (4.8)	8.1 (3.4)
With 0.015% SSGT	≥11.072	≥11.072	11.072 (0.00)	5.5 (4.9)	5.2 (3.1)
With 0.03% SSGT	≥11.072	≥11.072	11.072 (0.00)	5.6 (4.9)	5.7 (3.9)
With 0.06% SSGT	≥11.072	≥11.072	11.072 (0.00)	6.8 (4.9)	4.9 (3.4)
With 0.015% BB	≥11.072	≥11.072	11.072 (0.00)	6.4 (4.5)	4.9 (3.5)
With 0.03% BB	≥11.072	≥11.072	11.072 (0.00)	4.3 (3.8)	6.7 (3.3) [n=9]
With 0.06% BB	≥11.072	≥11.072	11.072 (0.00)	-	5.9 (2.5) [n=2]

L= lingzhi; LGT= loonjin green tea; SSGT= screw-shaped green tea; BB= bilberry

Table 6.3

Summary of MIC Values of Different MRSA, Standard MRSA and VISA Strains with Vancomycin (Alone and in Combination with Extracts)

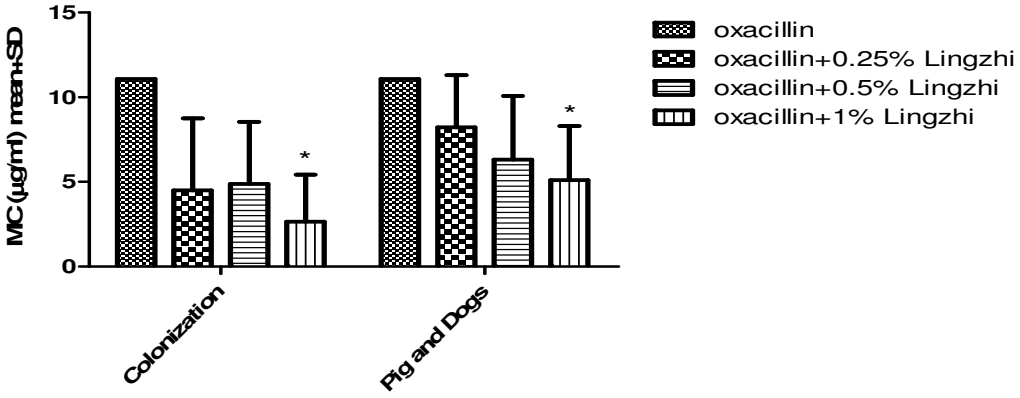
	Vancomycin MIC (µg/ml): Mean (SD)				
	Two Standard MRSA Strains	VISA (Mu50) Strain	30 clinical isolates	10 colonization isolates	14 animal isolates
Alone	2.396	≥4.487	1.8 (0.4)	1.5 (0.3)	1.3 (0.2)
	1.928	3.491	1.3 (0.3)	1.3 (0.5)	1.1 (0.3)
With 0.25% L					
With 0.5%L	1.29	2.114	1.3 (0.3)	1.3 (0.2)	1.0 (0.2)
With 1% L	0.996	1.645	1.0 (0.2)	1.2 (0.2)	1.2 (0.2)
	1.21	1.645	1.1 (0.2)	1.2 (0.3)	1.0 (0.2)
With 0.03% LGT					
With 0.06% LGT	1.372	1.865	1.0 (0.2)	1.1 (0.2)	1.0 (0.3)
With 0.12% LGT	1.928	2.717	0.9 (0.2)	1.2 (0.2)	1.1 (0.2)
	1.321	3.491	0.9 (0.2)	1.2 (0.2)	1.0 (0.2)
With 0.015% SSGT					
With 0.03% SSGT	0.996	2.717	0.8 (0.2)	1.1 (0.2)	1.1 (0.1)
With 0.06% SSGT	1.28	2.396	0.9 (0.2)	1.1 (0.2)	1.0 (0.2)
	1.78	3.08	1.1 (0.3)	1.1 (0.2)	0.9 (0.1)
With 0.015% BB					
With 0.03% BB	1.763	2.396	1.0 (0.2)	1.3 (0.2)	1.2 (0.2)
With 0.06% BB	0.775	0.775	0.7 (0.1) [n=13]	1.0 (0.2) [n=6]	0.9 (0.1) [n=6]

L= lingzhi; LGT= loonjin green tea; SSGT= screw-shaped green tea; BB= bilberry

In Figure 6.6, it can be seen that oxacillin together with lingzhi at 1% w/v significantly decreased (P<0.05) the MIC against colonization, animal MRSA isolates. Oxacillin together with 0.25% and 0.5% w/v lingzhi decreased the MIC against the isolates tested but it did not reach statistical significance.

Figure 6.6

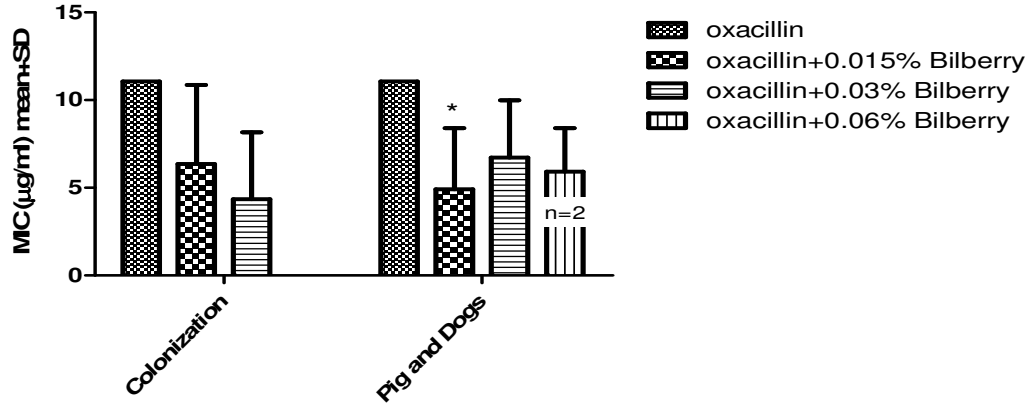
The Effect of Lingzhi Extract at Different Concentrations Against MRSA Isolates: Colonization n=10; Pig and Dog n=14



In Figure 6.7, it can be seen that oxacillin together with Bilberry extract at 0.15% w/v, significantly decreased (P<0.05) the MIC against animal MRSA isolates. Bilberry together with oxacillin at 0.06% w/v completely inhibited the growth of MRSA in colonization isolates.

Figure 6.7

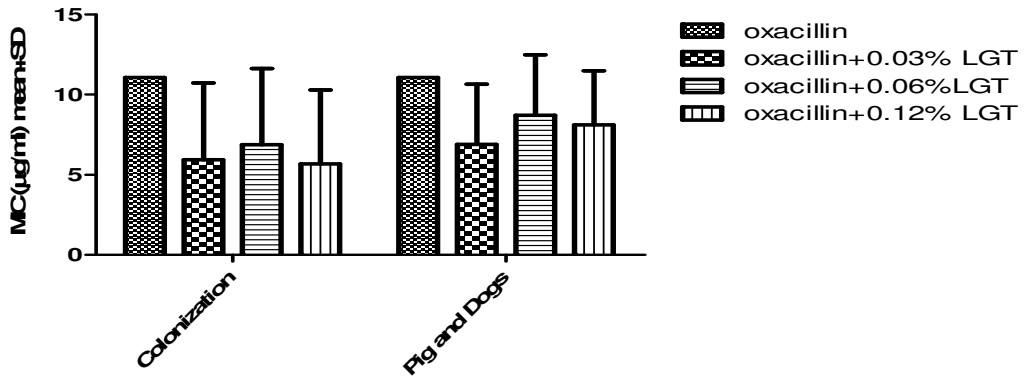
The Effect of Bilberry Extract at Different Concentrations Against MRSA Isolates: Colonization n=10; Pig and Dog n=14



In Figure 6.8, it can be seen that oxacillin in combination with LGT extract at different concentrations decreased the MIC against colonization and animal MRSA isolates but it did not reach statistically significance.

Figure 6.8

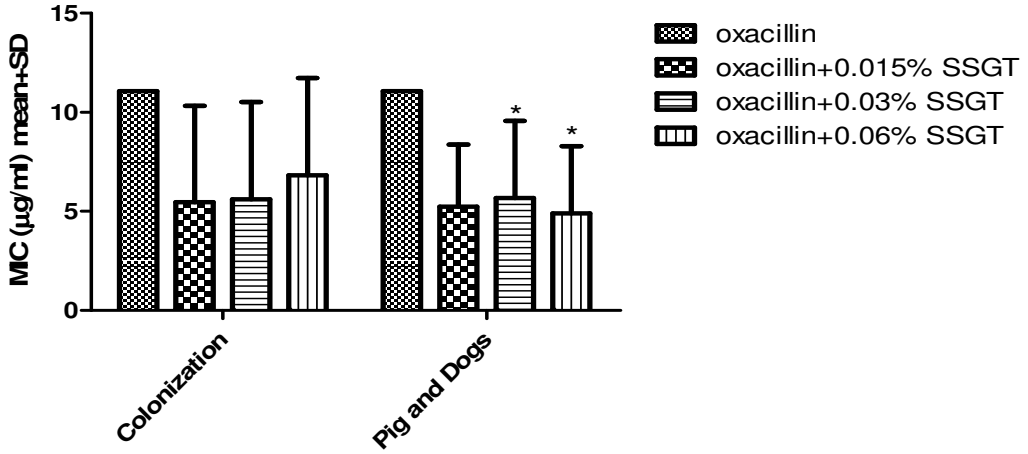
The Effect of Loongjin Green Tea (LGT) Extract at Different Concentrations Against MRSA Isolates: Colonization n=10; Pig and Dog n=14



In Figure 6.9, it can be seen that oxacillin in combination with SSGT at different concentrations decreased the MIC against colonization MRSA isolates. A significant decrease ($P<0.05$) was observed at 0.03% and 0.06% w/v SSGT together with oxacillin against animal MRSA isolates.

Figure 6.9

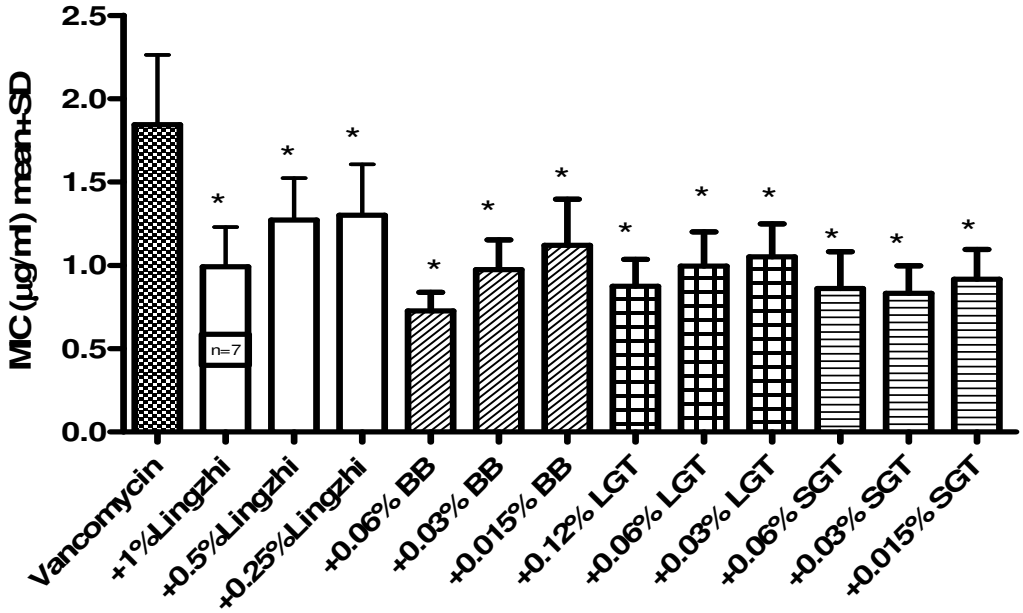
The Effect of Screw-shaped Green Tea (SSGT) Extract at Different Concentrations Against MRSA Isolates: Colonization n=10; Pig and Dog n=14



In Figure 6.10, vancomycin in combination with the four extracts at different concentrations significantly decreased (P<0.05) the MIC against clinical MRSA isolates when compared to the effect of vancomycin alone in a dose dependent manner.

Figure 6.10

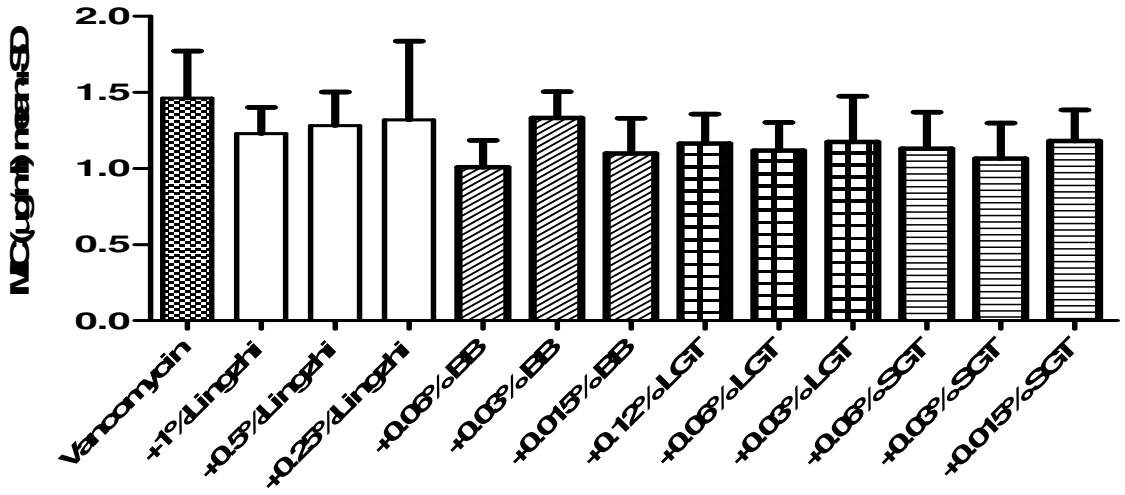
The Effect of Vancomycin in Combination with Lingzhi, Bilberry (BB), Loongin Green Tea (LGT) and Screw-shaped Green Tea (SSGT) Extracts at Different Concentrations Against Clinical MRSA Isolates (n=30)



In Figure 6.11, it can be seen that vancomycin in combination with the four extracts at different concentrations slightly decreased the MIC against colonization MRSA isolates when compared to the effect of vancomycin alone. However, results did not reach statistical significance.

Figure 6.11

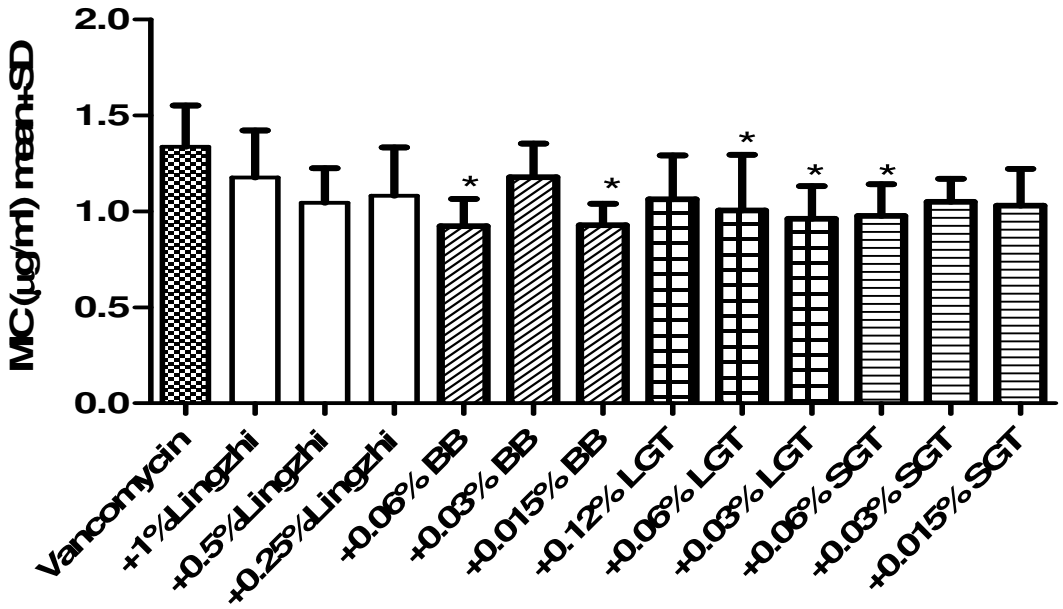
The Effect of Vancomycin in Combination with Lingzhi, Bilberry (BB), Loongin Green Tea (LGT) and Screw-shaped Green Tea (SSGT) Extracts at Different Concentrations Against Colonization MRSA Isolates (n=10)



In Figure 6.12, vancomycin in combination with the four extracts at different concentrations decreased the MIC against animal MRSA isolates when compared to the effect of vancomycin alone. A significant decrease ($P<0.05$) was observed when vancomycin was in combination with 0.06% and 0.015% w/v bilberry extract, 0.06% and 0.03% w/v loongjin green tea extract and 0.06% w/v screw-shaped green tea extract.

Figure 6.12

The Effect Vancomycin in Combination with Lingzhi, Bilberry (BB), Loongin Green Tea (LGT) and Screw-shaped Green Tea (SSGT) Extracts at Different Concentrations Against Animal MRSA Isolated from Pigs and Dogs (n=14)



Discussion

The main objective of this study was to evaluate the antimicrobial effects of lingzhi, bilberry and green tea extracts with and without oxacillin and vancomycin against a wide range of methicillin resistant *Staphylococcus aureus* (MRSA) isolates and a standard vancomycin intermediate *Staphylococcus aureus* (VISA). Increasing antibiotic resistance is a major threat for the treatment of many infectious diseases. MRSA and VISA are examples of resistant organisms that increase complications and fatal outcome for infected patients [Neu, 1992]. Indeed, it was reported that swine flu was complicated by bacteremic pneumonia due to community-acquired MRSA in immunocompetent adults, frequently resulting in death [Cheng et al., 2009]. Moreover, MRSA is increasing in the community and more people are being colonized with this organism. Transmission between animals and human can occur and MRSA may also be found in the environment [O'Donoghue and Boost, 2004]. In 1960s, vancomycin was introduced for the treatment of serious MRSA infections. However, vancomycin resistant *Staphylococcus aureus* emerged [Howden et al., 2010]. Subsequently, the Clinical and Laboratory Standards Institute (CLSI) defined organisms with an MIC of ≤ 2 µg/ml as susceptible to vancomycin, a MIC of 4-8 µg/ml as intermediate to vancomycin and a MIC of ≥ 16 as resistant to vancomycin [Howden et al., 2010]. Therefore, the definition for VISA is a *Staphylococcus aureus* with a vancomycin broth MIC of 4-8 µg/ml [Howden et al., 2010]. However, in a study which identified vancomycin MIC threshold range that is associated with an increased probability of treatment failure, a lower MIC value was recorded than the CLSI susceptibility range [Lodise et al., 2008]. Indeed it was reported that patients (n=66) with MRSA bloodstream infections with a vancomycin MICs ≥ 1.5 µg/ml responded poorly to vancomycin and had a 2.4 fold increase in treatment failure compared to patients with MICs ≤ 1.0 µg/ml. The patients with MICs ≥ 1.5 µg/ml were reported to have a longer duration of bacteremia, a higher likehood of recurrence and a longer hospital length stay [Lodise et al., 2008]. This means that an organism with an MIC ≥ 1.0 µg/ml is less susceptible to vancomycin and might lead to treatment failure.

Resistance to methicillin in *Staphylococcus aureus* has been found to be associated with a methicillin resistant gene, which is mediated via the *mec* operon, part of the staphylococcal cassette chromosome mec (SCC*mec*) [Enright, 2003]. Resistance is conferred by the *mecA* gene, which codes for an altered penicillin-binding protein (PBP2a or PBP2') that has a lower affinity for binding β-lactams such as penicillins, cephalosporins and carbapenems. This allows for resistance to all β-lactam antibiotics and thus impeding their clinical use during MRSA infections [Lencastre et al., 2007]. The exact genetic basis of the VISA phenotype is still unclear, however it has been hypothesized that it is associated with spontaneous mutations in genes involved in cell wall biosynthesis. In fact, MRSA isolates with decreased susceptibility to vancomycin such as VISA have been shown to have a thickened cell wall with decreased peptidoglycan cross-linking. This results in a structure that binds and sequesters vancomycin causing a reduction in the amount of antibiotic reaching the sites of cell wall synthesis and so lowering its efficacy [Enright, 2003].

To date, there is no antibiotic class that is uniformly effective against resistant *Staphylococcus aureus* and methicillin resistance has recently become a community phenomenom [Enright, 2003]. Therefore, alternative antibiotic therapy for the treatment of resistant organisms is sought and the use of natural products or herbs together with known antibiotics could be of great significance as there is evidence that some herbs can increase the potency of antibiotics, allowing lower doses to be used and thus avoiding undesirable side effects.

In the present study it was found that the lingzhi, bilberry and the two types of green teas extracts demonstrated activities against the bacteria tested. Direct antimicrobial effect was found in bilberry (MIC= 0.6 mg/ml), screw-shaped green tea (MIC= 0.6 mg/ml) and loongjin green tea (MIC= 1.2 mg/ml) against MRSA, MSSA and VISA. In addition, it has been shown that this direct effect observed was not due to the production of hydrogen peroxide of these extracts. Indeed, these extract were shown to produce very low level of hydrogen peroxide. However, no direct antimicrobial effect was found in lingzhi even at the highest concentration of 10 mg/ml. In addition, it was found that

bilberry has the highest antioxidant level (31340μM), followed by screw-shaped green tea (6380μM) and loongjing green tea (5740μM), while the lowest antioxidant level was found in lingzhi with only a total antioxidant level of 1620μM. Therefore, it is possible that the antimicrobial activity seen is related to the total antioxidant level observed in these extracts. However, these results have yet to be confirmed.

Green tea has been extensively investigated as an antimicrobial agent [Lai and Roy, 2004; Friedman, 2007; Shimamura et al., 2007; Song and Seong, 2007]. Green tea bioactive chemicals such as catechins and polyphenols have been shown to be responsible for the antimicrobial effect seen in green tea against MRSA [Si et al., 2006]. In addition, a commercial bilberry extract was found to significantly inhibit *Helicobacter pylori* and also to increase susceptibility of *Helicobacter pylori* to clarithromycin. Indeed, it has been shown that phenolic compounds found in bilberry possess antimicrobial effects against human gastroinstestinal pathogens in particular against *Salmonella* and *Staphylococcu*s [Puupponen-Pimia at al., 2005]. Phenolics have been shown to attack the cell wall and penetrate the cell, where they react with the cytoplasm and cellular proteins [Puupponen-Pimia at al., 2005]. In addition, EGCG was found to attach the same cell wall target site (peptidoglycans) and thus acts synergistically with β-lactams against MRSA, owing to interference with the integrity of the cell wall through direct binding to peptidoglycan [Zhao et al., 2001]. Therefore, results seen in this current study support these previous studies and further studies investigating these natural products antibacterial effects at cellular level are warranted.

Besides direct antimicrobial effects, synergistic effects of lingzhi, bilberry and green tea were found in combination with oxacillin and vancomycin against the bacteria tested.

Oxacillin in the presence of the extracts tested was found to decrease the MICs of MRSA isolates from human colonization strains and animal strains only. Significant decrease ($P<0.05$) of MIC at 1% lingzhi together with oxacillin was seen. Moreover, a significant decrease ($P<0.05$) of MIC at 0.05% bilberry, 0.03% and 0.06% screw-shaped green tea together with oxacillin was observed against MRSA isolated from pigs and

dogs. No effect was seen against standard MRSA, VISA and clinical MRSA isolates. Therefore, it can be seen that strains from human colonization and animals are more susceptible to the effect of the extracts in the presence of oxacillin while clinical isolates seems to be more resistant. The different susceptibilities shown in the different isolates might be due to the fact that the gene structure and cassette components are different between MRSA strains from colonizing organisms, animal and hospital acquired strains. Indeed the community acquired strains, which tend to be resistant to multiple antibiotics are predominantly characterized by SCCmec cassette type IV and or type V [Lencastre et al., 2007].Other strains found mainly in the hospital share different antibiotic resistance patterns and tend to have a variety of SCCmec cassette (mainly I, II and III) [Enright, 2003; Lencastre et al., 2007]. Therefore, further studies to determine differences between genetic material in these strains and how these affect their response to antimicrobial agents in combination with natural extracts are needed.

Results showed that, vancomycin in combination with the different extracts was found to decrease the MICs for all the bacteria tested including the two control ATCC strains of MRSA and VISA. A significant decrease ($P<0.05$) of MIC against human colonization isolates of MRSA was observed with all the extracts tested in the presence of vancomycin in a concentration dependent manner. In addition, the MICs of MRSA isolated from human colonization and animals were also decreased, however results did not reach statistical significance. The reason for this observation might be due to the fact that the MRSA isolated from human colonization and animals showed low MIC against vancomycin alone (i.e. the isolates were highly susceptible to vancomycin) and thus the effect seen was small. One interesting finding was the effect seen of the extracts together with vancomycin against the VISA; Mu50 strain. All the extracts at different concentrations when together with vancomycin decreased the MIC of VISA to a great extent. Indeed, an 82% decrease in MIC was observed with 0.06% bilberry together with vancomycin with a MIC < 1 µg/ml. Another interesting finding was the killing effect of 0.06 % bilberry together with vancomycin and oxacillin against the MRSA isolates. In addition, 0.06% bilberry together with vancomycin greatly increased the

susceptibility of the standard MRSA and VISA to vancomycin and decreased the MICs to a therapeutically useful level.

In conclusion, this study shows preliminary data supporting the potential use of bilberry and green tea alone as antibacterial agents. Moreover, this study supports the possible use of lingzhi, bilberry and green tea in combination with antibiotics to increase their potency. This type of finding could further boost the use of extracts and functional foods either alone, combined or together with antibiotics to treat resistant infections. This preliminary data need to be confirmed by further studies and should be followed by the identification of active compounds and determination of the mechanisms of inhibition. In addition, toxicity against animal or human cells should be evaluated, effects *in vivo*, and positive or negative interactions with other types of antibiotics, as well as potential clinical applications of these results could be further explored.

Nonetheless, the results presented show the potentially high impact of natural products for improving the approach to the treatment of infectious disease.

Chapter 7

RECAP OF FOCUS OF STUDY, MAIN FINDINGS, THEIR IMPLICATIONS, OVERALL CONCLUSIONS AND SUGGESTIONS FOR FURTHER STUDIES

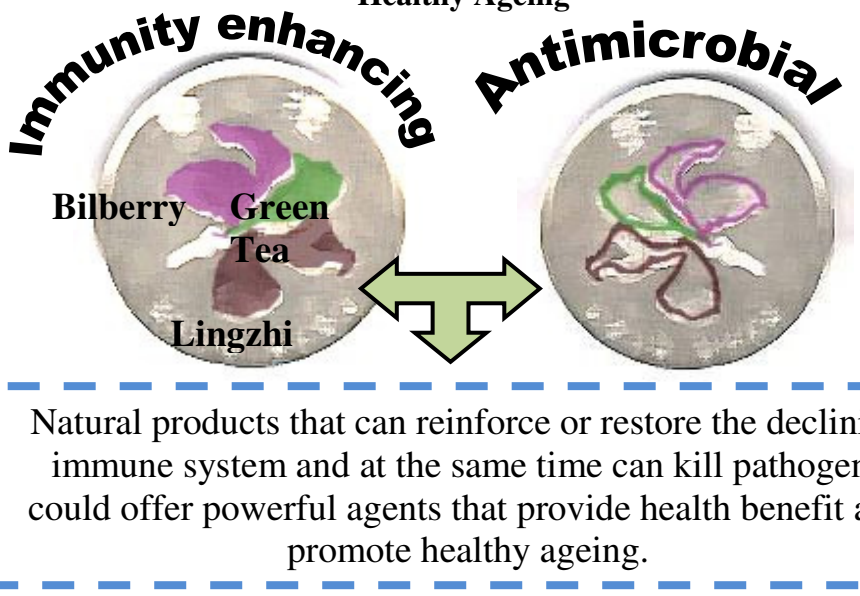
The immune system has the ability to protect the human body from diseases by killing or inactivating infectious agents such as bacteria, viruses, parasites and fungi, as well as identifying changed (including tumour) cells and removing them [Chaplin, 2006]. However, with advancing age there is a progressive decline in the function of the immune system, and this is a major contributor to the many deleterious changes that occur with ageing, such as the increased incidence of cancer, increased susceptibility to and poorer recovery from infection, and lowered efficacy of vaccination in elderly persons [Aw et al., 2007; Gruver et al., 2007]. Strategies that help maintain immune function are needed to promote healthy ageing. In relation specifically to infectious diseases, the efficacy of currently used antibiotics is decreasing with the emergence of antibiotic resistant strains and new microbes, and there is a lack of new antibiotics to treat infectious disease [Moellering et al., 2007]. Older people and those who are immuno-compromised are prone to acquire infectious diseases and are more likely to develop resistant bacterial infections due to the heavy use of antimicrobials [Livermore, 2007]. In addition, resistant bacterial infections are complicated to treat and often have fatal outcome [Livermore, 2007]. Therefore, a two-pronged approach in relation to immunomodulation and antibacterial therapy by natural products in order to prevent and cure diseases is a worthwhile area of research.

Currently, there is growing awareness of the role of diet and traditional herbal medicines in health promotion. Natural products, such as herbs, teas and other plant-based foods, could offer potential immunomodulatory treatments, and some are reported to have beneficial effects on biomarkers of cellular immunity as well as to have antimicrobial activities [Tan and Vanitha, 2004; Shan et al., 2007;]. There is some evidence that

lingzhi, green tea and bilberry have health-promoting effects through anti-inflammatory, immunomodulatory, anti-oxidant and antimicrobial properties [Watchtel-Galor et al., 2005; Cabrera et al., 2006; Zafra-Stone et al., 2007]. For example, lingzhi has been reported to have some beneficial effect on certain lymphocyte subsets in patients with colorectal cancer [Chen et al., 2006]; bilberry anthocyanins have been reported to have effects in relation to oxidative stress and inflammatory response in animal studies [Zafra-Stone et al., 2007]; green tea has been shown to enhance a certain type of T cell (gamma delta T cell) function in a human controlled trial [Rowe et al., 2007]. However, further study is needed for each of these as some effects were very small and none of these studies specifically measured biomarkers of cellular immunity. In this study the overall aim was to generate valid science-based evidence, adopting a two-pronged approach, in regard to immunomodulation and antimicrobial effects of these three dietary agents. An overview of this two-pronged approach for promotion of health is shown in Figure 7.1.

Figure 7.1

Overview of the Two-pronged Approach Used Here to the Study of Natural Products (i.e. Green Tea, Bilberry and Lingzhi) for Promotion of Health and Healthy Ageing



Therefore, in this study, there were two main areas of interest. One was to investigate the immunomodulatory effect of green tea, bilberry and lingzhi in target groups in three independent controlled human intervention trials. The other was to investigate if green tea, bilberry and lingzhi have direct or indirect antimicrobial effects against isolates of antibiotic resistant microorganisms (i.e. MRSA and VISA).

Recap of Main Findings and Their Implications

This study overall comprised four parts, each with its own specific objectives (as presented in Chapters 3-6). Three parts involved placebo-controlled human intervention trials of immunomodulatory effects. The fourth was an *in vitro* study of antimicrobial effects. The main objectives for each experimental study and the main findings and their implications are summarised below:

1. To investigate changes in selected biomarkers of immune status (lymphocyte subsets) and hsCRP (an inflammatory marker) after supplementation with two types of green tea and to find out if these effects were related to antioxidant status.

   Main findings: There was no statistically significant change in the immune markers measured after 4 weeks' supplementation with green tea in 18 healthy middle-aged subjects. However, a slight increase in the lymphocytes number and CD8+ cell number in response to green tea when compared with water response was seen. In addition, decreases in hsCRP averaged 30% after Loonjin and 23% after Screw-shaped green tea, in comparison to an average increase in hsCRP of 33% after water were observed. Furthermore, there were two potentially interesting, statistically significant, direct correlations of responses seen. One was between the supplementation-related changes in plasma FRAP and the change in CD4+ cell number. The other was between the change in plasma ascorbic acid and the change in % of CD19+ cells. This indicates that increased antioxidant status might help promote the circulating numbers of CD4+ (T helper) cells and CD19+ (B) cells. These cells are among the most affected lymphocytes with ageing [Lazuardi et al., 2005; Haynes and Maue, 2009]. Therefore, preserving or restoring T helper and B cells has important

implications in promoting healthy ageing as they play an important role in fighting infection and immunosurveillance, respectively. Overall, the new data presented here suggest a role for increased antioxidant intake, such as from green tea, in maintenance and restoration of cell-mediated immunity and, possibly, as an anti-inflammatory therapy.

2. To investigate changes in immune markers (white blood cell count and selected lymphocyte subsets) in 20 Type 2 DM subjects taking a bilberry supplement for 4 weeks, and to find out if these effects were related to antioxidant status, and to compare the immune status in Type 2 DM subjects with a group of apparently healthy subjects of similar age and sex.
   Main findings: There was no significant effect seen with 4 weeks' bilberry supplementation in Type 2 DM patients when compared with placebo response. However, there were fairly strong, inverse and statistically significant correlations between the change in monocytes % and the changes in fasting plasma FRAP and changes in plasma ascorbic acid after bilberry treatment. Moreover, direct significant correlations between changes in ascorbic acid and changes in neutrophils % and number after bilberry treatment were found. Type 2 DM subjects (n=20) were found to have lower NK cell % than healthy subjects ($P<0.05$; n=18). Total lymphocyte % and number were significantly higher and T helper (CD4+) cells number was also higher ($P<0.05$) when compared with healthy subjects. The high numbers of lymphocytes and helper T cells seen in Type 2DM patients are consistent with an inflammatory state, and inflammation is associated with increased oxidative stress (and lower antioxidants). The significant direct correlation found between baseline urine 8-oxodG and lymphocytes%, and a significant inverse relationship baseline 'total antioxidants' in plasma (as the FRAP value) and lymphocytes number support this scenario. A possible modulatory role for antioxidants in immune function is suggested by the inverse relationship seen between changes in monocyte % and plasma FRAP and an even stronger inverse relationship was seen with ascorbic acid after bilberry treatment. In addition, diabetic patients have been reported to have defects on

neutrophil chemotactic, phagocytic and microbicidal functions [Alba-Loureiro et al., 2007]. Therefore, it is reasonable to speculate that an antioxidant-rich food such as bilberry might help to restore or maintain certain cells of the immune system.

3. To investigate the effect of radiotherapy (RT) on selected biomarkers of cell-based immune status in a small group (n=15) Nasopharyngeal Carcinoma (NPC) patients; monitor the recovery of immune biomarkers after completion of RT (in a very preliminary human controlled trial); investigate supplementation with lingzhi to see if it can modulate RT-induced changes in immune biomarkers; compare the immune status in NPC patients with non-cancer patients.
   Main findings: RT was shown to have a detrimental effect on cellular immunity as seen by the profound depletion in the absolute numbers of all lymphocytes and subpopulations studied, in particular C19+ cells. Moreover, the impact of RT is of prolonged duration, as after 4 weeks post RT all the immune parameters remained very depressed. It was observed that for the total lymphocyte % and number, the lingzhi group has a less dramatic initial fall when compared to placebo group. Subjects taking lingzhi showed a less dramatic initial fall in the CD8+ cytotoxic T cell number when compared to placebo group. In addition, the CD56+ NK cells in the lingzhi group appeared to be rebounding faster 4 weeks after completion of RT when compared to placebo group. However, results did not reach statistical significance. It was found that NPC patients pre-RT had a significantly lower helper T cells % (CD4+) when compared with non-cancer patients of similar age and sex. This marked and prolonged immunosuppression effect of RT has important implications. Indeed, it has been reported that there is a strong correlation between the status of cellular immunity and survival of patients with head and neck cancers [Verastegui et al., 2003]. In addition, NPC patients have significantly lower helper T cells, which further predispose them to infection. Therefore, it is important to try to modulate the drastic effects RT has on cellular immunity, and as shown by this preliminary study, lingzhi might have some potential effects on counteracting the drastic negative effect of RT.

4. To determine if extracts (hot water) of lingzhi, two types of green tea and bilberry have direct antimicrobial effects on selected microbes (i.e. MRSA, VISA and susceptible *Staphylococcus aureus*) and to determine if these extracts can act synergistically to lower the MIC of oxacillin and vancomycin against MRSA and VISA.

   Main findings: Direct antimicrobial effect was found in bilberry (MIC= 0.6 mg/ml), screw-shaped green tea (MIC= 0.6 mg/ml) and loongjin green tea (MIC= 1.2 mg/ml) against MRSA, MSSA and VISA. However, no direct antimicrobial effect was found in lingzhi even at the highest concentration tested (10 mg/ml). Synergistic effects of lingzhi, bilberry and green tea were found when these were used in combination with oxacillin and vancomycin against VISA and MRSA. To date, there is no antibiotic class that is uniformly effective against resistant *Staphylococcus aureus,* and methicillin resistance has recently become a community problem, not just a hospital acquired phenomenom [Enright, 2003]. This current study provides some evidence that natural products (i.e. green tea, bilberry and lingzhi) can increase the potency of antibiotics, allowing lower doses to be used and thus avoiding undesirable side effects and enhancing efficacy. In addition, bilberry at the highest concentration tested (i.e. 0.06%) together with vancomycin greatly increased the susceptibility of the standard MRSA and VISA to vancomycin and decreased the MICs to a therapeutically useful level. These results have important implications for the treatment of resistant antibacterial infections.

Summary of Overall Conclusions for the Two Pronged Approach

a) In regard to effects of selected natural products on immune function.

Natural products and herbs have gained popularity for the promotion of health [Tan and Vanitha, 2004]. However, there are not many clinical studies available in the literature which confirms the claimed beneficial effects of natural products or herbs with science-based evidence. In this study, the effect of three antioxidant rich natural products (green tea, bilberry and lingzhi) on immunomodulation and antimicrobial were investigated in three independent controlled human intervention trials. Results suggest that green tea and bilberry might have immunomodulating activity in relation to antioxidant status as observed by the strong correlations in changes in markers of antioxidant status and certain lymphocytes subsets after treatment with the supplementation. Moreover, lingzhi showed some possible indications of effect in counteracting the drastic fall of certain lymphocytes subsets in NPC patients undergoing RT when compared with placebo response. Thus, this current study provides some rational basis for the use of these natural products in restoring or maintaining certain parameters of cellular immunity and further study is warranted. Further purification of active components may be required for further investigating clinical application.

b) In regard to antimicrobial effect of selected natural products

In recent years, there have been concerns about the greater frequencies of antibiotic resistant bacteria, in particular MRSA and the lack of available therapeutic options to treat it [Woodford and Livermore, 2009]. In addition, humans can be colonized with antibiotic resistant bacteria which may cause infections and lead to greater frequencies of treatment failure [Boost et al., 2007]. This study shows preliminary data supportive of the potential using of bilberry and green tea alone as an antibacterial agent. Moreover, this study supports the possible use of lingzhi, bilberry and green tea in combination with oxacillin and vancomycin to increase their potency. This type of finding could further boost the use of extracts and natural products either alone, combined or together with antibiotics to treat resistant infections.

Summary of Strengths and Limitations of Study

The strengths of the study in each clinical trial included the design (placebo controlled), a relatively homogenous group (all ethnic Chinese), a dietary-relevant dose of the natural product was used and a wide range of biomarkers were measured. However for all the clinical trials the limited sample size might have contributed to the lack of statistical significance observed. Also, there was a marked variability in the immune variables measured, making it difficult to detect small changes in these endpoints. Moreover, the intervention trials were of short period of time (only four weeks for bilberry and green tea study and seven weeks for the lingzhi study), so longer term effects could not be evaluated. Functions of immune cells and levels of inflammatory cytokines were not investigated as these tests are resource intensive, and so a wider range of effects on immune response could not be studied.

For the green tea study, it remains to be seen whether these results can be generalized to people who already show a marked immune decline. There was no measurement of biomarkers of humoral immunity function, which could indicate wider changes in immune status, so it is not clear whether there were other effects on other immune components. We did not measure tea polyphenols in plasma or urine and so the amount absorbed is unknown.

For the bilberry study, plasma anthocyanins were not measured, and thus their bioavailability or plasma response could not be evaluated. The supplement was quite low dose and was for only 4 weeks. A higher dose or a longer duration of supplementation may have been useful.

For the lingzhi study, NPC patients receiving RT usually encounter difficulties in eating and swallowing. Thus, it cannot be ignored that other factors such as poor nutrition, zinc deficiency, liver insufficiency and the cancer itself may contribute to low lymphocytes count and impaired cellular immunity. In addition, the recovery of cellular immunity may also depend on age and nutritional factors.

Suggestions for Further Studies

As stated above, function of immune parameters were not measured so wider effects were not evaluated. A possibly rewarding area of future research would be to study immune function and inflammatory cytokines together with cellular immune parameters in further clinical trials.

Regarding the microbiology part of this study, it is suggested that the obtained preliminary data should be followed by the identification of active compounds and determination of the mechanisms of inhibition against MRSA. In addition, positive or negative interactions with other types of antibiotics, as well as potential clinical applications of these results could be further explored.

Concluding Remarks

This is the first study to date which used a two-pronged approach investigating the effect of green tea, bilberry and lingzhi on immunomodulation and antimicrobial activity. Interesting results in regard to antimicrobial effects of these natural products were obtained providing useful insights to the development of potentially useful new pharmacological agents. Some evidence of immunomodulation in regard to cellular immunity was also shown by the selected agents. The new data presented here will support further studies in this important area, and have powerful implications for the promotion of healthy ageing.

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Appendix I

Bilberry capsules information

桂林莱茵生物科技股份有限公司
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Certificate of Analysis

Product Name:	**European Bilberry P.E.**	**Manufacture Date:**	**2007-JAN-08**
Latin Name:	**Vaccinium Myrtillus**	**Testing Date:**	**2007-JAN-08**
Batch Number:	**BIL01-070101**	**Expire Date:**	**2009-JAN-07**
Quantity:	**200kgs**	**Shelf Life:**	**2 Years**

ITEM	SPECIFICATION	TEST RESULT
PHYSICAL TESTS:		
DESCRIPTION:		
APPEARANCE	DEEP PURPLE POWDER	COMPLIES
ODOR	CHARACTERISTIC	COMPLIES
TASTE	CHARACTERISTIC	COMPLIES
COUNTRY OF ORIGIN	EUROPE	COMPLIES
PARTICLE SIZE	THROUGH 80 MESH	COMPLIES
PLANT PART USED	FRUIT	COMPLIES
SOLUBILITY	SOLUBLE (IN H_2O), SOLUBLE (IN C_2H_5OH)	COMPLIES
CHEMICAL TESTS:		
ANTHOCYANIDINS(UV)	≥25%	25.60%
EXTRACT SOLVENTS	ETHANOL & WATER	COMPLIES
SOLVENTS RESIDUE	<0.05%	COMPLIES
CARRIERS USED	NONE	COMPLIES
LOSS ON DRYING	< 5.0%	3.20%
ASH	< 5.0%	1.09%
HEAVY METALS	< 10PPM	COMPLIES
ARSENIC (As)	< 0.5PPM	COMPLIES
LEAD (Pb)	< 0.5PPM	COMPLIES
CADMIUM (Cd)	< 0.05PPM	COMPLIES
MERCURY (Hg)	NOT DETECTED	COMPLIES
PESTICIDE RESIDUE (GC)		
666	<0.1 PPM	COMPLIES
DDT	<0.1 PPM	COMPLIES
ACEPHATE	<0.1 PPM	COMPLIES
METHAMIDOPHOS	<0.1 PPM	COMPLIES
PARATHION	<0.1 PPM	COMPLIES
PCNB	<10 PPB	COMPLIES
MICROBIOLOGICAL TEST		
TOTAL PLATE COUNT	<1000CFU/G	COMPLIES
YEAST AND MOLD	<100 CFU/G	COMPLIES
SALMONELLA	NEGATIVE	COMPLIES
E.COLI	NEGATIVE	COMPLIES
STAPHYLOCOCCUS	NEGATIVE	COMPLIES
AFLATOXINS	<0.2 PPB	COMPLIES
EXTRACT METHOD	ETHANOL & WATER EXTRACTION AND SPRAY DRY.	
STORAGE	STORE IN COOL AND DRY PLACE. KEEP AWAY FROM STRONG LIGHT AND HEAT.	
PACKING	20KG/DRUM I.D.42CM × H52CM	

QC_____　　QA_____

Analysis Method of Anthocyanidins in European Bilberry Extract (UV)

1 Equipment

Electronic balance

Pipet, Volumetric flasks

Ultrasonic bath

Spectrophotometer

1cm quartz cell

2 Process

To about 10mg(W), accurately weighed, add 20ml of 2% hydrochloric acid Methanol solution and ultrasonic bath until sample has dissolved, Cool to room temperature, then diluting to volume with 2% hydrochloric acid Methanol solution to 50ml (L1) exactly. Then take 5ml(L2) of this solution , dilute with 2% hydrochloric acid Methanol solution to 50ml exactly.

3 Assay

Measure the extinction (A) of the solution at the maximum at about 540nm in 1cm cell, using 2% hydrochloric acid Methanol solution as the blank.

4 Calculation

$$\text{Content of ANTHOCYANIDINS (\%)} = \frac{A \times 50ml \times 0.5}{M\,(1 - m)\ 5ml \times 1020} \times 100\%$$

1020: coefficient of Delphinidin

M: Weight of sample (g)

m: content of moisture in sample

A: Absorbance of the sample solution

Appendix II

Lingzhi capsules information

SGS

Test Report No. 2014898/BS Date : May 19 2006 Page 1 of 5

POLYU MODERN TCM RESEARCH INSTITUTE LTD.
ROOM 601A 6/F
EMPIRE CENTRE
60 MODY ROAD
T.S.T., KOWLOON, HONG KONG

Job No. 工作編號 : 2047637

Report on the submitted sample identified by the client as Deerhorn Lingzhi
對據稱為"鹿角靈芝"之樣品之分析報告

Product Description 樣品名稱	:	Deerhorn Lingzhi 鹿角靈芝
Quantity Received 收到樣品數量	:	10 bottles X 21 g/bottle (60's/bottle)
Sample Appearance 樣品外觀	:	Capsule with grey cap / white body containing brown powder
Lot No. / Batch No. 批號	:	E06006
SGS Sample No. 樣品編號	:	2047637-101
Expiry Date 使用期限	:	04-2008
Sample Receiving Condition	:	In sealed plastic bottle under ambient condition
樣品收到時狀態	:	室溫下存放於密封塑膠樽中
Manufacturer / Supplier 製造商 / 供應商	:	Pharmtech (HK) Ltd.
Region of Origin 來源地	:	Hong Kong 香港
Region of Destination 目的地	:	Hong Kong 香港
Sample Receiving Date 樣品收到日期	:	May 10 2006 2006年5月10日
Testing Period 化驗日期	:	May 10 – 18 2006 2006年5月10日至18日

Test Requested, Test Method and Test Results 測試項目、分析方法及分析結果
Please refer to the following page(s). 請參考續頁。

****

Signed for and on behalf of
SGS Hong Kong Ltd.
香港通用公証行代表簽名

CHAN SUK-HANG, JOAN
TECHNICAL MANAGER - HEALTHCARE AND PHARMACEUTICAL SERVICES
陳淑恒 / 保健及藥劑品組技術經理

H12690681

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Test Requested 測試項目

1. To perform Arsenic, Lead, Mercury and Cadmium analyses on the submitted sample.
樣品中砷、鉛、汞及鎘之含量測定。

2. To perform Aerobic Bacteria Count, Mould & Yeast Count and Escherichia coli analyses on the submitted sample.
樣品中總細菌數，霉菌計數，酵母菌計數及大腸桿菌之分析。

3. To perform Pesticides Residue - Aldrin & Dieldrin, Chlordane (sum of cis-, trans- & oxychlordane), DDT (sum of p,p'-DDT, o,p'-DDT, p,p'-DDE & p,p'-TDE), Endrin, Heptachlor (Heptachlor & Heptachlorepoxide), Hexachlorobenzene, Hexachlorocyclohexane isomers (other than gamma), Lindane and Quintozene (sum of quinotzene, pentachloroaniline and methyl pentachlorophenyl sulphide) analyses on the submitted sample.
樣品中殘餘殺蟲劑 - 艾氏劑及狄氏劑、氯丹(順式-，反式及氧化氯丹)、滴滴涕及衍生物(4,4'-滴滴涕，2,4-滴滴涕，4,4'-滴滴伊及4,4'-滴滴滴)、異狄氏劑、七氯(七氯及環氧七氯)、六氯苯、六六六異構體(丙型除外)、林丹、五氯硝基苯(五氯硝基苯，五氯苯胺及甲基五氯硫基苯)之分析。

Test Method 分析方法

1. Heavy Metal and Toxic Elements 重金屬及有毒元素
The analyses were performed by in house method TCM HM1-001 employing the digestion of sample in acid mixture following with Inductively Coupled Argon Plasma Spectrometry measurement.
根據內部化驗方法 TCM HM1-001, 將樣品以酸分解再以電感耦合等離子體原子發射光譜法作檢定。

2. Microbial Examination 微生物檢查
The analyses were performed with reference to The Pharmacopoeia of the People's Republic of China 2000, Volume I Appendix XIII C.
測試乃參考中華人民共和國藥典，2000年版，第一冊，附錄XIII C。

3. Pesticides Residue 殘餘殺蟲劑
The analyses were performed by in house method TCM PEST1-001 employing solvent extraction of sample following with Gas Chromatography-Mass Spectrometry measurement.
根據內部化驗方法 TCM PEST1-001, 將樣品經溶劑萃取後再以氣相色譜-質譜儀法作檢定。

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Member of the SGS Group (SGS SA)

As_2O_3 197.84

SGS

Test Report No. 2014898/BS Date : May 19 2006

Test Results 分析結果

Parameter 測試項目		Deerhorn Lingzhi 鹿角靈芝 SGS Sample No. 樣品編號 2047637-101	
(1) Heavy Metal and Toxic Elements 重金屬及有毒元素			
Arsenic (As) 砷	1.4 ppm	2 mg/kg (ppm) 毫克/千克(百萬分率)	1 µg/day 微克/日*
Cadmium (Cd) 鎘	0.1 ppm	<0.04 mg/kg (ppm) 毫克/千克(百萬分率)	<0.03 µg/dose 微克/劑*
Lead (Pb) 鉛	6 ppm	0.4 mg/kg (ppm) 毫克/千克(百萬分率)	0.3 µg/day 微克/日*
Mercury (Hg) 汞	0.5 ppm	<0.1 mg/kg (ppm) 毫克/千克(百萬分率)	<0.08 µg/day 微克/日*

* The data was calculated based on the maximum dosage given by the client: 0.35 g /unit, 2 unit/dose, 1 dose/day 數據結果是根據客戶提供之最高服用量：每日1次，每次2單位，每單位0.35克計算。

(2) Microbiological examination 微生物檢查		
Aerobic Bacteria Count 菌落總數	<10 colony /g	<10 菌數 / 克
Mould & Yeast Count 霉菌及酵母菌計數		
- Mould Count 霉菌計數	<10 colony /g	<10 菌數 / 克
- Yeast Count 酵母菌計數	<10 colony /g	<10 菌數 / 克
Escherichia coli 大腸桿菌	Absent/ g	未檢出 / 克

(3) Pesticides Residue 殘餘殺蟲劑	
Aldrin & Dieldrin 艾氏劑及狄氏劑	< 0.02 mg/kg (ppm) 毫克/千克(百萬分率)
Chlordane (sum of cis-, trans- & oxychlordane) 氯丹(順式-，反式及氧化氯丹)	< 0.03 mg/kg (ppm) 毫克/千克(百萬分率)
DDT (sum of p,p'-DDT, o,p'-DDT, p,p'-DDE & p,p'-TDE) 滴滴涕及衍生物(4,4'-滴滴涕, 2,4-滴滴涕, 4,4'-滴滴伊及4,4'-滴滴滴)	< 0.10 mg/kg (ppm) 毫克/千克(百萬分率)
Endrin 異狄氏劑	< 0.01 mg/kg (ppm) 毫克/千克(百萬分率)
Heptachlor (Heptachlor & Heptachlorepoxide) 七氯(七氯及環氧七氯)	< 0.02 mg/kg (ppm) 毫克/千克(百萬分率)
Hexachlorobenzene 六氯苯	< 0.02 mg/kg (ppm) 毫克/千克(百萬分率)
Hexachlorocyclohexane isomers (other than gamma) 六六六異構體(丙型除外)	< 0.21 mg/kg (ppm) 毫克/千克(百萬分率)
Lindane 林丹	< 0.06 mg/kg (ppm) 毫克/千克(百萬分率)
Quintozene (sum of quinotzene, pentachloroaniline and methyl pentachlorophenyl sulphide) 五氯硝基苯(五氯硝基苯,五氯苯胺及甲基五氯硫基苯)	< 0.08 mg/kg (ppm) 毫克/千克(百萬分率)

Note 備注：

1. Results reported on the submitted sample on an as received basis. For capsule samples, heavy metals, toxic elements and pesticides residue analyses results are reported on powder inside the submitted sample on an as received basis. 分析結果乃根據所呈交之樣品於收到時之狀態計算及進行。然而，膠囊產品之重金屬及有毒元素和殘餘殺蟲劑分析結果乃根據所呈交之樣品膠囊內之粉末於收到時之狀態計算及進行。

2. < = less than 少於

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Test Results (Cont'd) 分析結果(續)

Note 備注 :

3. Chinese Medicine Council of Hong Kong, " Proprietary Chinese Medicine " Registration Application Booklet (Aug 2004 version) - Heavy Metals and Toxic Elements Content Limit Requirement 香港中醫藥管理委員會" 中成藥 "註冊申請手冊(2004年8月版)-重金屬及有毒元素含量限量要求

Parameter 測試項目	Upper Limit 上限
Arsenic (As) 砷	1500.00 µg/day 微克 / 日
Lead (Pb) 鉛	179.00 µg/day 微克 / 日
Mercury (Hg) 汞	36.00 µg/day 微克 / 日
Cadmium (Cd) 鎘	3500.00 µg/dose 微克 / 劑

4. Chinese Medicine Council of Hong Kong, " Proprietary Chinese Medicine " Registration Application Booklet (Aug 2004 version) - Microbial Limit Requirement
香港中醫藥管理委員會" 中成藥 "註冊申請手冊(2004年8月版)-微生物限度要求

Parameter 測試項目	Upper Limit 上限	
For Oral use – without crude drug powder (Medicinal granules / tablets / capsules) 口服用 - 不含原藥材粉 (顆粒劑 / 片劑 /膠囊劑)		
Aerobic Bacteria Count 菌落總數	1000 colony/ g	1000 菌數/克
Mould & Yeast Count 霉菌及酵母菌計數@	100 colony/ g	100 菌數 /克
Escherichia coli 大腸桿菌	Absent in 1 g	未檢出 / 克
For Oral use – with crude drug powder (Medicinal granules / tablets / capsules) 口服用 - 含原藥材粉(顆粒劑 / 片劑 /膠囊劑)		
Aerobic Bacteria Count 菌落總數	10000 colony / g	10000 菌數/克
Mould & Yeast Count 霉菌及酵母菌計數@	100 colony / g	100 菌數 /克
Escherichia coli 大腸桿菌	Absent in 1 g	未檢出 / 克

Other requirements 其他要求:

1. @ - Mould & Yeast Count : For dose in solid form and liquid form not containing sugar, royal jelly, honey or semi-solid form, mould count should be tested. For dose in liquid form containing sugar, royal jelly, honey or semi-solid form, both mould count and yeast count should be tested. 霉菌及酵母菌 : 固體製劑及不含糖、王漿、蜂蜜的液體製劑或半固體製劑檢查霉菌 ; 含糖、王漿、蜂蜜的液體製劑或半固體製劑檢查霉菌和酵母菌。
2. For oral preparations containing animal-origins raw ingredients, Salmonella should not be detected. For oral preparations containing animal horn, royal jelly, bee honey and Colla Corii Asini, test for Salmonella may be exempted
含有動物類原藥材的口服製劑，不得檢出沙門氏菌; 含動物角、王漿、蜂蜜、阿膠的口服製劑，則毋須進行有關檢定。

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Test Results (Cont'd) 分析結果(續)

Note 備注 :

5. Chinese Medicine Council of Hong Kong, " Proprietary Chinese Medicine " Registration Application Booklet (Aug 2004 version) – Pesticides Residues Content Limit Requirement 香港中醫藥管理委員會" 中成藥 "註冊申請手冊(2004年8月版)-殘餘殺蟲劑含量限量要求

Parameter 測試項目	Upper Limit 上限
Aldrin & Dieldrin 艾氏劑及狄氏劑	0.05 ppm 百萬分率
Chlordane (sum of cis-, trans- & oxychlordane) 氯丹(順式-, 反式及氧化氯丹)	0.05 ppm 百萬分率
DDT (sum of p,p'-DDT, o,p'-DDT, p,p'-DDE & p,p'-TDE) 滴滴涕及衍生物(4,4'-滴滴涕, 2,4-滴滴涕, 4,4'-滴滴伊及4,4'-滴滴滴)	1.0 ppm 百萬分率
Endrin 異狄氏劑	0.05 ppm 百萬分率
Heptachlor (Heptachlor & Heptachlorepoxide) 七氯(七氯及環氧七氯)	0.05 ppm 百萬分率
Hexachlorobenzene 六氯苯	0.1 ppm 百萬分率
Hexachlorocyclohexane isomers (other than gamma) 六六六異構體 (丙型除外)	0.3 ppm 百萬分率
Lindane 林丹	0.6 ppm 百萬分率
Quintozene (sum of quinotzene, pentachloroaniline and methyl pentachlorophenyl sulphide) 五氯硝基苯(五氯硝基苯,五氯苯胺及甲基五氯硫基苯)	1.0 ppm 百萬分率

6. The English version of this report shall prevail in case of discrepancy between the English version and the Chinese version.
本化驗報告之中英文版本如有岐異,概以英文版為準。

*** End of Report 報告完 ***

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Appendix III

SGE template

SGE™ Template

Spiral Biotech

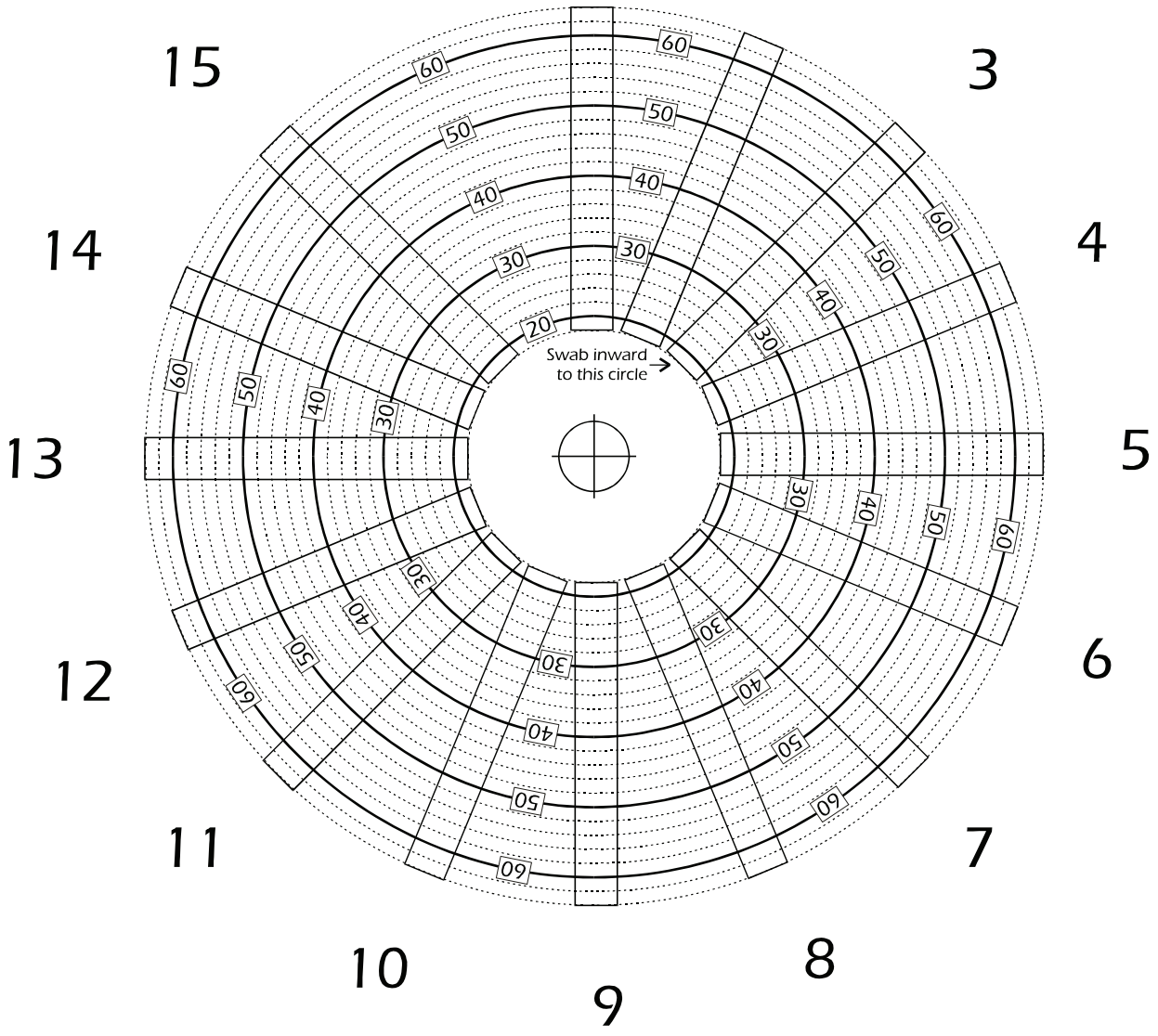

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Plate ID: _____

Antimicrobial 1: _____

Stock Concentration: _____

Deposition Mode:

❑Exponential ❑Proportional ❑Uniform

Antimicrobial 1: _____

Stock Concentration: _____

Deposition Mode:

❑Exponential ❑Proportional ❑Uniform

Agar Height: _____