CHAPTER I

INTRODUCTION

1.1 Statement of the problem

Mycotoxins are secondary metabolites of fungi that are commonly found in animal feed and foodstuffs. These metabolites can cause a variety of ill effects in humans, from allergic responses to immunosuppression and cancer (Pitt, 2000). The major mycotoxins found in Thailand include aflatoxins, trichothecenes (deoxynivalenol) and fumonisins. Aflatoxin B_1 (AFB_1) is the most potent member of the aflatoxin family; it induces liver cancer in every animal species tested so far and has been linked to liver cancer in humans. It is also an immunomodulating agent that acts primarily on cell-mediated immunity and phagocytic cell function (Bondy and Pestka, 2000). Deoxynivalenol (DON) was responsible for a large-scale human toxicosis in India in 1988, and human toxicosis has also been reported from China, Japan and Korea (Beardall and Miller, 1994). It can both suppress and stimulate immune function. Fumonisin B_1 (FB_1), the most abundant fumonisin in culture and naturally occurring in corn, has been shown to cause leukoencephalomalacia in horses (Marasas *et al.*, 1988), to be a liver and kidney carcinogen in rodents, and to inhibit ceramide synthase. In addition, consumption of corn contaminated with *F. moniliforme* has also been associated with esophageal cancer in the Transkei area of South Africa (Rheeder *et al.*, 1992) and China (Chu and Li, 1994). Fumonisin toxicity has been characterized relatively recently in comparison to aflatoxin, and fumonisin-induced immunotoxicity is an area of active research. The effects of these mycotoxins on the immune responses have been investigated; however, there is a major gap in our knowledge concerning on the immunotoxicity of fungal toxins and an absence of data on potential immunomodulation by commonly occurring mixtures of mycotoxins. Typically, several mycotoxins may be present in a contaminated foodstuff or animal feed. There is strong evidence for the co-occurrence of the two carcinogenic mycotoxins, AFB_1 and FB_1. Moreover, both Fumonisins and DON are produced from the *Fusarium* fungi. Therefore there may be co-contamination of the latter two mycotoxins. At present, little is known about the interaction of these mycotoxin mixtures with regard to their toxic and carcinogenic properties. Thus, this study aims to investigate the combined effects of AFB_1, DON and FB_1 on the immune system.

The realization that aflatoxins, DON and fumonisins are immunosuppressive agents has broad implications for the ability of human populations to resist disease. According to the concept of immunosurveillance, immunosuppressive activity of mycotoxins might be one mechanism of cancer susceptibility. Substances that have immunostimulating activity might reduce the toxicity of mycotoxins and subsequent risk of development of cancer. Medicinal plants have been used as foods and for medicinal purposes for centuries. Research interest has focused on various medicinal plants that have hypolipidemic, anti-platelet, anti-inflammatory, anti-tumor or immunomodulating properties and which may serve as useful adjuncts in helping reduce the risk of various diseases, including cardiovascular disease and cancer. Because the scientific data on immunomodulating activity of Thai medicinal plants is still lacking, this study aims to find Thai medicinal plants that could bolster the immune system. Thai medicinal plants that reportedly have anti-inflammatory, anti-carcinogenic or anti-oxidative activities were screened for immunomodulating activity. It is hypothesized that the immune stimulating activity of Thai medicinal plants might protect or reduce the immunotoxicity of these mycotoxins.

1.2 Literature review

1.2.1 The immune system

The immune system is composed of a variety of different cell types and proteins. The key process carried out by the immune system is recognition. The system must recognize and differentiate between self and non-self and eliminate potential pathogens such as viruses, bacteria, fungi, protozoa and multi-cellular parasites (Kuby, 1977). An effective immune response involves primary recognition of non-self and a response to eliminate the foreign agent (Huston, 1997). The immune system has traditionally been divided into innate and adaptive components, each having a different function and role (Fearon and Lockley, 1996). Although both responses have cellular and humoral components, two important features distinguish the innate from the adaptive immune response (Kuby, 1997; Huston, 1997). The first is the ability of the latter to recognize specific antigens (specificity) and the second is to recognize an antigen upon subsequent exposure. These features of the adaptive immune response permit responses which are more rapid, greater in magnitude, more specific and which persist for longer when exposure to the same antigen re-occurs (Kuby, 1997).

1.2.1.1 Innate immunity

Innate immunity is the first line of defense against attacking organisms. Innate immunity consists of cellular and biochemical defense mechanisms that are in place even before infection and poised to respond rapidly to infections. These mechanisms react only to microbes and not to noninfectious substances, and they respond in essentially the same way to repeated infections. The principal components of innate immunity are shown in Figure 1.1 and Table 1.1. The first component is physical and chemical barriers, such as epithelia and antimicrobial substances produced at epithelia surfaces (Abbas and Lichtman, 2003). Moreover, the range of humoral factors such as a C reactive protein, complement and cytokines, which have antibacterial and antiviral activities, are also important (Carroll, 1998; Male and Roitt, 1998). The innate immune response also has a strong cellular component, which involves the activity of mononuclear phagocytes (i.e. macrophages), granulocytes (i.e. neutrophils, basophils and eosinophils) and natural killer (NK) cells (Kuby, 1997). The main role of mononuclear phagocytes and neutrophils is to recognize, internalize and kill microbes (Kuby, 1997). Killing is affected via the release of anti-microbial and cytotoxic substances such as reactive oxygen intermediates (superoxide anion and hydrogen peroxide) and reactive nitrogen intermediates (nitric oxide and nitrogen dioxide) (Bogdan *et al.*, 2000). Eosinophils are mainly involved in protection against multi-cellular organisms (e.g., parasites), which cannot be phagocytosed, through the release of cytotoxic factors including reactive oxygen intermediates (Costa *et al.*, 1997). Finally, basophils and mast cells stimulate allergic reactions through the release of mediators such as histamine (Costa *et al.*, 1997; Galli *et al.*, 1999).

1.2.1.2 Adaptive immunity

Adaptive immunity develops as a response to infection and adapts to the infections. Adaptive immune responses are stimulated by exposure to infectious agents and increase in magnitude and defensive capabilities with each successive exposure to a particular microbe. The characteristics of adaptive immunity are exquisite specificity for distinct molecules and an ability to remember and respond more vigorously to repeated exposures to the same microbe (Abbas and Lichtman, 2003). The components of adaptive immunity are lymphocytes and their products (Figure 1.1 and Table 1.1). The main distinction between the innate and the adaptive immune systems lies in the mechanisms and receptors used for the immune recognition (Table 1.1).

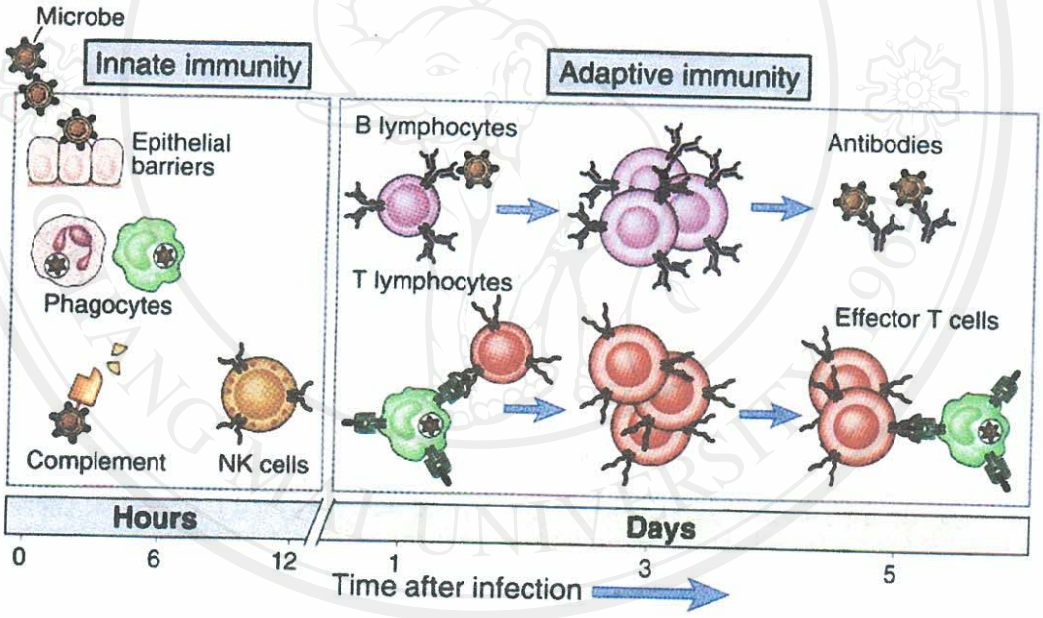
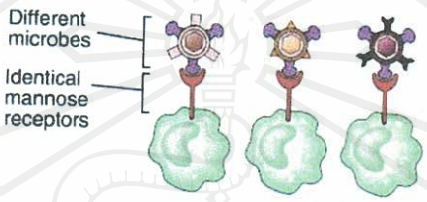
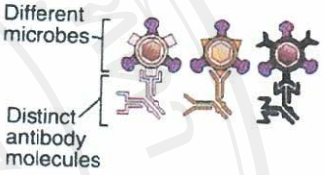
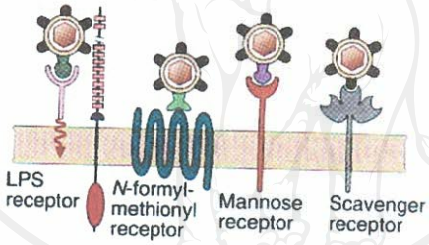
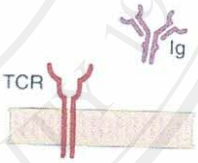


Figure 1.1 Innate and adaptive immunity. The mechanisms of innate immunity provide the initial defense against infections. Adaptive immune responses develop later and consist of activation of lymphocytes (Abbas and Lichtman, 2003).

Table 1.1 Features of innate and adaptive immunity (Adapted from Abbas and Lichtman, 2003).

	Innate immunity	Adaptive immunity
Characteristics		
Specificity	For structures shared by groups of related microbes 	For antigens of microbes and for nonmicrobial antigens 
Diversity	Limited; germline-encoded 	Very large; receptors are produced by somatic recombination of gene segments 
Distribution of receptors	Nonclonal: identical receptors on all cells of the same lineage	Clonal: clones of lymphocytes with distinct specificities express different receptors
Memory	None	Yes
Nonreactivity to self	Yes	Yes
Components		
Physical and chemical barriers	Skin, mucosal epithelia; antimicrobial chemicals	Lymphocytes in epithelia; antibodies secreted at epithelial surfaces
Blood proteins	Complement	Antibodies
Cells	Phagocytes (macrophages, neutrophils), natural killer cells	Lymphocytes

There are two types of adaptive immune responses, called humoral immunity and cell-mediated immunity (Figure 1.2). When a pathogen invades the body, lymphocytes of the humoral immune system (B cells) secrete antibodies that can bind to the pathogen, signaling its degradation by macrophages and other cells. The lymphocytes of the cellular system (T cells) carry out two major types of functions. Cytotoxic T lymphocytes (CTLs) develop the ability to directly recognize and kill cells infected by the pathogen. Helper T cells (Th cells) independently recognize the pathogen and secrete protein factors (lymphokines) that stimulate growth and responsiveness of B cells, T cells, and macrophages, thus greatly strengthening the power of the immune response.

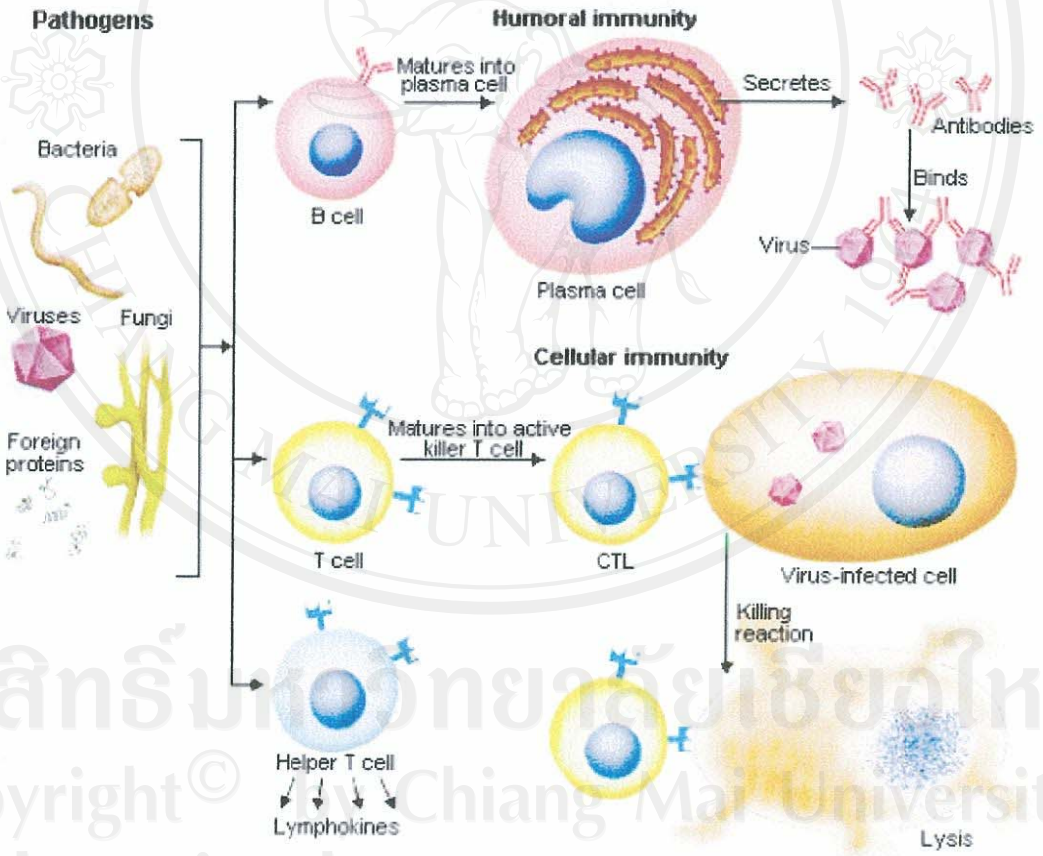


Figure 1.2 Types of adaptive immunity. In humoral immunity, B lymphocytes secrete antibodies that prevent infections by and eliminate extracellular microbes. In cell-mediated immunity, T lymphocytes either activate macrophages to kill phagocytosed microbes or cytolytic T lymphocytes directly destroy infected cells (Lodish *et al.*, 1995).

1.2.1.2.1 Humoral immunity

Humoral immunity is mediated by molecules in the blood and mucosal secretions, called antibodies, that are produced by cells called B lymphocytes (Abbas and Lichtman, 2003). Antibodies recognize microbial antigens, neutralize the infectivity of the microbes, and target microbes for elimination by various effector mechanisms (Figure 1.3). Antibodies, or immunoglobulins (Ig), are differentiated into different classes and subclasses, namely IgG (IgG1-IgG4), IgA (IgA1 and IgA2), IgM, IgE, and IgD according to the type of variable heavy polypeptide chain (Huston, 1997; Turner, 1998). The Ig molecule has two functional parts: the Fab portion that binds to only a specific antigen and Fc portion which binds to the Fc receptor on the surface of phagocytes or to one of the complement molecules thus mediating their effector functions (Male and Roitt, 1998).

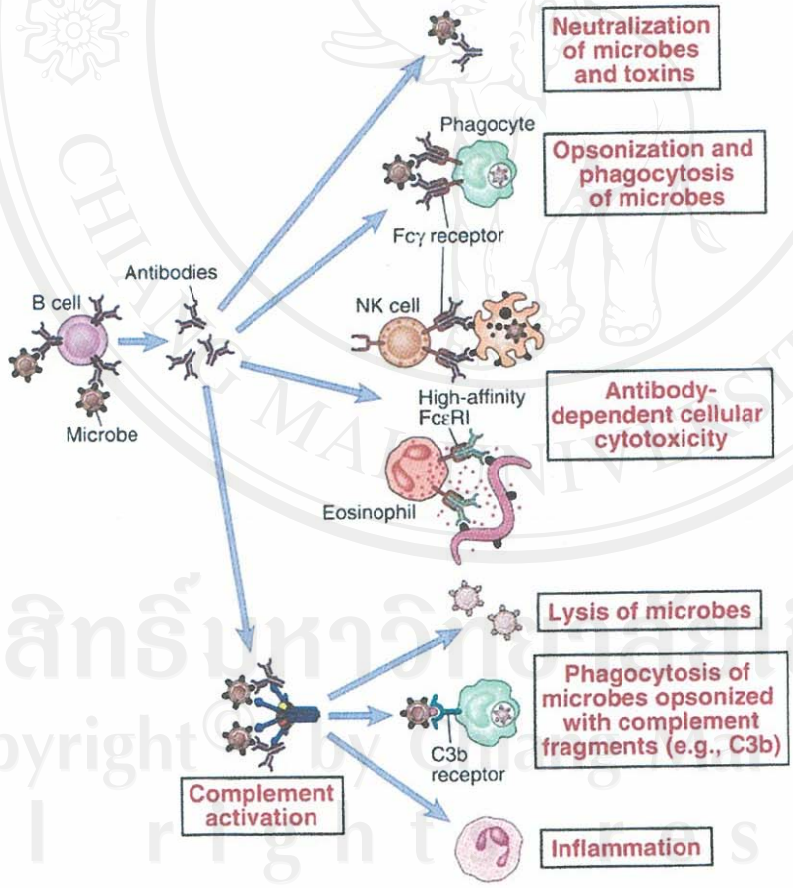


Figure 1.3 Effector functions of antibodies. Antibodies against microbes neutralize these agents, opsonize them for phagocytosis and antibody-dependent cellular cytotoxicity, and activate the complement system (Abbas and Lichtman, 2003).

The activation of B cells is initiated by specific recognition of antigens by the surface Ig receptors of the cells. In a primary immune response, naïve B cells are stimulated by antigen, become activated, and differentiate into antibody-secreting cells that produce antibodies specific for the eliciting antigen. Some of the antibody-secreting plasma cells survive in the bone marrow and continue to produce antibodies for long periods. Long-lived memory B cells are also generated during the primary response. A secondary immune response is elicited when the same antigen stimulates these memory B cells, leading to more rapid proliferation and differentiation and production of greater quantities of specific antibody than are produced in the primary response (Figure 1.4).

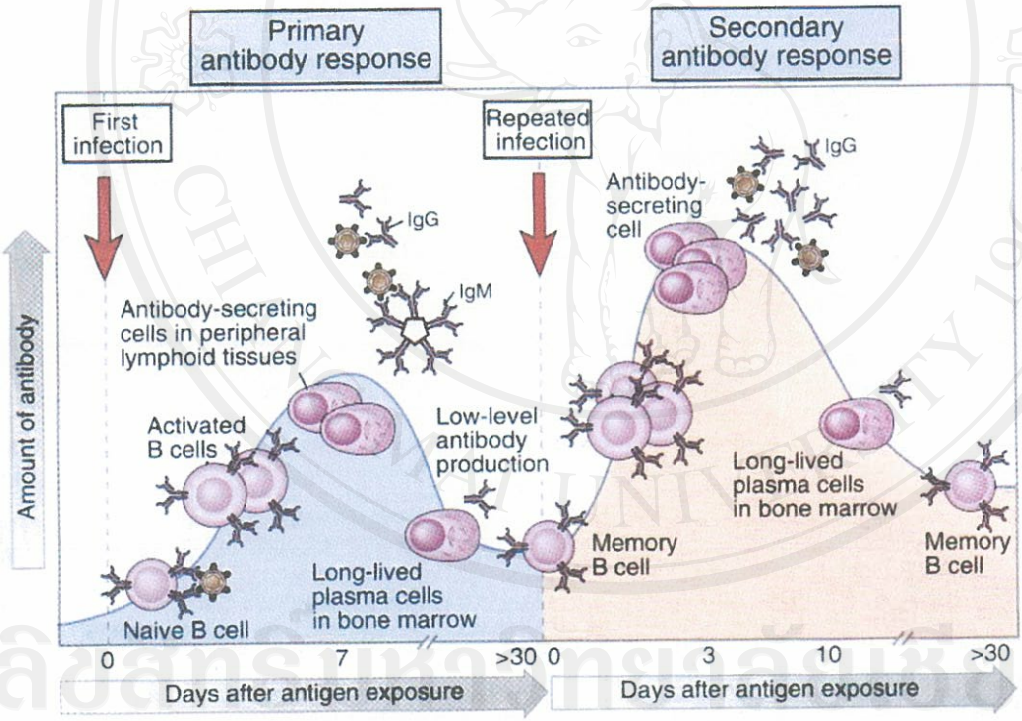


Figure1.4 Kinetics of primary and secondary humoral immune responses (Abbas and Lichtman, 2003).

1.2.1.2.2 Cell-mediated immunity

Cell-mediated immunity (CMI) is the effector function of T lymphocytes, and it serves as the defense mechanism against microbes that survive within phagocytes or infect nonphagocytic cells. The role of T lymphocytes is to directly attack foreign antigens such as viruses, fungi, or transplanted tissues, and to act as regulators of the immune system. The following discussion will emphasize the T_H1 and T_H2 system. The principal function of T_H1 cells is to eliminate intracellular microbes and this can occur in two ways (Abbas *et al.*, 1996). Firstly, the phagocytic and killing ability of macrophages against microbes is promoted via the action of IFN-γ and TNF produced by T_H1 cells (Figure 1.5). Secondly, $CD8^+$ cells are stimulated by IL-2 and IFN-γ and differentiated into active cytotoxic T cells (T_C), which recognize foreign antigens in association with class I MHC expressed on the surface of infected cells. The principal function of T_H2 cells is to enhance the production of IgM and IgG1 in mice or its analogue, IgG4 in humans (Figure 1.6). T_H2 cells also stimulate IgE production by B cells and can activate eosinophils in allergic and helminthic reactions (Romagnani, 1994). The former action is effected via IL-4 secreted by T_H2 cells, but the latter action is effected via IL-5. T_H2 cells have also been proposed to act as regulators of T_H1 functions because several T_H2 cytokines, such as IL-4 and IL-13, have anti-inflammatory actions thus limiting the injurious consequences of T_H1 mediated immunity (Abbas *et al.*, 1996). Macrophages also contribute to the specific immune response by processing and presenting antigens in association with class II MHC molecules, to T cells (Abbas and Lichtman, 2003).

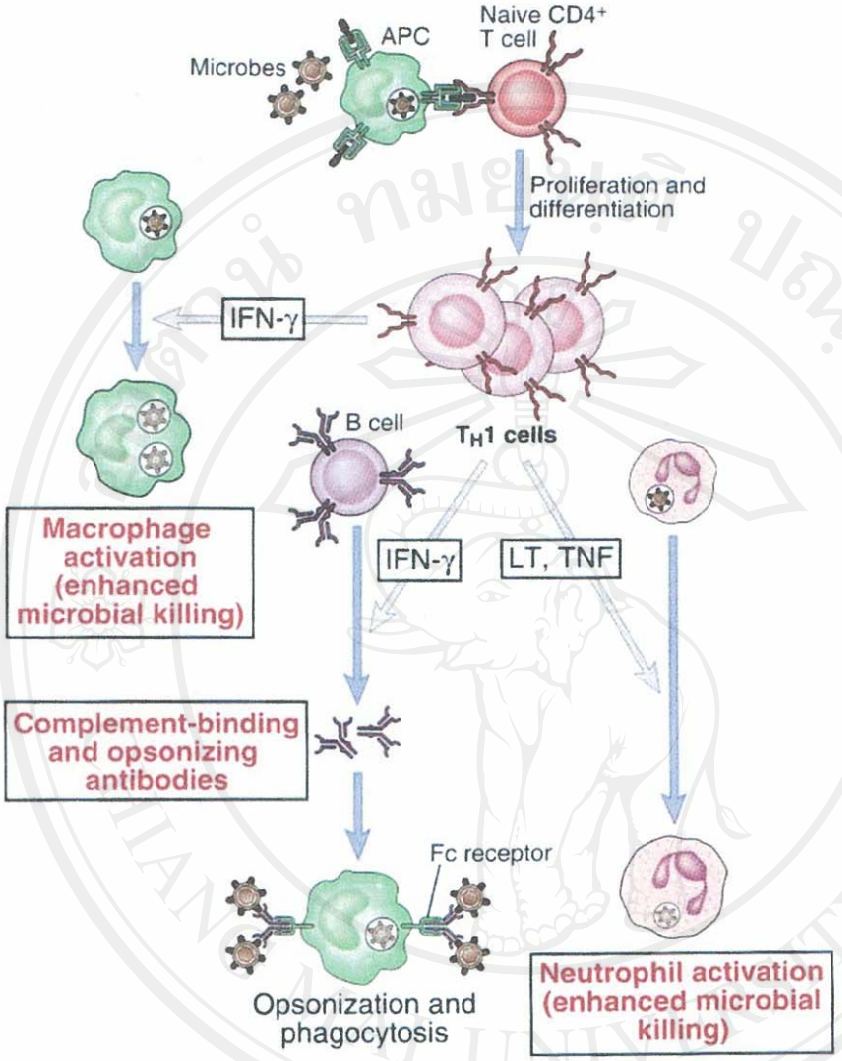


Figure 1.5 Effector functions of T_H1 cells (Abbas and Lichtman, 2003).

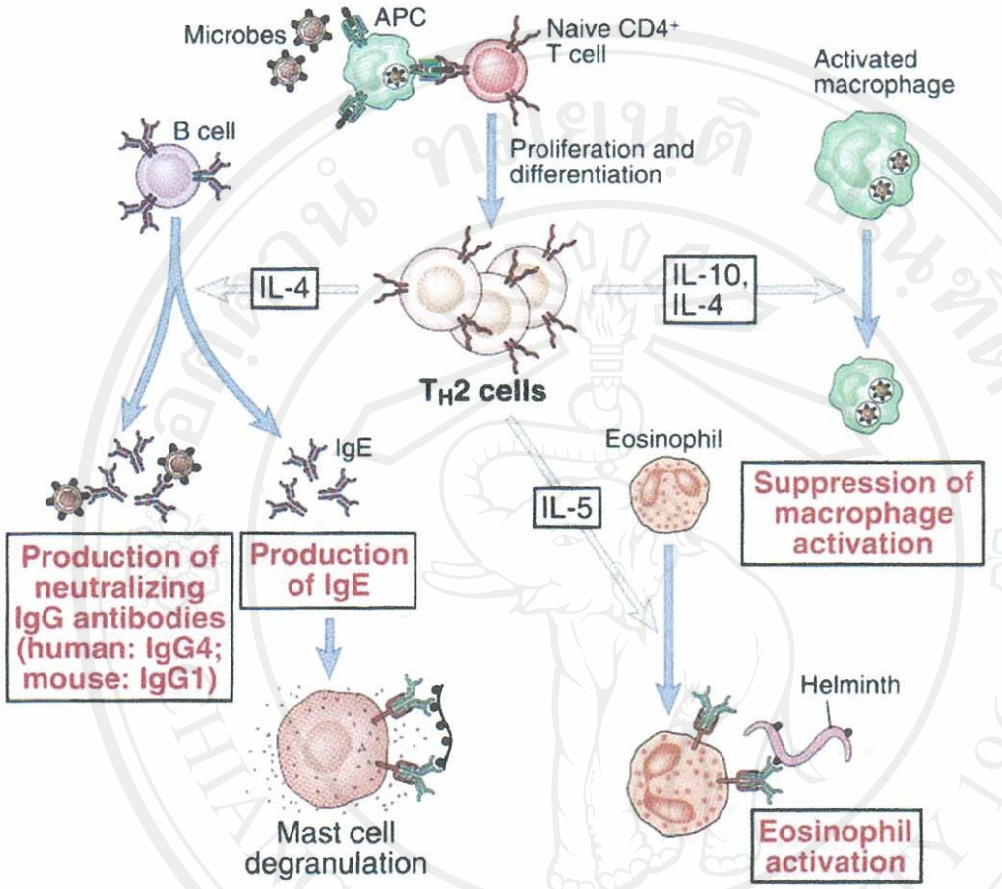


Figure 1.6 Effector functions of T_H2 cells (Abbas and Lichtman, 2003).

1.2.1.3 Cytokines

Cytokines are a group of low molecular weight proteins that are secreted by a range of cell types. Through their autocrine and paracrine activities, cytokines not only orchestrate communication between cells but also have a critical role in mediating immune responses. The following section will concentrate mainly on interleukin-2 (IL-2), which is produced by T lymphocytes, and tumor necrosis factor-α (TNF-α), which mainly produced by macrophages.

1.2.1.3.1 Interleukin-2

Interleukin-2 (IL-2) is a lymphokine synthesized and secreted primarily by T helper lymphocytes that have been activated by stimulation with certain mitogens or by interaction of the T cell receptor complex with antigen/MHC complexes on the surfaces of antigen-presenting cells. The response of T helper cells to activation is induction of the expression of IL-2 and receptors for IL-2 and, subsequently, clonal expansion of antigen-specific T cells. At this level IL-2 is an autocrine factor, driving the expansion of the antigen-specific cells. With respect to the specific role of IL-2 on the differentiation of T cells, the separation of $CD4^+$ T helper cells into the categories T_H1 and T_H2 according to their function in cell mediated or humoral immunity is a concept that is proving useful (Siegel *et al.*, 1987). In this system each category of cells secretes a characteristic set of cytokines that functions as a network to push the system either towards cellular immunity, associated with T_H1; or towards humoral immunity, associated with T_H2. IL-2, along with IFN-γ and TNF-β, is a defining product of the T_H1 subset. IL-2 also acts as a paracrine factor, influencing the activity of other cells. It enhances the cytolytic activity of T_C cells and secretion of other cytokines such as IL-4, IFN-γ and TNF-α by T cells (Thrope, 1998). B cells (Pfeffer *et al.*, 1993) and natural killer (NK) cells (Tartaglia *et al.*, 1993; Mozes *et al.*, 1991) respond, when properly activated, to IL-2. The so-called lymphocyte activated killer, or LAK cells, appear to be derived from NK cells under the influence of IL-2. Monocytes and macrophages also respond to IL-2, with elevated IL-1 and IL-6 production and enhanced cytotoxicity in these cells.

1.2.1.3.2 Tumor necrosis factor-α

Tumor necrosis factor-α (TNF-α) is mainly produced by macrophages, but also by a broad variety of other tissues including lymphoid cells, mast cells, endothelial cells, fibroblasts and neuronal tissue. It is cytotoxic to a variety of tumor cells and can induce hemorrhagic necrosis of tumors. In addition to triggering apoptotic effects on tumor cells, TNF-α induces inflammatory response and regulates immune function. Large amounts of soluble TNF-α are released in response to lipopolysaccharide (LPS) and other bacterial products. Inappropriate production of TNF-α or sustained activation of TNF-α signaling has been shown to be responsible for the pathogenesis of a wide spectrum of diseases, including sepsis, cerebral malaria, diabetes, cancer, osteoporosis, allograft rejection, autoimmune disease such as rheumatic arthritis, and inflammatory bowel disease (Mannel and Echtenacher, 2000). TNF-α once produced and secreted will bind to the TNF-α receptors (TNF-α R1 and TNF-α R2), located on the plasma membrane of most cells throughout the body. It has been reported that TNF-α R1 is responsible for mediating LPS toxicity and cell cytotoxicity and TNF-α R2 is involved in cellular proliferation (Wajant *et al.*, 2003). Exposure to TNF-α results in activation of activator protein (AP)-1 and nuclear factor-κB (NF-κB) (Mercurio *et al.*, 1997; DiDonato *et al.*, 1997). These transcription factors mediate induction of other cytokines and immunoregulatory molecules. Several kinases have been found to be activated rapidly in response to TNF-α, including IκB kinase, P38 mitogen-activated protein kinase (MAPK), Jun N-terminal kinase (JNK), and sphingosine kinase (Yang *et al.*, 2001; Baud *et al.*, 1999; Kim *et al.*, 2001). TNF-α is a pleiotropic inflammatory cytokine that can induce disease through TNF-toxicity and improve host defense mechanisms by stimulating inflammation and increasing immune cell function (Wajant *et al.*, 2003). In the future, therapies may be developed by blocking TNF-α harmful effects and enhancing TNF-α beneficial effects.

1.2.1.4 The immune cells

1.2.1.4.1 Lymphocytes

Lymphocytes are the main set of leukocytes that are involved in mediating the adaptive immune response (Huston, 1997). Lymphocytes comprise three major populations; T cells, B cells, and NK cells (Huston, 1997; Hardy *et al*, 2000). B cells originate from and mature in the bone marrow, T cells originate from the bone marrow but mature in the thymus (Huston, 1997; Hardy *et al*, 2000). B cells recognize specific antigens on the surface of pathogenic organisms (extracellular) through surface immunoglobulin (Huston, 1997). Upon recognition of antigens, B cells proliferate and differentiate into plasma cells, which can produce large quantities of specific antibodies (Huston, 1997). Other B cell surface markers include the class II MHC molecules and complement receptors.

Most T cells can be distinguished by the presence of T cell antigen receptor (TCR-CD3 complex) on the cell surface (Lydyard and Grossi, 1998). T cells are further subdivided into two main subsets: T helper (T_H) cells which express CD4 antigen, and T cytotoxic cells (T_C), which express the CD8 antigen (Zamoyska, 1998). T_H cells play a central role in the adaptive immune response, stimulating the killing functions of macrophages, antibody production by B cells and clonal expansion of T_C cells (Fearon and Locksley, 1996). The principal function of T_C cells is the recognition of surface changes on tumor and virus infected cells through antigen presentation in association with class I MHC molecules and elimination of these cells (Kuby, 1997).

Natural killer (NK) cells are a subpopulation of lymphocytes, which express neither B nor T cell surface antigens. NK cells play a similar role to that of T_C cells, but without the need for antigen presentation in association with class I MHC molecules (Lydyard and Grossi, 1998; Biron *et al.*, 1999).

1.2.1.4.2 Macrophages

Macrophages are one of the most important cells in the immune system. They can display very diverse functions as shown in Figure 1.7. They can function as accessory cells, presenting antigen and providing costimulatory ligands (e.g. CD80, CD86, and CD48) and co-stimulatory cytokines (e.g. IL-1 and IL-12) to the infiltrating T cells. Macrophages can be activated to produce prodigious amounts of pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6, chemoattractant cytokines such as IL-8 and macrophage inflammatory protein (MIP)-1 alpha/beta, and pro-inflammatory products of arachidonic acid metabolism. Macrophage activity is regulated through the generation of toxic mediators, particularly reactive nitrogen species. The following section will be described on nitric oxide, the main reactive nitrogen species produced by macrophages.

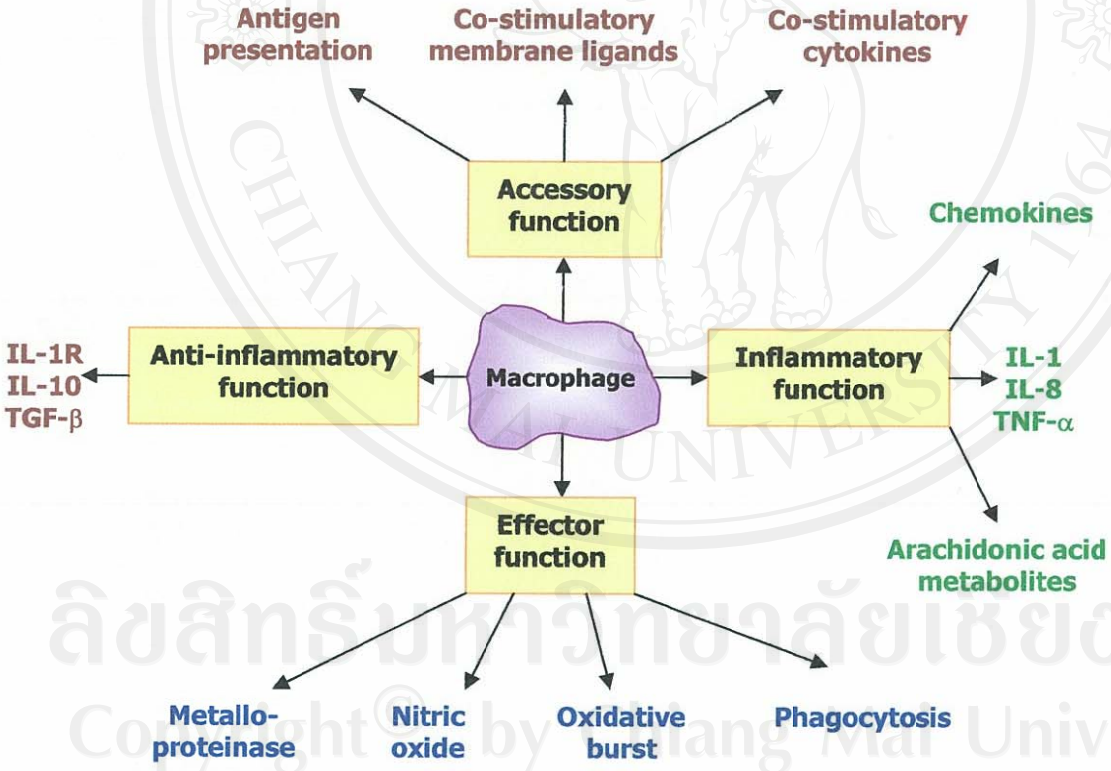


Figure 1.7 The diverse functions of macrophages. Macrophages are capable of many functional activities and contribute both to the initiation of cell-mediated immune response and to the effector limb of those responses. During the course of the response, macrophages can display, at different times, both inflammatory and anti-inflammatory activities (Adapted from Stout and Suttles, 1997).

1.2.1.5 Nitric oxide

Nitric oxide (NO) is a short-lived molecule capable of diffusing across membranes and reacting with a variety of targets. It is involved in many important biological processes such as neurotransmittion, vasodilation, immune modulation, and regulation of apoptosis (Nathan, 1992). NO is produced by the group of enzymes called nitric oxide synthase (NOS). These enzyme catalyze the production of NO and L-citrulline from L-arginine and O2. This process requires five essential cofactors ($FMNH_2$, FADH, NADPH, calmodulin, and tetrahydrobiopterin) and two divalent cations (calcium and heme iron) as shown in Figure 1.8.

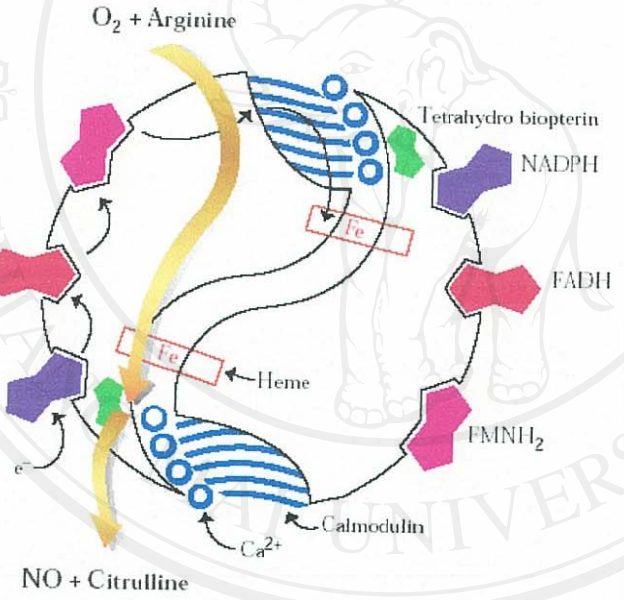


Figure 1.8 Biosynthesis of nitric oxide (www.ibl-hamburg.com).

Three isoforms of NOS have been identified and categorized into constitutive and inducible isoforms. Two distinct constitutive NOS isoforms are neuronal NOS (nNOS or NOS1) and endothelial NOS (eNOS or NOS3), which produce nanomolar amounts of NO. A third isoform, inducible NOS (iNOS or NOS2) is expressed in response to inflammatory cytokines in many cell types, and produces micromolar levels of NO. The three main isoforms share structural similarities and have nearly identical catalytic mechanisms as shown in Figure 1.9. Three distinct regions are necessary for catalytic activity; reductase domain, calmodulin-binding domain, and oxidase domain. The reductase domain transfers electrons from NADPH to the oxidase domain. The oxidase domain actually catalyzes the conversion of arginine into citrullin and NO. Calmodulin binding is required for activity of all of the NOS isoforms. The NOS isoform display a number of differences related to their individual functions. Transcriptional regulation and post-translational regulation of catalytic activity is distinct for each isoform. The following section will emphasize on iNOS isoform as it is produced mainly by macrophages.

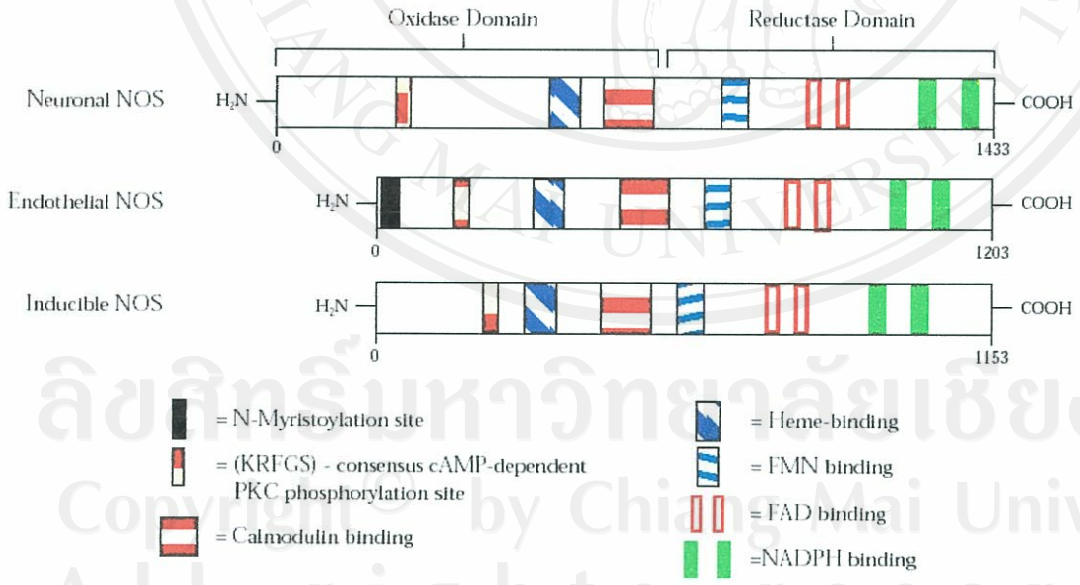


Figure 1.9 Nitric oxide synthase isoforms (www.ibl-hamburg.com).

Table 1.2 Comparison of three isoforms of nitric oxide synthase.

Name	Other name	Type	Regulated by	Present in
NOS1	Neuronal NOS (nNOS)	Constitute	Ca^{2+}/calmodulin	Brain, cerebellum Other neuronal tissue
NOS2	Inducible NOS (iNOS)	Inducible	Endotoxin Cytokines	Macrophages Neutrophils Chondrocytes Hepatocytes
NOS3	Endothelium NOS (eNOS)	Constitute	Ca^{2+}/calmodulin	Endothelial cells

The iNOS was found in the cytoplasm of many cell types as shown in Table 1.2, including macrophages. Main function of iNOS in macrophage is mediated the response of macrophages to infectious agents (Nathan, 1997). In normal condition, this enzyme is normally not expressed. However, in response to stimulation with microbial product and/or cytokines, cells can express iNOS. The iNOS is bind to calmodulin tightly even at very low concentrations of Ca^{2+} (Gorren and Mayer, 1998; Stuehr, 1999). Activity of iNOS is not responsive to changes in intracellular Ca^{2+} levels and thus it is capable of a high output and long-lasting release of NO. The iNOS gene is under transcriptional control although activity is also influenced by a variety of other control mechanisms that affect mRNA stability, translation and degredation of the protein, and availability of substrate and cofactors (Hemmens and Mayer, 1998; Taylor, 1998). The overproduction of NO by iNOS is implicated in a number of pathologies. Septic shock, which is often fatal, is caused by the over activation of macrophages in response to bacterial infection in the blood (Titheradge, 1999). The resulting overproduction of NO leads to a severe drop in blood pressure and subsequently dysfunction of multiple organs. NO generated by iNOS is present in a number of inflammatory conditions including rheumatoid arthritis and asthma (Moncada, 1999). Because of its role as both an immune mediator and an effector molecule, NO can have deleterious or beneficial roles in inflammatory conditions depending on the setting (Nathan, 1997).

1.2.2 Mycotoxins

Mycotoxins are secondary fungal metabolites. Fungal metabolites are compounds that are not necessary for growth. Fungi need organic compounds such as protein, fat and carbohydrates to obtain energy and produce and maintain their cellular components. To achieve this task, organic compounds are metabolized into intermediate compounds and then either to primary metabolites such as amino acids, saccharides, fatty acids and proteins or secondary metabolites, including mycotoxins (Smith and Moss, 1985). Primary metabolites are essential for the survival of organisms. Although the role of secondary metabolites is not clearly defined, they specify species and strain of fungi, and they can be toxic to other living organism including plants, animals and human beings (Smith and Moss, 1985). The mycotoxins of major concern for human health are produced by three main genera of fungi; *Aspergillus*, *Penicillium*, and *Fusarium*.

The impact of mycotoxins on animals and crops is relevant to both developed and developing countries (Miller, 1998). This impact is a result of direct or indirect economic losses. Direct losses are due to decreased performance and productivity of animals, their increased susceptibility to infections and the decreased quality and quantity of crops. Indirect losses come from the millions of dollars paid by animal and crop producers, as well as the food processing industry, in order to prevent of mycotoxin contamination (Charmley *et al*, 1995). Economic losses are probably the main effect of mycotoxins in developing countries. In developing countries, people are exposed to high levels of mycotoxins, often to mixtures of two or more mycotoxins in their staple foods. In addition, there is usually no regulation of these food contaminants (Miller, 1998). The impact of these exposures on human health can be inferred from estimates of the social costs related to aflatoxin in South East Asia. This estimate, termed disability adjusted life years, is calculated from the loss in productivity of an individual due to disease and/or premature death. Miller (1998) remarked that 40% of the given causes of years lost in developing countries was due to diseases that could be related to mycotoxins. It should be stated that this calculation assumes that mycotoxins contribute to immune suppression in developing countries and hence to infectious disease morbidity and mortality.

1.2.2.1 Aflatoxin B_1

1.2.2.1.1 Classification and chemical structure

Aflatoxins are difuranocoumarin derivatives produced by many strains of *Aspergillus flavus* and *Aspergillus parasiticus*; in particular, *Aspergillus flavus* is a common contaminant in agriculture. *Aspergillus bombycis, Aspergillus ochraceoroseus*, *Aspergillus nomius*, and *Aspergillus pseudotamari* are also aflatoxin-producing species, but they are encountered less frequently (Goto *et al.*, 1996; Klich *et al.*, 2000; Peterson *et al.*, 2001). The four major aflatoxins are called aflatoxin B_1 (AFB_1), AFB_2, AFG_1 and AFG_2 based on their fluorescence under UV light (blue or green) and relative chromatographic mobility during thin-layer chromatography. AFB_1 is the most potent natural carcinogen known (Squire, 1981) and is usually the major aflatoxin produced by toxigenic strains. In addition other metabolites are found in body fluids and these include AFM_1, AFM_2 found in milk and urine and the urinary metabolites, AFP_1 and AFQ_1.

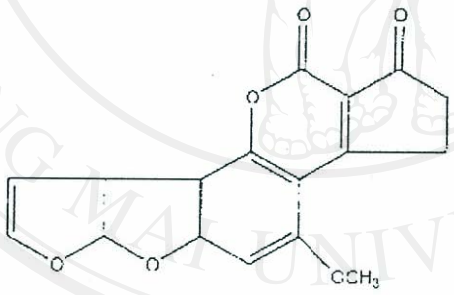


Figure 1.10: The chemical structure of aflatoxin B_1 (Bennett and Klich, 2003).

1.2.2.1.2 General toxic effects

Aflatoxin B_1 is associated with both toxicity and carcinogenicity in human and animal populations (Eaton and Groopman, 1994; Newberne and Butler, 1969; Peers and Linsell, 1973; Shank *et al.*, 1972). The diseases caused by aflatoxin consumption are called aflatoxicosis. Acute aflatoxicosis results in death; chronic aflatoxicosis results in cancer, immune suppression, and other slow pathological conditions. The liver is the primary target organ of aflatoxin B_1. There are substantial differences in species susceptibility. Moreover, within a given species, the magnitude of the response is influenced by age, sex, weight, diet, exposure to infectious agents, and the presence of other mycotoxins and pharmacologically active substances.

1.2.2.1.3 Metabolism and mechanism of action

The extent of biological activity of AFB_1 depends on the balance between enzyme systems involved in activation and detoxification of AFB_1. AFB_1 is bioactivated to a highly reactive metabolite, AFB_1-8,9-epoxide, by several cytochrome P450 (CYP450) enzymes, in particular CYP1A2 and CYP3A4 (Eaton *et al.*, 1994; Guengerich *et al.*, 1998). Other enzymes such as prostaglandin H synthase and lipoxygenase have been demonstrated to activate AFB_1 to the epoxide form but with much lower activity than the CYP450 (Eaton, 1994). AFB_1-8,9-epoxide exists in two isomers; exo- and endo-epoxide and only the exo form of this epoxide, can bind to the N7 position of deoxyguanosine in deoxyribonucleic acid (DNA) to form AFB_1-N7-guanine adduct. CYP1A2 oxidises AFB_1 to endo-epoxide as well as to other hydroxylated metabolites such as AFM_1 and AFQ_1 whilst CYP3A4 oxidises AFB_1 to exo-epoxide and AFQ_1 (Guengerich *et al.*, 1998). With the exception of exo-epoxide, all other aflatoxins metabolites have less biological activity than the parent compound (Eaton *et al.*, 1994).

The AFB_1 8,9-epoxide can also be hydrolysed spontaneously or possibly by epoxide hydrolase (EPXH) to the AFB_1-8,9-dihydrodiol, which can bind to proteins (Eaton *et al.*, 1994). AFB_1 has been shown to inhibit protein synthesis both in in vitro and in vivo studies in several species including rats, mice and monkeys (Busby and Wogan, 1984). The inhibition of protein synthesis by AFB_1 required the metabolic activation of this toxin, and occurred over a longer period and to a lesser extent than inhibition of ribonucleic acid (RNA) synthesis. Disaggregation

of the polyribosomes by AFB_1 may be one mechanism by which this toxin inhibits protein synthesis (Pong and Wogan, 1970).

Enzymes involved in the detoxification of AFB_1 include glutathione S-transferases (GSTs) and aldehyde reductase (AR). Both endo and exo forms of AFB_1-8,9-epoxide are inactivated by conjugation of glutathione. The activity of GST as well as cytochrome P450 varies among species and this partially explains the interspecies differences in susceptibility to the toxic and carcinogenic effect of AFB_1 (Eaton and Groopman, 1994; Eaton and Ramsdel, 1992).

1.2.2.1.4 Toxicity and carcinogenicity

Exposure to aflatoxins in the diet is considered an important risk factor for the development of primary hepatocellular carcinoma, particularly in individuals already exposed to hepatitis B. In epidemiology, several studies have linked liver cancer incidence to estimated aflatoxin consumption in the diet (Li *et al.*, 2001; Peers and Linsell, 1973; Van Rensburg *et al.*, 1985). The incidence of liver cancer varies widely from country to country, but it is one of the most common cancers in China, the Philippines, Thailand, and many African countries. The presence of hepatitis B virus infection, an important risk factor for primary liver cancer, complicates many of the epidemiological studies.

In molecular epidemiology, it is possible to demonstrate the association between putative carcinogens and specific cancers. Monitoring of aflatoxins can be done by analyzing for the presence of aflatoxin metabolites in blood, milk, and urine; moreover, excreted DNA adducts and blood protein adducts can also be monitored (Sabbioni and Sepai, 1994). The aflatoxin B_1-N7-guanine adduct represents the most reliable urinary biomarker for aflatoxin exposure but reflects only recent exposure. Numerous studies have shown that carcinogenic potency is highly correlated with the extent of total DNA adducts formed in vivo (Eaton and Gallagher, 1994; Eaton and Groopman, 1994). Inactivation of the p53 tumor suppressor gene may be important in the development of primary hepatocellular carcinoma. Studies of liver cancer patients in Africa and China have shown that a mutation in the p53 tumor suppressor gene at codon 249 is associated with a G-to-T transversion (Hsu *et al.*, 1991; Bressac *et al.*, 1991). Mechanistically, it is known that the reactive aflatoxin epoxide binds to the N7 position of guanines. Moreover, aflatoxin B_1-DNA adducts can result in GC to TA transversions. The specific mutation in codon

249 of the p53 gene has been called the first example of a "carcinogen-specific" biomarker that remains fixed in the tumor tissue (Eaton and Gallagher, 1994). There is also considerable evidence associating aflatoxin with neoplasms in extra hepatic tissues, particularly the lungs. For example, one early epidemiological study of Dutch peanut processing workers exposed to dust contaminated with aflatoxin B_1 showed a correlation between both respiratory cancer and total cancer in the exposed group compared with unexposed cohorts (Hayes *et al.*, 1984).

It has been hypothesized that kwashiorkor, a severe malnutrition disease, may be a form of pediatric aflatoxicosis (Hendrickse, 1997). Kwashiorkor children who were positive for serum aflatoxins when entering hospital had a poorer prognosis with a significant decrease in haemoglobin level, longer duration of edema, longer duration of stay in hospitals and increased number of infection than those children who are negative for aflatoxin (Adhikari *et al.*, 1994). The author proposed that the poor prognosis in kwashiorkor children with high serum levels of aflatoxin was due to the immunosuppressive activity of this mycotoxin. Conversely, the disease itself may impair aflatoxin metabolism. Ramjee *et al.* (1992) reported that kwashiorkor children had a higher prevalence and higher concentrations of aflatoxins in the sera compared to other children (marasmas, underweight, and normal healthy controls). Further speculation, e.g. that aflatoxin might be involved in Reye's syndrome, an encephalopathy, and fatty degeneration of the viscera in children and adolescents (Hayes, 1980) have not been substantiated. The latter idea is based on a study in Thailand suggested that aflatoxin exposure is one of the risk factor for this disease, although other risk factors have since been identified (Hall and Wild, 1994).

1.2.2.2 Deoxynivalenol

1.2.2.2.1 Classification and chemical structure

Deoxynivalenol (DON) is included in a group of mycotoxins called trichothecenes. Trichothecenes are mostly produced by Fusarium species. *F. graminearum* which produces DON and/or nivalenol (NIV) and zearalenone (ZEA) is the most important Fusarium species in terms of plant pathology, followed by *F. culmorum* which produces DON and ZEA (Miller, 1995).

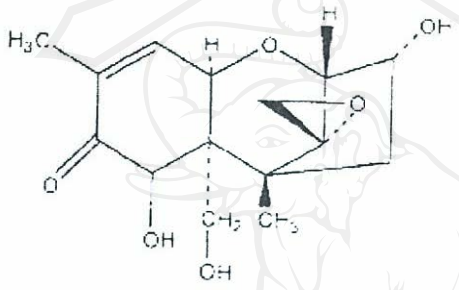


Figure 1.11: The chemical structure of deoxynivalenol (Bennett and Klich, 2003).

1.2.2.2.2 General toxic effects

DON is one of the most common mycotoxins found in grains. When ingested in high doses by agricultural animals, it causes nausea, vomiting, and diarrhea; at lower doses, pigs and other farm animals exhibit weight loss and food refusal (Rotter *et al.*, 1996). For this reason, DON is sometimes called vomitoxin. Although less toxic than many other major trichothecenes, it is the most prevalent and is commonly found in barley, corn, rye, safflower seeds, wheat, and mixed feeds (Miller *et al.*, 2001). The symptoms produced by various trichothecenes include effects on almost every major system of the vertebrate body; many of these effects are due to secondary processes that are initiated by often poorly understood metabolic mechanisms related to the inhibition of protein synthesis.

1.2.2.2.3 Mechanism of action

DON inhibits the synthesis of DNA and RNA and protein at the ribosomal level by binding to the 60S subunit of the eukaryotic ribosome and consequently interfereing with the activity of peptidyl transferase (Rotter *et al.*, 1996). The toxin has a haemolytic effect on erythrocytes. An acute dose of DON can induce vomiting (emesis) in pigs, whereas at lower concentrations in the diet it reduces growth and feed consumption (anorexia). Both effects are mediated by affecting the serotonergic activity in the CNS or via peripheral actions on serotonin receptors (Rotter *et al.*, 1996; Eriksen and Alexander, 1998). Trichothecenes including DON are cytotoxic in human lymphoid cell lines (Visconti *et al.*, 1991; Ueno *et al.*, 1995). Recently these toxins were shown to induce a ribotoxic stress response in a human T-lymphoid cell line activating c-jun terminal kinase and p38 mitogen-activated protein kinase, and stimulating apoptosis (Shifrin and Anderson, 1999).

1.2.2.2.4 Toxicity and carcinogenicity

The effects of DON vary depending upon dose, duration of exposure and species involved. Acute and subacute toxicities of DON are characterized by vomiting, feed refusal, weight loss and diarrhea. After acute intoxication necrosis in various tissues such as gastrointestinal tract, bone marrow and lymphoid tissues is also observed (Rotter *et al.*, 1996). After subchronic oral exposure of various species (mouse, rat and pigs) several effects were found, including reduced feed intake, reduced weight gain, and changed levels in some blood parameters including serum immunoglobulins (Eriksen and Alexander, 1998).

In a long-term study with B6C3F1 mice, the animals were administered with DON 0, 1, 5 or 10 mg/kg feed daily during 2 years. Survival was not significantly changed. A significantly reduced weight gain was observed. DON did not cause biologically relevant effects in haematological and clinical-chemical parameters. In females serum immunoglobulins showed some increase of IgA and IgG. Relative liver weights in males were increased. At this latter dosage males showed decreased relative spleen weights. No increase in incidence of preneoplastic or neoplastic changes was observed (Iverson *et al.*, 1995). In a two-stage experiment with Sencar mice, DON did not cause any apparent skin tumor initiation or promotion (Lambert *et al.*, 1995).

1.2.2.3 Fumonisin B_1

1.2.2.3.1 Classification and chemical structure

Fumonisin B_1 (FB_1) belongs to the recently (1988) discovered toxins known as fumonisins. Fumonisins are produced by *Fusarium* species including *Fusarium verticilloides* (an older synonym is *F. moniliforme*) and *F. proliferatum,* fungi that commonly contaminate maize (Miller, 1995). *F. moniliforme* has been shown to produce six types of fumonisins of which FB_1, FB_2, and FB_3 are the most abundant in nature. Other members of this family include FB_4, FA_1 and FA_2; the hydrolysed products of FB_1 and FB_2 include the aminopentol (AP_1) and aminotetraol respectively (Gelderblom *et al.*, 1992; 1993). FB_1 has been found as natural contaminant in maize and maize-based food from many parts of the world, e.g. the US, Canada, South Africa, Nepal, Australia, Thailand, Philippines, Indonesia, Mexico, France, Italy, Poland, and Spain (Eriksen and Alexander, 1998).

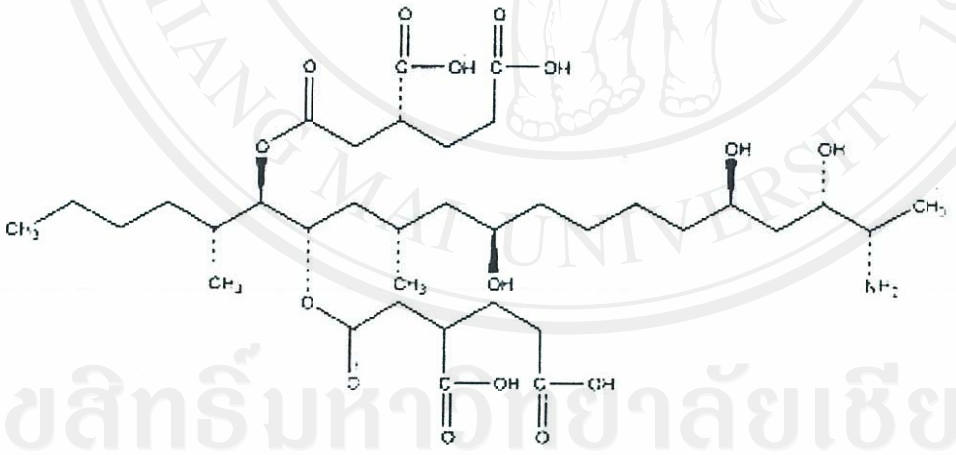


Figure 1.12: The chemical structure of fumonisin B_1 (Bennett and Klich, 2003).

1.2.2.3.2 General toxic effects

Fumonisins affect animals in different ways by interfering with sphingolipid metabolism (Dutton, 1996; Marasas, 1995; Merrill *et al.*, 2001; Wang *et al.*, 1991). They cause leukoencephalomalacia in equines (Marasas *et al.*, 1988) and rabbits (Bucci *et al.*, 1996); pulmonary edema and hydrothorax in swine (Harrison *et al.*, 1990); and hepatotoxic and carcinogenic effects (Gelderblom *et al.*, 1991; 1996a; 1996b) and apoptosis in the liver of rats (Pozzi *et al.*, 2000). In humans, there is a probable link with esophageal cancer (Sydenham *et al.*, 1991). The occurrence of FB_1 is correlated with the occurrence of a higher incidence of esophageal cancer in regions of Transkei (South Africa), China, and northeast Italy (Peraica *et al.*, 1999). It has been isolated at high levels in corn meal and corn grits, including seven samples from a supermarket in Charleston, S.C., which has the highest incidence of esophageal cancer among African-Americans in the United States (Sydenham *et al.*, 1991).

1.2.2.3.3 Mechanism of action

The mode of action of fumonisins is primarily explained by interference with the *de novo* synthesis of complex glycosphingolipids. This results in disturbances of cellular processes such as cell growth, cell differentiation and cell morphology, endothelial cell permeability and apoptosis. Inhibition of biosynthesis of sphingolipids is seen at different levels of the process (Merill *et al.*, 1996; Riley *et al.*, 1996), and is reflected in changes of the ratio sphinganine/sphingosine (Sa/So). Marginal effects on the Sa/So ratio were seen from 0.2mg/kg bw level onwards. The inhibition of biosynthesis of glycosphingolipids is already seen a few hours after oral exposure to FB_1.

Recently it has been shown that FB_1 administered to different animal species is able to produce increased apoptosis in various tissues. Increased apoptosis in particular seems to play an important role in the toxic effects, including tumour induction by FB_1. In animal experiments (when determined), apoptosis is seen at all dose levels of FB_1, causing other toxic including carcinogenic effects. In most studies apoptosis is one of the observations on which the NOAEL is based. The dose level causing apoptosis depends on the duration of exposure and can vary in rodents from 0.9 to 12 mg FB_1/kg bw (respectively in long-term and short-term experiments). Oxidative damage has also been indicated in the etiology of the toxic effects (Abel and Gelderblom, 1998).

1.2.2.3.4 Toxicity and carcinogenicity

FB_1 has a low acute oral toxicity in several animal species (Eriksen and Alexander, 1998). In addition to the studies with rodents, pigs and horses, there are several subacute toxicity studies performed with many animal species (poultry, rabbits, hamsters, nonhuman primates, lamb, mink, cattle) not directly useful for quantitative dose response assessment. Many of these studies were performed with contaminated feed rather than pure fumonisin (Javed *et al.*, 1995; Bryden *et al.*, 1987; Weibking *et al.*, 1993; Kuiper-Goodman *et al.*, 1996; Norred *et al.*, 1996). The major target organs are liver and kidney in almost all animal species, but particularly in mouse and rat. For the rat, whether the liver or the kidney is the most sensitive organ may depend on the strain or gender (Gelderblom *et al.*, 1996a). In addition, in pigs and horses some other typical effects are seen, like porcine pulmonary edema (PPE) in pigs (Kriek *et al.*, 1981; Colvin *et al.*, 1993; Diaz and Boermans, 1994; Fazekas *et al.*, 1998; Casteel *et al.*, 1993,1994; Rotter *et al.* 1996; Gumprecht *et al.*, 1998; Haschek-Hock, 1999) and equine leukoencephalomalacia (ELEM) in horses (Marasas *et al.*, 1988a; Kriek *et al.*, 1981; Kellerman *et al.*, 1990; Wilson *et al.*, 1992, Ross *et al.*, 1993,1994; Uhlinger, 1997). The minimum dose that causes ELEM ranges from between 0.2 and 0.44 mg FB_1/kg bw/day (Ross *et al.*, 1994). The dose for horses is thus estimated at 0.2 mg FB_1/kg bw/day. The lowest dose for FB_1 in a subchronic study with rats was 0.2 mg FB_1/kg bw/day and the lowest dose for mice was 1.8 mg FB_1/kg bw/day (Voss *et al*, 1995). The doses established in the long-term toxicity/carcinogenicity studies in mouse and rats were are 0.25 mg FB_1/kg bw/day (male rats) for kidney lesions and 0.7 mg FB_1/kg bw/day (female mice) for liver lesions. The kidney adenomas and carcinomas in rats were only seen at higher doses than those that caused the other toxic effects, including apoptosis, similarly the hepatocellular neoplasms were only seen at the higher dose levels in female mice. The lowest dose level at which increased kidney tumor incidences were observed in rats (males) is 2.5 mg/kg bw, and was 7.0 mg/kg bw in mice (females) for increased liver tumor incidence.

1.2.3 Immunotoxicity of mycotoxins

Immunomodulation is one of the most significant toxic effects of mycotoxins in livestock. Many veterinarians noticed that these toxins could alter the immune system of animals and in particular could cause immunosuppression at dose levels lower than those, which cause over toxicity (Pestka and Bondy, 1990; Oswald and Comera, 1998). One outcome of this immunosuppression is increased susceptibility to pathogens. A secondary effect of lowered immunity is that animals are more frequently treated with antibiotics, and thus levels of these drugs are increased in animal tissues and milk consumed by humans (Pestka and Bondy, 1990; Oswald and Comera, 1998). In the same vein, Pestka and Bondy (1990) have pointed out that direct exposure of humans to mycotoxins in the diet might increase the incidence of infectious diseases, autoimmune diseases and tumors. Mycotoxins do have a complex effect on the immune system, with elements of both suppression and stimulation. The toxins can suppress both innate and adaptive immunity, the former including reduced complement activity and impaired macrophage and neutrophil function and the latter including suppression of T and B cell activity and of Ig production (WHO, 1990; Corrier, 1991; Oswald and Comera, 1998). However, immunostimulation can cause hypersensitivity, heightened allergic reactions and autoimmune diseases (Oswald and Comera, 1998). These contrasting aspects are described below for trichothecenes (with an emphasis on DON), aflatoxins and fumonisins.

1.2.3.1 Effect of AFB_1 on the immune system

1.2.3.1.1 Cellular effects

Cell mediated immunity is the primary target for aflatoxins, including diminished responses in DTH, graft-versus-host reaction, leukocyte migration and lymphoblastogenesis (Pier, 1992). Phagocytic activity is also modified by aflatoxin and consequently clearance of microbial agents and presentation of antigens to other cellular components in the immune system are affected (Pier, 1992). However, much higher aflatoxin concentrations are required for modulation of humoral immunity compared to cell mediated immunity; in addition there are differences among species in this respect (Pestka and Bondy, 1994).

The effect of aflatoxins on lymphocytes

In mice treated orally with aflatoxin (0-0.7 mg/kg bw) every day for two or four weeks, there was a dose related decrease in the number of circulating white blood cells at higher doses (0.14, and 0.7 mg/kg bw) (Reddy *et al.*, 1987; Reddy and Sharma, 1989). In C57B1/6 mice treated with AFB_1 daily for four weeks a significant decrease in the splenic T helper cells as a percentage of the total splenic cells was observed only at the highest concentration of AFB_1 (0.75mg/kg) (Hatori *et al.*, 1991). A similar decrease was observed in splenic B cells, but this decrease was also observed at lower doses. In chicks that received AFB_1 in the diet (0.3 and 1 mg/kg bw) for six weeks, there was also decrease in the number of T lymphocytes in the circulation (Ghosh *et al.*, 1990).

AFB_1 does not only alter the number of lymphocytes, but it can also affect the function of lymphocytes as measured by blastogenic response and DTH. This effect on blastogenic response was not totally consistent between different species. Three different strains of mice exhibited different responses in both T and B cells. In a study by Reddy *et al.* (1987), CD-1 mice were orally exposed for two weeks on alternate days to AFB_1 (0-0.7mg/kg bw). Splenic cells were isolated and stimulated with a specific T cell mitogen, specific B cell mitogen or non-specific (T and B cells) mitogen. A significant decrease in [^{3}H]-thymidine incorporation was found with T cell and non-specific mitogens only at the highest concentration of AFB_1. A decrease was also found in splenic cells stimulated by B cell mitogen but all AFB_1 concentrations. Subsequently, Reddy and Sharma (1989) studied the same effect on BALB/c mice exposed orally to AFB_1 (0-0.7mg/kg bw) every other day for four weeks and a significant decrease was found in splenic cells with T and/or B cell mitogens only at the highest concentration of AFB_1. A marked suppressive effect of AFB_1 in unstimulated cells was demonstrated in both studies, which could be a direct cytotoxic effect of this mycotoxin (Reddy *et al*, 1987; Reddy and Sharma, 1989). A further study by Hatori *et al.* (1991), using C57B1/6 mice treated orally with AFB_1 (0-0.75mg/kg bw) daily for four weeks, showed that spleenic B cells were more sensitive to the suppressive effects of aflatoxin than T cells. Whereas B cells exhibit a decrease in mitogenic response at the medium and high doses of AFB1, T cells showed the same effect only at the higher doses of the toxin. These apparent contradictions among the three studies maybe related to the differences in the strain of mice used and the timing of exposure to the toxin. In contrast to these in vivo studies in

mice, an in vitro study showed that mouse splenic B cells required 2-fold higher AFB_1 concentrations for suppression of [^{3}H]-thymidine incorporation compared to T cells (Reddy and Sharma, 1989).

In other species of rodents, oral exposure of rats to AFB_1 (0.06 to 0.6 mg/kg bw) suppressed the blastogenic response of T cells, whereas B cells required a five times higher dose to exhibit the same effect (0.3 to 0.6 mg/kg bw) (Raisuddin *et al.*, 1993). In guinea pigs, in vitro exposure of peripheral blood lymphocytes to AFB_1 resulted in a significant reduction in proliferation of mitogen stimulated T cells (Pier *et al.*, 1989). As regards the effect of AFB_1 on human peripheral blood lymphocytes, this was only demonstrated in one study (Savel *et al.*, 1970). The authors reported reduced incorporation of [^{3}H]-thymidine to DNA in cells stimulated with PHA or potent antigens including tuberculin and mumps.

The effect of AFB_1 on NK cells was examined in three studies (Reddy *et al.* (1987); Reddy and Sharma (1989) and Hatori *et al.* (1990)) and contradictory results were obtained. Whilst in the earlier study by Reddy *et al.* (1987) and the one by Hatori *et al.* (1990), no effect on the NK cell function was observed, Reddy and Sharma (1989) found marked depression in NK cell function. Again this apparent contradiction may be due to the different strains of mice used; Reddy and Sharma (1989) used BALB/c mice whilst the other two studies used CD-1 or C57B1/6. Different time courses of exposure were also used i.e. every other day for four weeks (Reddy and Sharma, 1989) or two weeks (Reddy *et al.*, 1987) or daily for four weeks (Hatori *et al.*, 1990).

DTH is another test to evaluate the function of lymphocytes specifically on T cells. In contrast to the inconsistency of the data on blastogenic response, AFB_1 in this test always gives a consistent suppressive effect among different species such as mice, rats, guinea pigs and chickens. DTH to keyhole limpet hemocyanin was significantly suppressed in CD-1 mice receiving 0.14 to 0.70 mg/kg bw AFB_1 (Reddy *et al.*, 1987). In chickens receiving 0.3 ppm AFB_1 in the diet for two months, DTH response to dinitrofluorobenzene was reduced as early as two weeks after the start of treatment and was reduced almost to zero after six weeks treatment. In the same study, following withdrawal of the toxin from the feed, the depressive effect on delayed hypersensitivity persisted for at least three days (Kadian *et al.*, 1998). In guinea pigs the DTH response to *Nocardia asteroids* was depressed upon oral administration of 0.045 mg/kg bw AFB_1. Similarly,

in weanling rats oral exposure to 0.3 to 0.6 mg/kg bw AFB_1 significantly suppressed the DTH to sheep erythrocytes (Raisuddin *et al.*, 1993).

The effect of aflatoxins on macrophages

AFB_1 can also affect phagocytes quantitatively and qualitatively. Oral exposure of chickens to 0.3ppm AFB_1 in the diet for three months reduced the phagocytic activity of reticuloendothelial cells as measured by the rate of clearance of colloidal carbon in the blood. As with the DTH assay the effect was observed as early as two weeks into treatment, lasted for the six weeks and persisted three days after withdrawal of the toxin from the feed (Kadian *et al.*, 1988). Direct exposure of chicken peritoneal macrophages to 5-20 μg/ml AFB_1 in vitro caused cell damage and a two-to three-fold reduction in the number of adhered cells compared with untreated cells. However, there was no effect on phagocytic activity under these conditions (Neldon-Ortiz and Qureshi, 1992). In the same study, the importance of metabolism of AFB_1 after activation of the toxin with a mixed function oxidase system (MFO) caused a dramatic suppressive effect on the adherence ability of macrophages at markedly lower concentrations of AFB_1 (1μg/ml) than in the absence of activation. Almost complete loss of adherence, but also of phagocytic activity of macrophages, was observed at an AFB_1 concentration of 5 μg/ml. The effect of AFB_1 on the same parameters was also assessed on turkey peritoneal macrophages (Neldon-Ortiz and Qureshi, 1991). Activation of AFB_1 by the MFO system was again a prerequisite to seeing a significant reduction in cell adherence, phagocytic activity of macrophages and cytotoxicity. Overall these data suggested that AFB_1 requires metabolic activation in order to exert these immunotoxic effects.

In addition to the effect of AFB_1 on phagocytic activity in avian species, the phagocytic and microbicidal activity from other species such as rat and human macrophages was suppressed at very low concentrations of AFB_1 in vitro (0-0.01 pg/ml) (Cusumano *et al.*, 1995, 1996).

1.2.3.1.2 Humoral effects

In chickens and turkeys, the antibody response to bacterial and viral challenge was unaffected at low levels of aflatoxin in the feed (0.2-0.5 mg/kg bw). However, higher levels (0.6-10 mg/kg bw) suppressed Ig production particularly IgG or IgA in chickens and antibody response to Salmonella and SRBC (reviewed by Corrier, 1991). In chicks receiving AFB_1 (0.3 and 1 mg/kg bw) for six weeks, there was a decrease in albumin and globulin levels in the peripheral blood (Ghosh *et al.*, 1990).

Guinea pigs orally exposed to a mixture of aflatoxins (79% AFB_1 and 21% AFB_2, AFG_1 and AFG_2) at doses of 0.045 mg/kg exhibited no apparent effect on the titer of antibody against *Brucella abortus* after intramuscular injection, although a significant decrease in the serum complement titre was found (Pier *et al.*, 1989). In CD-1 mice orally exposed to AFB_1 (0, 0.03, 0.145, 0.70 mg/kg bw) every other day for two weeks, there was a dose related decrease in IgM class antibody-producing cells against T cell dependent antigens (SRBC) whereas no such effect against T independent antigens such as LPS was reported (Reddy *et al.*, 1987). These latter results indicate that the effect of AFB_1 on B cells is mediated by its effect on T cells. Hatori *et al.* (1991) also found a dose-related decrease in anti-SRBC antibody producing cells and anti-SRBC antibody titer in the plasma in C57B1/6 mice orally exposed to 0-0.75 mg/kg AFB_1 daily for four weeks. In contrast, in weanling rats, no effect was reported for AFB_1 (0.3 to 0.6 mg/kg bw) on humoral immunity as assessed by a plaque forming cell assay (Raisuddin *et al.*, 1993).

1.2.3.1.3 Host resistance

The broad immunosuppressive effects on the cellular and humoral elements described above would be expected to affect the resistance of the host to invading pathogens. The effect of mixtures of aflatoxins (76% AFB_1+24% AFG_1) on host resistance after vaccination was investigated in rabbits (Venturini *et al.*, 1990). Rabbits were divided into three groups; two groups were vaccinated with *Bordetella bronchioseptica* vaccine with one of the two also receiving daily aflatoxin treatment (0.05 mg/kg bw) whilst the third group was neither vaccinated nor treated with aflatoxins. All three groups were challenged by *Bordetella bronchioseptica* intranasally at day forty-four. Decreased resistance to the challenge in terms of the percentage of the lung occupied by pneumonic lesions was observed between the unvaccinated control group

(13.4%) and the vaccinated group not treated with aflatoxin (2.3%). However aflatoxin treatment increased the pneumonic lesions to 7% in the second vaccinated group. Furthermore, in terms of microscopic lesions in the lung, the percentage of grade 3 microscopic lesions in the alveoli (defind as mild to moderate thickening in the alveolar walls due to inflammatory cell infiltration, more mononuclear infiltration in the alveolar lumen and alveolar necrosis) was 21.7 % in rabbits vaccinated and aflatoxin treated whilst no such lesions were found in the alveoli of rabbits vaccinated but not aflatoxin treated. No significant difference in antibody titre to *Bordetella* was found between the three groups. Based on these results the authors suggested that the decreased resistance to bacteria is due to suppression of cell-mediated immunity (Venturini *et al.*, 1990).

Resistance to *Toxoplasma gondii* infection was studied in mice treated or not with aflatoxin daily to 0.1 mg/kg bw for 50 days. Infection with *Toxoplasma gondii* resulted in meningoencephalitis and formation of cysts around the parasites. Fifty-nine percent of these cysts were ruptured in aflatoxin treated mice infected with the parasite compared with only 15% in mice not receiving aflatoxin. Ruptured cysts were associated with severe brain lesions in the aflatoxin treated group (Venturini *et al.*, 1996). Increased suppression of cell-mediated immunity by AFB1 was again one of the proposed mechanisms to explain these results.

1.2.3.1.4 Mechanism of immunosuppression

Rainbow et al. (1994) observed that splenic lymphocytes isolated from mice receiving AFB_1 (9mg/kg bw) orally each day for three weeks showed disruption of the inner membrane of the mitochondria where oxidative phosphorylation and generation of energy occur. This finding could provide a mechanism for the immunodysfunction noted in animals exposed to AFB_1. Another possible mechanism for the immunomodulatory effect of AFB_1 on macrophages and lymphocytes could be through interference with gene expression and protein production e.g. cytokines, produced by the aforementioned cells. Dugyala and Sharma (1996) investigated the above assumption in four groups of mice receiving 0, 0.03, 0.14 and 0.7 AFB_1/kg bw orally every other day for two weeks. Superinduction of IL-1α mRNA at the low AFB_1 concentration and IL-6 and TNF mRNA at the intermediate concentration was accompanied by suppression of the supernatant level of these cytokines in the exudates of peritoneal macrophages. With respect to cytokines produced by splenic lymphocytes including IL-2, IL-3 and IFN-γ, inhibition of genetic

expression of IL-2 mRNA only was reported and was particularly evident at low doses of AFB_1 (Dugyala and Sharma, 1996). Human monocytes were more sensitive to AFB_1 than murine monocytes in this respect. Human monocytes exposed to a very low concentration of AFB_1 (0.05-1 pg/ml) for 2-12 hours, followed by mitogenic stimulation, showed a decrease in IL-1, IL-6 and TNF-α mRNA levels. This was reflected in a severe decrease in cytokine levels to 10% for IL-1, 20% for IL-6 and 13% for TNF- α of controls at 1pg AFB_1/ml at 12 hours (Rossano *et al.*, 1999). A significant decrease in IL-2 level was reported in mice receiving 0.14 to 0.7 mg AFB_1 orally each day for four weeks (Hatori *et al.*, 1991). Recently a decrease in IL-2 production was shown to be due to decreased IL-2 mRNA expression in murine thymocytes and in human jurkat T cell line. Furthermore, the decrease in IL-2 mRNA levels was explained by a decrease in transcription factors for the aforementioned cytokine, and particularly the nuclear factor of activated T cell and activator protein-1 were affected (Han *et al.*, 1999).

Aflatoxin is transformed in vivo into active metabolites such as AFB_1-8,9-epoxide that bind to DNA and RNA and inhibit RNA and protein synthesis. AFB_1 was demonstrated to inhibit DNA-mediated RNA synthesis in rat liver nuclei and nucleoli in vivo by inhibition of RNA polymerase type II (Yu, 1997, 1981). RNA synthesis is also inhibited upon incubation of synthetic AFB_1-8,9-epoxide with plasmid RNA and RNA polymerase in vitro (Yu *et al.*, 1994). Inhibition of protein synthesis could occur by binding of the dihydrodiol derivative of the epoxide to the cellular proteins. AFB_1-8,9-epoxide can also be detoxified by GST enzymes to a glutathione conjugate. The activity of these enzymes varies among species and as explained above this partially determines the difference in interspecies susceptibility to the toxic and carcinogenic effects of aflatoxin. The effects of variations in these enzymes on immune parameters are unexplored. A bulky adduct can be formed when the imidazole portion of the nucleic acid adduct is positively charged and results in a ring-opened formamidopyrimidine (AFB_1-FAPY) (reviewed by Wang and Groopman, 1999). This bulky adduct is resistant to spontaneous and enzymatic processes of adduct removal and as a consequence can accumulate in DNA and RNA. If this bulky adduct accumulated in the immune cells such as lymphocytes or macrophages, it could inhibit nucleic acid synthesis and indirectly lead to immunosuppression. Corrier (1991) has concluded that inhibition of DNA, RNA, and protein synthesis directly or indirectly impairs the continual proliferation and differentiation of the cells of lymphoid system,

the synthesis of monokines and interleukin polypeptides that regulate the communication network of the immune system and the synthesis of Igs and antibodies.

Genetic susceptibility could possibly modulate the suppressive effect of AFB_1 on cell-mediated immunity although the evidence for this is quite limited. In one report the presence of leukocyte antigen HLA-A on the surface of human peripheral lymphocytes increased the suppressive effect of AFB_1 on the blastogenic response of lymphocytes in vitro compared to lymphocytes without that antigen (Cheng-Ya *et al.*, 1987).

Overall, AFB_1 has a broad effect on both elements of the immune system, but whilst effects on the cellular element occur at low concentrations of AFB_1, the effects on humoral immunity generally require much higher concentrations. The toxic effects on the immune system appear to be directly related to inhibition of DNA, RNA, and protein synthesis. There are however limited data on the effects of AFB_1 on the human immune system.

1.2.3.2 Effect of DON on the immune system

1.2.3.2.1 Cellular effects

Acute exposure to trichothecenes including DON, T-2 toxin and DAS has a radiometric effect with results in depletion of the activity dividing cells in tissues such as bone marrow, lymph nodes, spleen, thymus, and intestinal mucosa (reviewed by Corrier, 1991). T-2 toxin was the most extensively studied in this respect and the radiomimetic effects were demonstrated in numerous laboratory animals, and in animals raised for meat production such as cattle, sheep, turkey and poultry. The same effects were shown in animals and poultry with DON and DAS but effects were less severe.

Low and chronic exposure to trichothecenes can affect the two major cellular elements in the immune system, the lymphocytes and macrophages. The toxic effects can however extend from these mature cells of the immune system to the progenitor cells in the primary lymphoid organs including the thymus and bone marrow where maturation of these cells occurs. The effects of trichothecenes on both lymphocytes and macrophages are specifically discussed below.

The effect of DON on lymphocytes

The effect of DON on lymphocyte blastogenic responses has been studied *in vitro* in several species, including rats, mice, cattle and humans, and also *in vivo* in the first of these species. DON can impair or stimulate lymphocyte proliferation at lower doses (reviewed by Pestka and Bondy, 1990). The blastogenic response of isolated thymic lymphocytes to phytohaemagglutinin (PHA) (T cell antigen) and splenic lymphocytes to either PHA or lipopolysaccharide (LPS) (B cell mitogen) was assessed following daily oral administration of DON (5-50 ppm) for a week to mice, by assessing incorporation of [^{3}H] thymidine into DNA in vitro. A dose dependent decrease in mitogenic response was observes at ≥ 10 ppm in all cells examined with a more pronounced effect in thymic than splenic cells (Robbana-Barnat et al., 1998). DON also inhibited T or T and B lymphocyte proliferation in *in vitro* studies using cells from mice and cattle (Mekhancha-Dahel *et al.*, 1990; Charoenpornsook *et al.*, 1998). Murine splenic lymphocytes which were exposed to DON at concentrations of 25-800 ng/ml and stimulates with PHA *in vitro*, showed a 50% reduction in thymidine incorporation into DNA at DON concentrations above 120 ng/ml (Mekhancha-Dahel *et al.*, 1990). Similarity, the effect of in vitro exposure to DON on bovine peripheral blood lymphocytes, stimulated with either PHA or concanavalin A (Con A) (T cell mitogen) or pokeweed mitogen (PWM) (non specific mitogen for T and B cells) was assessed by many tests including [^{3}H]-thymidine incorporation (Charoenpornsook *et al*, 1998). A 50% reduction in the thymidine incorporation into DNA was obtained at DON concentrations between 70 and 90ng/ml for T cells and at 40ng/ml for T and B cells. In earlier studies by Miller and Atkinson (1986, 1987), exposure of rat lymphocytes to low concentrations of DON (0.005-1ng/ml) enhanced proliferation, however higher concentrations (>50ng/ml) caused a decrease in the proliferation of these cells. In addition, the timing of exposure to DON in relation to mitogen exposure resulted in either enhancement or an inhibition of the blastogenic response of rat lymphocytes (Miller and Atkinson, 1986). Whilst the exposure of lymphocytes to 90ng/ml for 1 hour prior to adding mitogen caused an increase in thymidine incorporation to 130% of control levels, the co-exposure of mitogen and toxin at the same concentration induced a 50-60% reduction in thymidine incorporation.

In summary, DON inhibited both T and B cell functions as measured by lymphoblastogenic response in many species including mice, rat and cattle. However, a short

exposure to a low concentration of DON prior to adding mitogen could have an enhancing effect, as demonstrated in rats.

Human lymphocytes have been shown to be sensitive to the effects of trichothecenes. DON inhibited human T lymphocyte proliferation and was cytotoxic to two human cell lines; K-562 (a human erythroleukemia lymphoblastic cell line), and MIN-GL1 (an EBV transformed human lymphoid B-cell line) at concentrations of DON of 300 and 400ng/ml, respectively using the MTT assay (Visconti *et al.*, 1991). Mekhancha-Dahel *et al.* (1990) showed that DON inhibited human lymphocyte proliferation as measured by thymidine incorporation into DNA. A 50% inhibition was achieved with 100 to 250ng/ml for T or B mitogen stimulated cells and 150ng/ml for unstimulated cells. In another study, human lymphocytes were stimulated with either PWM or PHA and then treated with DON at a range of concentrations (60-1184ng/ml) and proliferation was again measured using thymidine incorporation and lymphocyte proliferation was inhibited in a dose dependent manner (Thuvander et al., 1999). Combinations of DON with other trichothecenes, including T-2 toxin and DAS, were tested at concentrations causing 25% inhibition of thymidine incorporation; in this study there was an antagonistic effect. The inhibitory effect of co-exposure of DON and T-2 toxin or DAS was less marked or similar to the inhibition caused by exposure to T-2 toxin or DAS alone respectively (Thuvander et al., 1999). The authors suggested that this antagonism might result from DON interfering with T-2 toxin and DAS in binding to common sites on cell membranes and as a consequence reducing their toxic effects. Recently, comparative studies of DON, T-2 toxin, DAS and NIV were performed on human lymphocytes and the potency of the inhibitory effects were in the following order T-2 toxin >DAS>NIV>DON (Visconti *et al.*, 1991; Thuvander *et al.*, 1999).

The effect of DON on other cellular immune effects such as delayed type hypersensitivity (DTH) and the time for allograft rejection was assessed mainly in the mouse model. DTH is an immune response mediated by sensitized T lymphocytes. Mice exposed to 25ppm DON in the diet for three weeks were injected subcutaneously in the back of the footpad with keyhole limpet haemocyanin at three different time points. Twenty-four hours after the last injection, the degree of swelling as a percentage of control was measured and found to be decreased to about half that of untreated mice on diets restricted in intake to match intakes in the DON treated group (Pestka *et al.*, 1987). This study is important in confirming the significant

direct effect of DON on DTH and cell mediated immunity whilst permitting exclusion of the hypothesis that feed refusal was the cause of DON related effects. In another study, a decrease in DTH against *Salmonella enteritidis* was found in mice exposed to 2 ppm DON in drinking water for three weeks (Sugita-Konishi *et al.*, 1997).

The effect of DON on macrophages

Phagocytic cells, either the mature or progenitor forms, are affected by trichothecene exposure. The effect of DON (20-640μg/ml) on turkey macrophages isolated from the peritoneal cavity in terms of viability, percentage of damages cells and phagocytic ability was investigated (Kidd *et al.*, 1995). A 50% decrease in macrophage viability was reported at DON concentration of 320 μg/ml; however, at a lower concentration (50 μg/ml), effects were already evident on the adherence of these cells (52% below the control). The phagocytic ability toward both unopsonised (Unops) and opsonised (Ops) SRBC decreased to 58% and 77% of controls respectively at a DON concentration of 400 μg/ml. Furthermore, at the same concentration, the number of Ops SRBCs per macrophage, but not Unops SRBC, was reduced to 50% that of control cultures (Kidd *et al.*, 1995). Parent-Massin and his colleagues (1994 and 1995) tested the proposed mechanism of the toxicity of trichotecenes on haemopoeitic cells using rat and human progenitor cells in vitro. The authors found that T-2 toxin, DON and other related trichothecenes reduced the growth of the aforementioned cells compared to control cells. T-2 toxin was the most potent toxin whereas DON was the least toxic among the trichotecenes studied which also included HT-2 toxin and DAS. In a subsequent study by Lautraite *et al.* (1997), human haemopoeitic progenitor cells were found to be ten times more sensitive than rat progenitor cells based on the concentration of DON required to produce 50% growth inhibition.

1.2.3.2.2 Humoral effects

In addition to the cellular effects of trichothecenes, these toxins also affect humoral immunity. In particular, DON was demonstrated to cause hyperelevation of serum IgA levels coupled with a decrease in serum IgG and IgM both *in vivo* and in *in vitro* studies. In addition, DON causes a decrease in specific serum proteins such as α-globulin (Rotter *et al.*, 1996).

Spleen cells were isolated from B6C3F1 mice fed a diet containing 25ppm DON for two weeks and incubated with T dependent antigen SRBC (Pestka *et al.*, 1987). The number of splenic cells forming plaques decreased to 37 and 38% of two control groups fed either the toxin-free diet or the same diet at a restricted rate. Robbana-Barnat *et al.* (1988) reported a significant decrease in thymus weight of BALB/c mice occurred after addition of DON (>10ppm) to the diet for one or two weeks and this effect was associated with a decrease in antibody production to SRBC *in vitro*. In contrast, Tryphonas *et al.* (1986) observed no effect on SRBC response but using a lower level of DON <4ppm in the diet administered to Swiss Webster mice. The effect of DON on serum albumin and subtypes of globulin was investigated in mice (Tryphonas *et al.*, 1986). A marked decrease in α2-globulin was observed, together with a slight decrease in α1-globulin, and a slight increase in albumin level in mice fed a diet containing 1 mg DON/kg bw. Furthermore, mice fed a diet containing 0.5mg DON/kg bw showed a significant reduction in both α2-globulin and b-globulin. Interestingly, withdrawal of DON and a return to a normal basal diet for 40 days resulted in a reduction of α1-globulin whilst α2-globulin levels returned to those of control mice (Tryphonas *et al.*, 1986). The cause and significance of these changes in globulin fraction upon withdrawal of the toxin from the diet is not known.

A striking finding in the mouse model is that although treatment with DON causes a decrease in total serum IgM and IgG, at the same time there is an increase in total serum IgA (Pestka *et al.*, 1989). IgA production is regulated in Payer's patches in the sub mucosa of the gastrointestinal tract. Upon exposure to antigen in the lumen of gastrointestinal tract there is activation, proliferation, and differentiation of B cells into IgA secreting progenitor cells. These processes are regulated, in the Payer's patches, by T helper and accessory cells. The IgA secreting progenitor cells migrate into systemic compartments (including the lymph nodes and spleen) and the mucosa of the intestine where maturation into IgA secreting plasma cells takes place (reviewed by Pestka and Bondy, 1990). It is postulated that the Payer's patches are the targets for

DON toxicity upon oral exposure. In Payer's patches, DON could interfere with the cytokine production of the T helper cells or accessory cells or act directly on the B cells. As a consequence a premature differentiation of IgA committed progenitor cells into IgA secreting plasma cells happens in the Payer's patches. The possibility of a dysregulation hypothesis in this process is supported by the observation of a preponderance of the polymeric form of IgA in the serum of DON treated animals (Peska *et al.*, 1989). Further support for this dysregulation comes from studies of Payer's patches and splenic lymphocytes isolated from DON treated mice. Both Payer's patches and to lesser extent splenic lymphocytes from DON treated mice produced high levels of IgA and IgA secreting cells when cells were either unstimulated or mitogen stimulated compared with those from untreated mice (Pestka *et al.*, 1989; Bondy and Pestka, 1991). Furthermore, in the same study a significant increase in serum IgA, with a slight elevation in IgG levels, was found in mice treated with 25ppm DON for 8 weeks. The increase in serum IgA was associated with increase in IgA immune complexes, mesangial IgA accumulation and haematuria, signs which persisted up to three months after withdrawal of the toxin from the diet (Dong and Pestka, 1993).

The difference between continuous feeding and intermittent feeding with DON contaminated diet on Ig production in mice was investigated by Banotai *et al.* (1999). In this study three groups of mice were included; a control group fed uncontaminated diet, a second group fed DON contaminated diet (20ppm) for 13 weeks, and a third group fed alternately with DON contaminated and control diet on a weekly basis for the same length of time (intermittent diet). In the intermittent feeding regime the decrease in IgG and IgM was not accompanied by an elevation in serum IgA as was observed in the condition of continuous treatment. In addition, whereas haematuria did occur during the intermittent regime no accumulation of immune complexes was found in the mesangia of kidneys.

In vitro studies have also demonstrated an increase in IgA production coupled with a decrease in IgM and IgG. In an in vitro study by Minervini *et al.* (1993) the effect of DON on Ig production was tested on a murine CH12LX B cell line that mainly express membrane (m) bound Ig of the IgM class (mIgM+). DON caused an increase in IgA production in a dose dependent manner at concentrations of 5-50ng/ml but a decrease was observed at concentrations of 100ng/ml or higher. Whilst low concentrations of DON failed to stimulate IgM, concentrations ≥ 50ng/ml inhibited IgM production. In the same study the authors evaluated the effect of DON on

DNA synthesis, cell proliferation and protein synthesis using [^{3}H] thymidine incorporation, the MTT assay and leucine incorporation respectively (Minervini *et al.*, 1993). The 50% inhibition for these three parameters was at concentrations of DON of 130, 120 and 110 ng/ml respectively. Furthermore, partial protein synthesis inhibition was observed at concentrations of DON as low as 10ng/ml. From these findings the authors suggested that the reduction in Ig secretion was due to protein synthesis inhibition. However, Ig stimulation is more difficult to explain as it occurs even with partial protein inhibition. The authors suggested that DON may inhibit the synthesis of a selective protein, with enhancement of switching and differentiation of mIgM+ into mIgA+ and increased IgA production. In a further in vitro experiment DON, at concentration between 10-1000ng/ml, inhibited IgA, IgG and IgM production in both unstimulated and mitogen stimulated Payer's patches and spleen lymphocytes (either crude preparation or purified B cells) and also decreased DNA and protein synthesis in the crude lymphocyte cultures (Warner *et al.*, 1994). Recently the effect of DON on Ig producton in PWM stimulated human lymphocytes was evaluated in vitro and a decrease in IgA, IgM and IgG was detected (Thuvander *et al.*, 1999). There was no significant difference between the dose needed to cause 50% inhibition of lymphocyte proliferation or Ig production (Thuvander *et al.*, 1999).

1.2.3.2.3 Host resistance

The overall result of inhibition of cellular functions, e.g. suppression of T and B cell activity, depression of DTH, increased time for allograft rejection and decreased phagocytic activity and humoral immunity, would be a decrease in the host resistance towards pathogens. In relation to in vivo studies, mice fed a diet containing DON (0.5 and 1 mg/kg bw) for five weeks showed a dose dependent decrease in time to death following exposure to *Listeria monocytogenes* (Tryphonas *et al.*, 1986). Similarly Pestka *et al.* (1987) found that dietary exposure of mice to DON (25mg/kg bw) for two weeks significantly increased splenic *Listeria* counts. This was attributed in part to the feed refusal caused by the toxin, which can be an immunosuppressive effect in itself.

1.2.3.2.4 Effect of DON on lymphocyte subsets, macrophages and cytokine expression and production

DON and other trichothecenes such as T-2 toxin modulate the transcription and translation of cytokines produced by mitogen stimulated T helper cells in vitro. As a consequence of modulation of cytokine levels, DON can deregulate Ig production by B cells. Purified $CD4^{+}/CD8^{-}$ T cells pulsed with DON and mitogen for 24 hours resulted in a 3- to 5-fold increase in IgA production with no concomitant increase in IgG and IgM by B cells in co-culture with the T cells for 7 days. A possible mechanism for enhanced T cell helper activity by DON is increased interleukin production (IL-4, IL-5 and IL-6), cytokines which are responsible for proliferation and differentiation of B cells into IgA secreting cells (Warner *et al.*, 1994). In vitro exposure to DON, T-2 toxin and other trichothecenes for either two or seven days modulated Il-2, IL-4 and IL-5 protein production and messenger RNA (mRNA) expression in murine CD4+ cells (Ouyang *et al.*, 1995). T-2 toxin was more potent than DON, exerting its effect at a concentration 250 times lower than DON. A continuous 7 days exposure of murine CD4+ cells to DON led to increased mRNA expression of IL-2, IL-4 and IL-5 followed by increased protein levels of the same cytokines. In contrast, decreased IL-6 was observed but without an effect on mRNA expression level (Azcona-Olivera *et al.*, 1995a). Inhibition of cell proliferation occurred at the same DON concentrations, which led to superinduction of interleukins (Azcona Olivera *et al.*, 1995a). In most previous in vitro CD4+ models, Con A was the only mitogen used, and this only partially activates T cells via its receptor (Ouyang *et al.*, 1996a). Partial activation of T cells derives from the occupancy of the TCR complex by peptide antigens presented by MHC on the surface of antigen presenting cells followed by hydrolysis of phosphatidylinositol-4-5 biphosphate to inositol 1, 4, 5-triphosphate and 1,2-sn-diacetylglycerol (Mueller *et al.*, 1989). The former product increases intracellular calcium and the latter product stimulated protein kinase C, but this pathway is insufficient for maximal production of cytokine in purified CD4+ cells. For complete activation of naïve T cells by antigen and maximal production of cytokines, two signals are required. The first signal is the one mentioned above, derived from the occupancy of TCR, however the second co-stimulatory signal is derived from the interaction of CD28 on the surface of T cells with the B-7 molecule on the surface of the antigen binding cells (Kuby, 1997b). Ouyang *et al.* (1996a) sought to clarify whether cytokines are superinduced by DON when T cells

are already fully activated by phorbol ester and ionomycin. DON can superinduce the mRNA and protein expression of cytokines including IL-2, IL-4 and IL-5 under the former system of activation of T cells. This superinduction was much greater in terms of the kinetics and magnitude of cytokine production than cytokine superinduction produced by other mitogens such as Con A. In addition, the superinduction of cytokine mRNA was specific in that there was an increase in IL-2 mRNA but not in that of the housekeeping gene studied. Ouyang *et al.* (1996a) also demonstrated that increase in the transcription and translation of these cytokines occurred at concentrations of DON that impair cell proliferation and partially inhibit protein synthesis.

An important observation, in the in vivo mouse studies with DON is that superinduction of cytokines did not require the presence of mitogenic stimulant as was the case in the in vitro studies. In mice, a single oral exposure to DON (0-25mg/kg bw), without mitogen, cause a non specific elevation in expression of mRNA of both Th1 (IFN-γ and IL-2), Th2 cytokines (IL-4 and IL-10) and proinflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-12p40). This elevation in expression of mRNA of the aforementioned cytokines occurred only at concentrations $\geq$ 5 mg/kg bw in both spleen and Payer's patch, although the effect was more pronounced in spleen (Zhou *et al.*, 1997). Subsequently Zhou *et al.* (1998) investigated cytokine mRNA superinduction in mice under a number of dosing regimes with DON including a gavage for 1 or 2 days (5 or 25mg/kg); short term repetitive exposure every other day for a week (0-5mg/kg) and chronic exposure by feeding mice a diet mixed with DON for four weeks (10 and 25 mg/kg). The authors found that, prior exposure to DON can induce a state of tolerance to a second exposure on the second day with respect to superinduction of cytokine mRNA. However in either short, repeated or chronic exposure to DON a selective superinduction in cytokines was reported.

The stimulation of cytokine production in vivo in the absence of mitogen stimulation could result from exposure to low doses of endotoxins, e.g. LPS, that arise from gram-negative bacterial flora, which normally inhabit the lumen of the intestine and can pass through the mucosa of the gastrointestinal tract. However, this level of LPS does not disturb the homeostasis in the body in an unstressed individual not exposed at the same time to other environmental stimuli (reviewed by Roth *et al.*, 1997). In contrast, co-exposure to LPS with other environmental stimuli such as trichothecenes culminates in a serious insult to the immune system. This has been demonstrated in a mouse model where co-exposure to an oral dose of DON and ip exposure to

different doses of LPS superinduced proinflammatory cytokine mRNA expression, particularly TNF-α, in a synergistic manner (Zhou *et al.*, 1999). Furthermore in this latter study, severe injury in most of the lymphoid tissues occurred with marked apoptosis of cells at the highest doses of both toxins (5mg/kg LPS and 25 mg/kg DON). The reason suggested for the severe injury was that TNF-a might mediate apoptosis either directly in the lymphocytes or indirectly by activating other mediator of apoptosis such as glucocorticoids and prostaglandin E2 (Zhou *et al.*, 1999).

1.2.3.2.5 Mechanism of immunomodulation

The cellular-molecular basis for trichothecenes-induced immunosuppression is thought to be either directly or indirectly related to inhibition of protein synthesis (reviewed by Corrier., 1991). In eukaryotics cells, initiation of protein synthesis, and elongation and termination of the polypeptide chain takes place on ribosomes and requires peptidyl transferase. All trichothecenes inhibit protein synthesis through inhibition of peptidyl transferase activity. As a consequence of protein synthesis inhibition, DNA and RNA synthesis are also inhibited (Mclaughlin *et al.*, 1977; Ehrlich and Daigle, 1987). Other possible mechanisms such as increased apoptosis, impaired membrane function, and altered intercellular communication could also play contributory roles in the suppressive effect of trichothecenes on the immune system (reviewed by Rotter *et al.*, 1996). The immunostimulatory effect of trichothecenes, particularly DON, is probably affected through perturbation of the regulatory mechanisms of cytokine production. The stimulation of cytokine production in mice by DON has been ascribes to both transcriptional and post-transcriptional effects (Ouyang *et al.*, 1996b; Li *et al.*, 1997). At the post-transcriptional level, treatment of murine lymphocytes with DON appears to stabilize IL-2 mRNA in a dose dependent manner (Li *et al.*, 1997). DON also appears to inhibit IκB, a cytoplasmic inhibitor of the NF-κB/Rel family of transcription factors which regulate transcription of a number of cytokines including IL-2, and IFN-g and cytokine receptor genes (Ouyang *et al.*, 1996a). Among the NF-κB/Rel family of proteins, DON appears to specifically upregulate c-Rel (Ouyang *et al.*, 1996b). Rotter *et al.* (1996) proposed that cytokine superinduction and elevation of IgA production may occur through the following cascade of vents; DON selectively inhibits the synthesis of proteins that dowregulate the expression of T helper cytokine mRNA, leading to enhanced expression and elevated T helper cytokine production. The end result is cytokine switching and terminal

differentiation of B cells into IgA secreting cells. The authors also suggested that Payer's patches in particular, may be susceptible to this cascade of events, because of the high levels of exposure to DON through the enterohepatic circulation.

1.2.3.3 Effect of FB_1 on the immune system

Fumonisins are a family of toxins of which only FB1 and FB2 have been investigated for immunotoxic effects Overall there are few studies and these are limited to mouse model and avian species with no corresponding studies in human cells.

1.2.3.3.1 Cellular effects

The effect of FB_1 on lymphocytes

The effect of FB_1 on lymphocytes viability, morphology and lymphoblastogenic response to mitogens was investigated in several species including chickens, turkeys, barrows, calves and mice. Broiler chicks exposed to a diet mixed with *Fusarium proliferatum* culture containing FB_1 and FB_2 and moniliformin showed a dose and time dependent decrease in lymphocyte viability using the MTT assay. In the same study morphological changes of both lymphocytes and erythrocytes and even the immature form of the latter cells were revealed by microscopic examination (Dombrink-Kurtzman *et al.*, 1993). In another in vitro study, turkey lymphocytes exposed to FB_1 or FB_2 showed a decrease in proliferation compared to control cells (Dombrink-Kurtzman *et al.*, 1994). The 50% inhibition of proliferation in response to mitogen was achieved with a dose of FB_1 three to four fold higher than FB_2 (1.4 ug FB_1/ml compared with 0.4 ug FB_2/ml). Turkey lymphocytes also showed morphological changes including vacuolation of cytoplasm at exposures to 12.5 ug/ml FB_1 or 0.8 ug/ml FB_2 (Dombrink-Kurtzman *et al.*, 1994)

Barrows fed a diet containing FB_1 (100ppm) for four weeks showed a decrease in PHA stimulated lymphoblastognic response as measure by [^{3}H] thymidine uptake (Harvey *et al.*, 1996). Calves fed a diet contaminated with FB_1 at 15-148ppm also showed a decrease in lymphoblastogenic response at the highest concentration (Osweiler *et al.*, 1993). In mice, Martinova and Merril (1995) and Martinova (1996) reported FB_1 not to be mitogenic, rather it inhibited incorporation of thymidine into DNA of murine lymphocytes in culture.

The effect of FB_1 on macrophages

The effect of FB_1 on macrophages has been studied in chickens. FB_1 decreased the viability and phagocytic activity of peritoneal chiecken macrophages. However, the same toxin was stimulatory to phagocytic activity in a chicken macrophage cell line. In vitro studies by Qureshi and Hagler (1992) have shown that FB_1 (0.5 to 10 ug/ml) could decrease the viability of chicken peritoneal macrophages. Significant morphological alterations in the form of cytoplasmic blebbing and varying degrees of nuclear disintegration were observed in the same study and the effects were dose related up to 20ug/ml. As regards phagocytic activity, chicken macrophages exposed to FB_1 exhibited decreased activity toward opsonised and unopsonised SRBC but this effect was not dose related. In apparent contrast, a chicken macrophage cell line, exposed to FB_1 but without mitogen stimulation, showed an increased capacity for cytotoxicity towards a chicken B lymphoblastoid tumor cell line (Qureshi and Hagler, 1992). The above differences may arise from the differences in the type of cells studied or the different assays employed.

1.2.3.3.2 Humoral effects

FB_1 can alter the humoral immune response in BALB/c mice depending on the schedule of treatment. A single i.p. injection of FB_1 (0-2.5mg/kg bw) within 20 hours of administration of SRBC caused a reduction in the primary immune response to these cells, with a decrease in the number of antibody secreting cells at FB_1 concentrations $\geq$ 0.25 mg/kg bw. (Martinova and Merrill, 1995). Similarly, concurrent ip administration of single dose of FB_1 (0.25-2.5 mg/kg bw) with SRBC caused a decrease in the number of antibody secreting cells in a dose dependent manner (Martinova, 1996). In contrast multiple daily injections of FB_1 starting from the day of administration of SRBC, caused an increase in the number of antibody secreting cells at concentration $\geq$ 0.1mg/kg bw (Martinova and Merrill, 1995). In the same study, the ability of FB_1 to induce antigen binding cells was assayed by measuring the number of rosette forming cells in the mouse spleen, after a single and a second exposure to FB_1 (Martinova and Merrill, 1995). After a single exposure to FB_1 (0-5 mg/kg bw, ip), there was a significant increase in the percentage of rosette forming cells at the two highest doses of FB_1 (2.5, 5 mg/kg bw) compared with those from control mice. Moreover, a second exposure to FB_1 caused a significant increase in the percentage of rosette forming cells at much lower doses of FB_1. Based on this finding, it

was concluded that this toxin could induce antigen binding cells in spleen, and also affect the cell memory formation to FB_1 (Martinova and Merrill, 1995).

1.2.3.3.3 Mechanism of immunomodulation

Sphingolipid metabolism plays a pivotal role in controlling the development, differentiation, activation and apoptosis of immune cells. FB_1 can disrupt sphingolipid metabolism by inhibition of ceramide synthase and as result lead to an accumulation of sphinganine (Sa) and to lesser extent of sphingosine (So). So has been shown to activate the retinoblastoma protein (a tumor suppressor protein) by dephosphorelation and this causes inhibition of cell growth and arrest of the cell cycle at the G0/G1 phase (Dbaibo *et al.*, 1995). Furthermore, activated retinoblastoma protein results in inhibition of E2F transcription factor, inhibiting its binding to DNA and consequently resulting in reduced gene transcription. Arrest of the cell in G0/G1 phase may perturb DNA synthesis and culminate in apoptosis (Martinova, 1998). Concurrent ip administration of a single dose of either So or Sa at concentrations of 0.25-3.5mg/kg bw reduced the number of antibody secreting cells in dose dependent manner (Martinova, 1996). This may indicate that the inhibition of antibody secreting cells by a single exposure to FB_1 occurred via accumulation of So and Sa in lymphocytes.

Sphingomyelinase is a plasma membrane enzyme that catalyses the hydrolysis of sphingomyelin into second lipid messenger molecules including ceramide and initiated to sphingomyelin cycle (Martinova, 1998). This cycle is associated with signaling from the antigen-specific T cell receptor to stimulate the proliferation of T cells. Ip injection of mice with FB_1 (1mg/kg bw) caused a 2-fold increase in the activity of the former enzyme coupled with a 2.5-fold decrease in sphingomyelin concentration in the thymus (Martinova *et al.*, 1995). Moreover, a doubling in the concentration of cellular ceramide was observed in both thymus and spleen. Accumulation of this product has been shown to inhibit T human cell proliferation in vitro (Borchardt *et al*, 1994). Therefore this could be one of the mechanisms by which FB_1 inhibits cell proliferation.

FB_1 disrupts the expression of CD4, CD8 and CD45 molecules on the surface of T lymphocytes and consequently leads to apoptosis of these cells. These molecules are involved in the inhibition of the signaling process, by phosphorylation of tyrosine in the cytoplasmic domain

of the TCR complex, following antigenic stimulation (Martinova, 1998). It was found that ip administration of FB_1 (1mg/kg bw) to mice increased the expression of CD45 and CD8 and decreased the expression of CD4 in lymphocytes within two hours of treatment and this effect was associated with increased apoptosis (Martinova, 1996).

1.2.4 Natural co-occurrence of mycotoxins

Common factors such as drought, poor irrigation, and damage of plants by birds or insects cause plant stress, and hence increases the susceptibility to infection by more than one fungus, the result being co-occurrence of mycotoxins. The natural co-occurrence of mycotoxins has been reported worldwide. Corn samples collected from Philippines, Thailand and Indonesia have been surveyed for natural occurrence of aflatoxins and fumonisins. FB_1 were found in over 50 % of corn samples in individual countries, and their co-occurrences with aflatoxins at the incidence of 48 % were noted (Yamashita *et al.*, 1997). Additional studies have found that as many as 67-72 % of corn samples collected from Thailand was coincidentally contaminated with aflatoxins and fumonisins (Yoshizawa *et al.*, 1996). Recently, all the aflatoxin-positive corn samples from Brazil were found to be co-contaminated with fumonisin (Vargas, 2001; Ono, 2001). Moreover, the natural co-occurrence of Fusarium mycotoxins including fumonisin and deoxynivalenol has been found in corn from Korea (Sohn, 1999) and Indonesia (Ali, 1998).

The natural co-occurrence of aflatoxins, fumonisins and trichothecenes including DON and NIV has been reported, for example in corn samples in North Vietnam (Wang *et al.*, 1995). The co-occurrence of fumonisins and aflatoxins was also reported in corn from Southeast Asia (Thiland, Philippines and Indonesia) (Yamashita *et al.*, 1995; Yoshizawa *et al.*, 1996) and the state of Georgia in the USA (Chamberlain *et al.*, 1993). Fumonisins were also found to co-occur with DON in corn samples from South Africa (Sydenham *et al.*, 1990). There have been reports of synergistic effects of mycotoxins in animals (Harvey *et al.*, 1996; Miller, 1995). The fact that human are likely to be to exposed to mixtures of toxins means potential interactions are of prime importance for investigation.

1.2.5 Medicinal Plants

Plants have played a significant role in maintaining human health and improving the quality of human life for thousands of years, and have served humans well as valuable components of seasonings, beverages, cosmetics, dyes, and medicines. Herbal medicine is based on the premise that plants contain natural substances that can promote health and alleviate illness.

1.2.5.1 Immunomodulation by medicinal plants

Compounds that are capable of interacting with the immune system to upregulate or downregulate specific aspects of the host response can be classified as immunomodulators or biologic response modifiers. In recent years, natural products from the plant kingdom have been investigated for their immune modulating potential against infectious and neoplastic diseases. Herbal therapy, or "phytomedicine", the therapeutic use of plants, plant parts, or plant-derived substances, is generally considered a form of complementary medicine (Israelsen, 1995). In traditional medicine different plant parts are believed to have specific medicinal properties that were identified over centuries of trial-and-error observation (Jones, 1996). Among these properties is the ability to stimulate the body's disease fighting mechanisms, including those now considered facets of the immune system.

Herbal treatments that affect the immune system may be also classified as either adaptogens or immunostimulants, or both (Block and Mead, 2003). Adaptogens include substances that are reputed to increase the body's resistance to physical, chemical and biological stressors. Immunostimulants (immunopotentiators, immune enhancers), as opposed to immunosuppressors, are agents that activate the body's nonspecific defense mechanism against infectious organisms such as viral and bacterial pathogens or against neoplastic cells. The primary goal of immunotherapy is to stimulate the activity of immunologic cells that are indirect local contact with neoplastic cells or infectious agents (Wolf, 1994). In general, it is clamed that herbal immunostimulants have minimal effects on normal immune response, but may help rectify the moderately compromised cell-mediated immune response (Parnham, 1996; Schulz *et al.*, 1998).

The usage of medicinal plants appears particularly high among patients with chronic diseases, including cancer (Ernst and Cassileth, 1998), autoimmune diseases (Pal, 1998), asthma (Andrews *et al.*, 1998; Ernst, 1998), and acquired immunodeficiency syndrome (AIDS) (Ernst,

1997). This along with the fact that Echinacea and goldenseal, putative immune-enhancers, are among the top-selling herbal products in the United States (Scimone and Scimone, 1998), suggests that many patients turn to herbals in order to boost their immune system.

Several herbal products that may enhance the function of the immune system are available. These include Echinacea, astragalus, ginseng, licorice, cat's claw and garlic. Herbs that are rich in flavonoids, vitamin C, or the carotenoids may enhance immune function. The flavonoid-rich herbs may also possess mild anti-inflammatory action (Craig, 1999). Among the medicinal plants thought to function (at least in part) as immunostimulants, Echinacea, astragalus and ginseng have been thoroughly studied (reviewed by Block and Mead, 2003). Based on early studies, some of these plant extracts appear to affect humoral (acquired) immunity, but most appear to enhance cellular (innate) immunity. Changes in humoral immunity would include mitogenic effects on B lymphocytes (increased proliferation) and production of specific types of antibodies. Changes in cell-mediated immunity, the more common mechanism found in phytochemical studies, are measured in terms of NK cell number and activity, macrophage activation, phagocytic activity, and proliferation of specific T lymphocyte subsets.

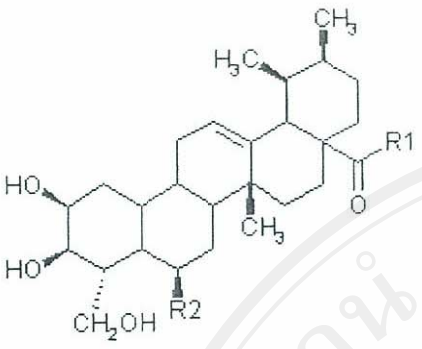
1.2.5.2 *Centella asiatica* (Linn) Urban

Centella asiatica is known as gotu kola, Indian Pennywort, Mandookaparni and Buobok. It is a small herbaceous plant, belonging to the family Umbelliferae. *C. asiatica* is a prostrate, faintly aromatic, perennial herb, up to 2m long, commonly found in abundance on moist, sandy or clay soils, often in large clumps forming a dense green carpet. It has been used for centuries as a medicinal herb. Both the leaves and the entire plants can be used therapeutically.

Figure 1.13 A *Centella asiatica* (Linn.) Urban.

1.2.5.2.1 Chemical composition

Centella asiatica contains a variety of biologically active chemical groups. It contains triterpenoid glycosides (saponins), phytosterols and a volatile oil consisting of vallerin, camphor, and unidentified terpene acetate that comprises 35% of the total oil content. The triterpenoid glycosides include asiaticoside, asiatic acid, madecassic acid, madecassoside, oxyasiaticoside, brahminoside, brahmoside, centelloside. These triterpene saponins and their sapogenins are mainly responsible for the wound healing and vascular effects. Brahmoside and brahminoside may be responsible for CNS and utero relaxant actions, but are yet to be confirmed by clinical studies. The phytosterols include campesterol, stigmasterol and sitosterol. In addition, the total extract contains vitamins B and C, tannins, several amino acids, an alkaloid named hydrocotylin, flavonoids, and other components with no known pharmacological activity.



Compound Name	R1	R2
Asiatic acid	OH	H
Madecassic acid	OH	O
Asiaticoside	O-glu-gluc-gluc	H
Madecassoside	O-glu-gluc-gluc	OH

Figure 1.14. The main active components of *C. asiatica*; asiatica acid, madecassic acid, asiaticoside, and madecassoside.

1.2.5.2.2 Pharmacological activities

Wound healing activity

C. asiatica has been documented to support wound healing in several scientific studies (Suguna *et al.*, 1996; MacKay and Miller, 2003). One of the primary mechanisms of action of this plant appears to be the stimulation of type-1 collagen production (Maquart *et al.*, 1990; Tenni *et al.*, 1988). The drug "Titrated Extract from Centella asiatica" (TECA), which is a mixture of 3 terpenes extracted from a tropical plant: asiatic acid (30%, w/w), madecassic acid (30%, w/w) and asiaticoside (40%, w/w), was tested on human skin fibroblast monolayer cultures. TECA increased collagen synthesis in a dose-dependent fashion (Maquart *et al.*, 1990). The principal terpenoids extracted from *C. asiatica*, include asiatic acid, madecassic acid and asiaticoside, were found to be the components responsible for stimulation of collagen synthesis (Maquart *et al.*, 1990, 1999; Shukla *et al.*, 1999; Widgerow *et al.*, 2000).

Animal studies have consistently shown topical application of *C. asiatica* to a sutured wound significantly increased the breaking strength of the wound. Shukla *et al.* (1999) showed a 0.2% asiaticoside solution applied topically twice daily for seven days to punch wounds in guinea pigs resulted in 56% increase in hydroxyproline, 57% increase in tensile strength, increased collagen content, and better epithelialization compared to controls. Using the same punch wound model the researchers demonstrated an oral dose of 1 mg/kg for seven days produced a 28%

reduction in wound area and a significant increase of tensile strength and hydroxyproline content of the wound.

Recent studies have shown that changes in gene expression in human fibroblast induced by *C. asiatica* (Coldren *et al.*, 2003; Lu *et al.*, 2004). Fibroblast cells grown in culture were used as a model system to evaluate the stimulation of wound healing by TECA as well as by the four principal triterpenoid components of *C. asiatica*. TECA treatment effects the expression of genes involved in angiogenesis and the remodeling of extracellular matrix, as well as diverse growth factor genes. The extent of expression change of TNFAIP6, an extracellular hyaluronan binding protein, was found to be largely dose-dependent, to respond most strongly to the free acids asiatic acid and madecassic acid, and to increase in expression over 48 hours of treatment (Coldren *et al.*, 2003). Using cDNA microarray technology, the alteration of gene expression profiles was determined for human dermal fibroblasts in vitro in the presence of asiaticoside (30 μg/ml). Fifty-four genes, with known functions for cell proliferation, cell cycle progression and synthesis of ECM, were significantly upregulated (Lu *et al.*, 2004).

Other studies on wound healing actions emphasized the role of asiaticoside in the increased levels of antioxidants, which can also be responsible for accelerated wound healing (Shukla *et al.*, 1999). Angiogenesis plays an important role in wound healing since the newly formed blood vessels help the hypoxic wounds to attain normoxic conditions. Asiaticoside prompted angiogenesis in both *in vivo* and *in vitro* models (Shukla *et al.*, 1999). In cases of vascular injury, thrombosis, acute myocardial infarction, and other peripheral vascular diseases, a higher number of circulating endothelial cells was detected. For example, in one study, patients with postphlebetic syndrome (PPS) showed a greater number of circulating endothelial cells compared to the normal subjects. During a three-week treatment with *C. asiatica* triterpenic fraction (CATF), PPS patients who received 90 mg CATF daily in three divided dosages showed a significant decrease in circulating endothelial cells, thereby indicating the effectiveness of *C. asiatica* in protecting the integrity of vascular intima. The lower number of circulating endothelial cells could be attributed to the protective effect of CATF on vascular intima integrity (Montecchio *et al.*, 1991).

Anti-tumor activity

The crude extract (CE) of *C. asiatica* and its partially purified fractions (AF) was tested for their anti-tumor activity. AF significantly inhibited the proliferation of the transformed cell lines in Ehrlich ascites tumor cells and Dalton's lymphoma ascites tumor cells with no toxic effects on normal human lymphocytes. AF was also found to inhibit the development of mouse lung fibroblast. Oral administration of both CE and AF retarded the development of solid and ascites tumors, and increased the life span of tumor-bearing mice. Tritiated thymidine, uridine and leucine incorporation assays suggested that the fraction acts directly on DNA synthesis (Babu *et al.*, 1995). A recent study has shown chemopreventive effect of *C. asiatica* extract on colon tumorigenesis. Treatment with *C. asiatica* extract decreased the number of aberrant crypt foci (ACF), increased the induction of apoptotic cells in the colonic mucosa, reduced the numbers and sizes of neoplasms, and reduced the multiplicity of neoplasms (Bunpo *et al.*, 2004).

Anti-gastric ulcer activity

An extract of *C. asiatica* significantly inhibited gastric ulceration induced by cold and restraint stress (Chatterjee *et al.*, 1992), by ethanol (Cheng and Koo, 2000), and by acetic acid (Cheng *et al.*, 2004). Fresh juice of *C. asiatica* also protected aspirin- and pyloric ligation-induced gastric ulceration (Sairam *et al.*, 2001). The mechanism of action may be due to strengthening of the mucosal barrier and reducing the damaging effects of free radicals. Cheng *et al.* (2004) examined effect of *C. asiatica* water extract (CE) and asiaticoside, an active constituent of CE, on acetic acid induced gastric ulcers in rats. They were found to reduce the size of the ulcers, with a concomitant attenuation of myeloperoxidase activity at the ulcer tissues. Epithelial cell proliferation and angiogenesis were on the other hand promoted. The expression of basic fibroblast growth factor, an important angiogenic factor, was also upregulated in the ulcer tissues in rats treated with extracts.

Anti-psoriatic activity

Aqueous extracts of *C. asiatica* inhibited keratinocyte replication prior and after treatment with polyvinylpolypyrrolidone (PVPP). The effect produced by *C. asiatica* is due to its two constituent triterpenoid glycosides madecassoside and asiaticoside (Sampson et al., 2001).

Antioxidant activity

The administration of asiaticoside, an isolated constituent of *C. asiatica*, significantly increased the levels of superoxide dismutase, catalase, glutathione peroxidase, vitamin E and ascorbic acid in excision-type cutaneous wounds in rats. The level of antioxidant activity was highest during the initial stages of treatment (Shukla *et al.*, 1999). Another study found that oral treatment with 50 mg/kg/day of crude methanol extract of *C. asiatica* for 14 days significantly increased the anti-oxidant enzymes, like superoxide dismutase, catalase and glutathione peroxidase, and anti-oxidants like glutathione and ascorbic acid decreased in lymphoma-bearing mice (Jayashree *et al.*, 2003).

Immunomodulating activity

Methanol extracts of whole plant of *C. asiatica* (0.18% of asiaticoside) significantly increased the phagocytic index and total white blood cell count (Jayathirtha and Mishra, 2004). A recent study showed that the degraded derivatives of pectin isolated from *C. asiatica* had immunostimulating activities (Wang *et al.*, 2003).

Anti-depressant activity

Antidepressant activity of total triterpenes from *C. asiatica* was evaluated in a forced swimming test. Total triterpenes from *C. asiatica* reduced the immobility time and ameliorated the imbalance of amino acid levels (Chen *et al.*, 2003).

Central nervous system activity

Oxidative stress or an impaired endogenous anti-oxidant mechanism is an important factor that has been implicated in Alzheimer's disease (AD) and cognitive deficits seen in the elderly. An aqueous extract of *C. asiatica* is effective in preventing the cognitive deficits, as well as the oxidative stress (Veerendra Kumar and Gupta, 2002, 2003; Gupta *et al.*, 2003). For example, the aqueous extract has been tested in paradigms of learning and memory. All doses of aqueous extract (100-300 mg/kg) improved learning and memory in rats. An aqueous extract also significantly decreased the brain levels of malondialdehyde (MDA) and simultaneously increased levels of glutathione and catalase (Veerendra Kumar and Gupta, 2002). Asiatic acid and its semi-

synthesized derivatives have also been evaluated in regard to neuroprotective activity. They significantly mitigated the neurotoxicity induced by glutamate and attenuated decreases in the levels of glutathione, glutathione peroxidase and other enzymes, which participate in the cellular defense mechanisms blunting oxidative stress. Furthermore, they significantly reduced the overproduction of NO induced by glutamate. These results showed that asiatic acid and its derivatives exerted significant neuroprotective effects on cultured cortical cells by their potentiation of the cellular oxidative defense mechanism (Lee *et al.*, 2000).

Radioprotection activity

A few studies have been conducted on radioprotective activity of *C. asiatica*. Radiations are known to cause behavioral perturbations, even at very low doses. Administration of *C. asiatica* (100 mg/kg bw) rendered significant radioprotection against radiation-induced body weight loss and CTA. *C. asiatica* could be useful in preventing radiation-induced behavioral changes during clinical radiotherapy (Shobi and Goel, 2001). The same dose of *C. asiatica* increased the survival time of the mice significantly after treatment with a sublethal dose of Co 60 gamma radiation (Sharma and Sharma, 2002). Another study showed that madecassol, one of the active components from *C. asiatica*, is able to reduce acute radiation reactions by its anti-inflammatory activity (Chen *et al.*, 1999).

Effect on hypertension and microangiopathy

Many clinical studies have evaluated the microcirculatory alteration effects of the total triterpenic fraction of *C. asiatica* (TTFCA) in patients with hypertensive microangiopathy (Incandela *et al.*, 2001a; 2001b; 2001c; 2001d; De Sanctis *et al.*, 2001; Cesarone *et al.*, 2001a; 2001b; 2001c; 2001d; Belcaro *et al.*, 1990a; 1990b). TTFCA is effective in improving venous wall alterations in chronic venous hypertension and in protecting the venous endothelium. TTFCA is active in connective tissue modulation, improves the synthesis of collagen and other tissue proteins by modulating the action of fibroblasts in the vein wall, and stimulates collagen remodeling in and around the venous wall. TTFCA is active on the microcirculation in venous and diabetic microangiopathy by improving microcirculation and decreasing capillary permeability. Signs and symptoms of venous hypertension and edema are improved by treatment

of TTFCA. The remodeling of collagen synthesis could be one of the possible mechanisms of actions of TTFCA in the remodeling of echolucent (soft; therefore, with risk of thrombosis and embolization) plaques at the carotid and femoral bifurcation. Moreover, The effects of the TTFCA on enzymes involved in mucopolysaccharide metabolism supported the hypothesis that the extract acts on basic metabolism in the connective tissues of the vascular wall. The levels of basal serum uronic acid and enzymes involved in mucopolysaccharide metabolism (β-glycuronidase, β-N-acetylglucosaminidase, and arylsulfatase) were elevated in patients with varicose veins, indicating an increased mucopolysaccharide turnover. After treatment (60 mg/day for three months) the above enzyme levels fell progressively (Arpaia *et al.*, 1990).

Anxiolytic activity

The anxiolytic activity of *C. asiatica* was studied in healthy subjects. *C. asiatica* significantly attenuated the peak ASR amplitude 30 and 60 minutes after treatment. *C. asiatica* had no significant effect on self-rated mood, heart rate, or blood pressure. These findings suggest that *C. asiatica* has anxiolytic activity in humans (Bradwejn *et al.*, 2000). It remains to be seen whether this herb has therapeutic efficacy in the treatment of anxiety syndromes.

Anti-viral activity

C. asiatica in combination with other Thai medicinal plants was investigated for its effects against herpes simplex virus type 1 and types 2 (HSV-1 and HSV-2). Vero cells, originating from an African green monkey kidney, were cultivated in growth medium and infected by HSV-1 and HSV-2. The plague inhibition assay was used to detect antiviral activities of the extracts. *C. asiatica* extract showed inhibitory effect on plague formation and also reduced the yield of HSV-1 and HSV-2 (Yoosook *et al.*, 2000).

1.2.5.3 *Rhinacanthus nasutus* (Linn.) Kurz

Rhinacanthus nasutus (Linn) Kurz belongs to the family Acanthaceae. *R. nasutus* is a shrub widely distributed in Southeast Asia. It is named "Tong Punchang" in Thailand.

Figure 1.15 A *Rhinacanthus nasutus* (Linn.) Kurz (www.hktree.com).

1.2.5.3.1 Chemical composition

Although flavonoids, anthraquinones, triterpenes, sterols, and other components have been isolated from *R. nasutus*, the only biologically active constituents reported are naphthoquinones. Figure 1.15 shows some naphthoquinones that have been reported biological activities.

1.2.5.3.2 Pharmacological activities

There are limited scientific studies on the pharmacological activities of *R. nasutus*. The literature reported that extracts of various parts of *R. nasutus* are used for treatments against ringworm and other fungus derived skin diseases, as well as eczema, pulmonary tuberculosis and cancer (Farnsworth and Bunyapraphatsara, 1992; Rojanapo *et al.*, 1990).

Extracts of the aerial parts of *R. nasutus* were identified as being potentially useful against herpes virus infections. Indigenous medical practitioners in Thailand use the roots and leaves, which are pounded with vinegar or alcohol and applied to herpes infections and other skin

eruptions. The active component that responsible for antiviral activity is naphthoquinones. Sendl *et al.*, (1996) isolated two new naphthoquinones, rhinacanthin-C and rhinacanthin-D. They exhibited inhibitory activity against cytomegalovirus (CMV) (Sendl *et al.*, 1996). Then later, Kernan *et al.* (1997) isolated another two new lignans, rhinacanthin E and rhinacanthin F, from the aerial parts of the plant *R. nasutus*. These compounds showed significant antiviral activity against influenza virus type A (Kernan *et al.*, 1997). A recent study showed that rhinacanthin C exerted an *in vitro* antiproliferative activity, which was comparable with or slightly weaker than that of 5-FU, and antiproliferative activity for MDR1-overexpressing Hvr100-6 cells, similarly to parent HeLa cells. The authors also found that the *in vitro* antiproliferative activity of the ethanol extract of the root of *R. nasutus* was due to rhinacanthin C, whereas that of the aqueous extract of leaves of *R. nasutus* was due to constituents other than rhinacanthin C. Moreover, both of the *R. nasutus* extracts showed *in vivo* antiproliferative activity after oral administration once daily for 14 days (Gotoh *et al.*, 2004). The potential anticancer activity of *R. nasutus* was reported. Rhinacanthin Q was isolated and tested for its biological activities. It showed significant cytotoxicity against various cancer cell lines including P-388, A-549, HT-29 and HL-60. It also demonstrated 36-100% inhibition of the rabbit platelet aggregation induced by arachidoic acid (100 mM) or collagen (10 μg/ml) or platelet activation factor (2 ng/ml) (Wu *et al.*, 1998). In another study, the cytotoxicity of synthesized rhinacanthin-M, -N and -Q and 39 novel naphthoquinone esters were studied. Almost all the naphthoquinone esters that contain a C-3 hydroxy group showed significant cytotoxicities against KB, HeLa, and HepG2 cell lines. In contrast, ester derivatives lacking the C-3 hydroxy group were inactive to the cancer cell lines (Kongkathip *et al.*, 2004). A related naphthoquinone, rhinacanthone, isolated from R. nasutus, has been shown to have potent in vitro antifungal activity (Kodama *et al.*, 1993).

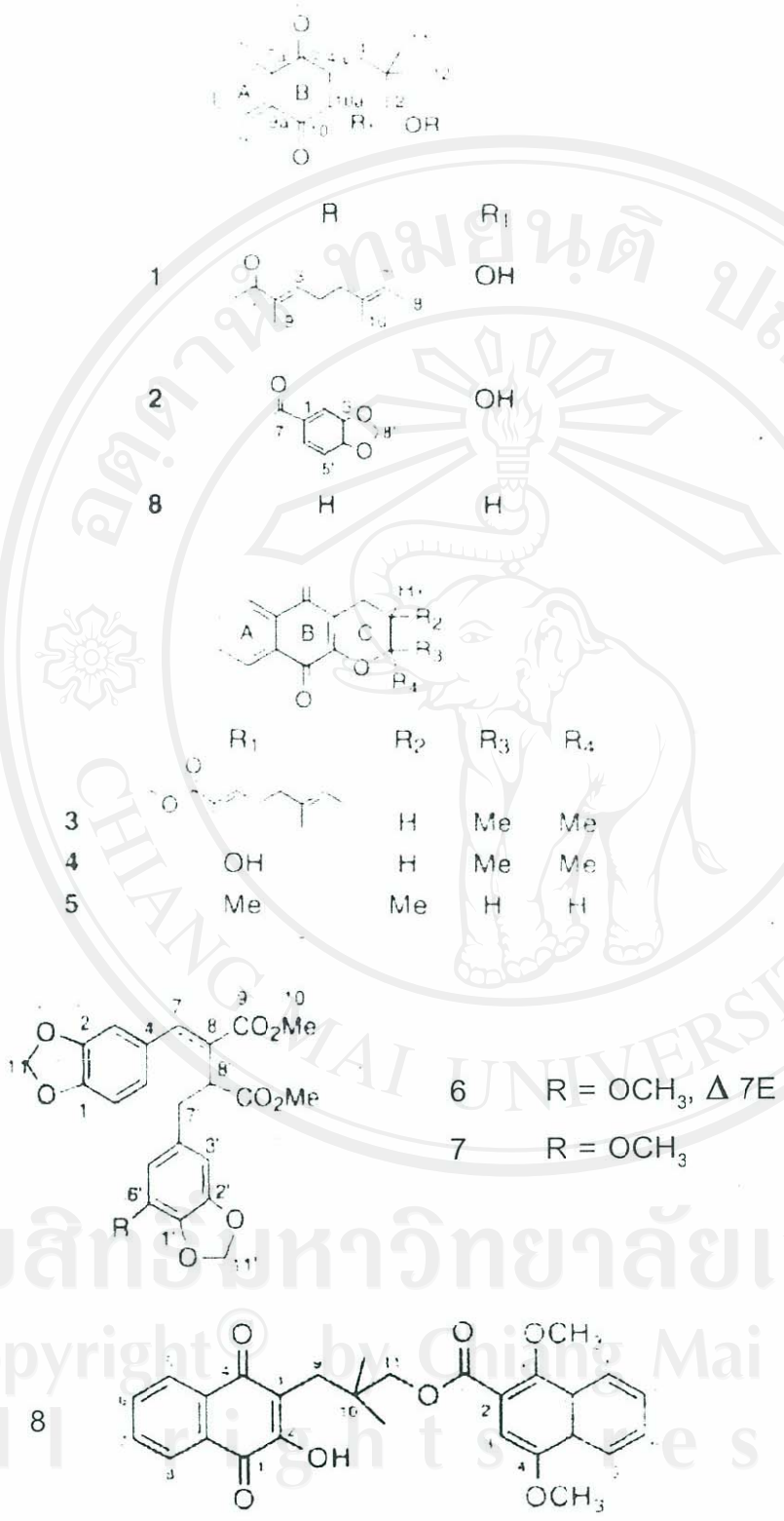


Figure 1.16. The structure of some naphthoquinones isolated from *R. nasutus*; rhinacanthin-A (3), -B (4), -C (1), -D (2), -E (6), -F (7), -Q (8), and rhinacanthone (5) (Sendl *et al.*, 1996; Kernan *et al.*, 1997; Wu *et al.*, 1998).

1.3 Objectives

1. To search for Thai medicinal plants that show immunomodulating activities
2. To study the combined effects of aflatoxin B_1, deoxynivalenol and fumonisin B_1 on the immune system
3. To study the effect of *Centella asiatica* and *Rhinacanthus nasutus* extracts on mycotoxin-induced immunotoxicity