CHAPTER 1

GENERAL INTRODUCTION

Asthma is a chronic inflammatory disease of the airways in which many different cells and cellular mediators are involved and play a role in the complex inflammatory process in asthma. Asthma is considered to be a global health problem that affects millions of people worldwide (Steinbacher and Glick, 2001).

There are many different treatment approaches to inflammatory diseases such as asthma, of which the recognized standard therapy is inhaled corticosteroids and is also currently the most effective (Sumi and Hamid, 2007). However, complementary and alternative medicine are studied extensively by many different researchers over the world and many allergies and allergic diseases such as asthma are frequently treated with alternative medication, including herbal as well as homeopathic products. Homeopathic products have been proved to exhibit biological activity in *in vivo* and *in vitro* animal studies (Piemonte and Buchi, 2002).

Modul8® is a complex homeopathic product of natural origin, composed of a mixture of plants and minerals and its effect on the immune system is investigated to determine whether this product might be an immunomodulator. Immunomodulators are defined as agents that enhance the immunity of an individual to favor a specific immunological response. In this thesis, the anti-inflammatory properties of this homeopathic product are investigated by using the BALB/c murine asthmatic animal model. The BALB/c asthmatic animal model is a known model, which has been shown to be useful in testing novel therapeutics. Since there is a correlation between the allergic response in humans and that of OVA-challenged animals, this animal model was used to investigate the anti-inflammatory properties of this homeopathic product (Epstein, 2006; Blyth *et al*., 1996; Hogan *et al*., 1996).

The main focus of this thesis is ultrastructure and morphology and this feature in the majority of the chapters. However, other parameters that also form an important part of this thesis include hematology and immunology. On ultrastructural level, platelets and fibrin networks, as well as tissue remodeling in the lungs of mice, were investigated by using the BALB/c asthmatic animal model and the morphology of peritoneal macrophages were investigated *in vitro*. Hematological studies involved the investigation of the white blood cell counts in the blood, as well as bronchial lavage. Platelet distribution in the bronchial lavage was also investigated. The production of the inflammatory mediators, nitric oxide and TNF-alpha, were investigated by the *in vitro* activation of peritoneal macrophages by lipopolisacharides (LPS) and Modul8®. The different parameters investigated in this thesis will be discussed in more detail in the following paragraphs.

Platelets and fibrin networks were also investigated ultrastructurally. Both play an important role in allergic processes involved in asthma as platelets act as inflammatory cells by releasing inflammatory mediators (Camera *et al*., 1999; Butenas and Mann, 2002; Lazarus *et al*., 2003). The effect of alternative (herbal) products on the ultrastructure of platelets and fibrin networks has been studied previously to show that asthma has an effect on the ultrastructure of platelets and fibrin networks (Pretorius *et al*., 2007a). Therefore, in this thesis the effect of Modul8® on the ultrastructure of platelets and fibrin networks were investigated and compared to the control and untreated asthmatic animals. Since the question always arises whether an animal model adequately mimics human disease, the results found in the above-mentioned study were compared to that of human controls and asthmatic subjects.

A wide range of inflammatory cells and mediators are involved in the inflammatory process in asthma. An influx of inflammatory cells into the airways of asthmatics is a

common feature in an asthmatic response. Therefore, this thesis also includes a chapter on the histological and ultrastructural changes that occur in the lungs of asthmatic animals. Remodeling in the lungs of untreated asthmatic animals were compared to control animals as well as asthmatic animals treated with the homeopathic product Modul8® and hydrocortisone. Hydrocortisone was used throughout the study as positive control, since it is known that hydrocortisone is used in the treatment of asthma.

White blood cells also play an important role in the inflammatory response in asthma. Eosinophil numbers in particular are increased in asthmatic subjects and these cells contribute to the airway remodeling observed in asthma (Kay *et al*., 2004). Since it is thought that Modul8® might have a positive influence on the immune system, the number of white blood cells in the blood as well as bronchial lavage was counted and the results of the asthmatic animals were compared to the control and treatment groups.

Macrophages are important mediators of the immune response and they become activated in response to microenvironment to release a number of inflammatory mediators. Also, the morphology of macrophages changes when they become activated. It was previously found by researchers that an immunomodulator has the ability to activate macrophages *in vitro* (Piemonte and Buchi, 2002). Therefore, in this thesis mouse peritoneal macrophages were stimulated with LPS, which is a known activator of macrophages, and Modul8® to investigate whether Modul8® has the ability to activate macrophages to produce inflammatory mediators.

Therefore, the overall aim of this study was to investigate the possible effects of the homeopathic product Modul8® on ultrastructural, hematological and immunological

parameters by using the BALB/c murine asthmatic animal model. The research objectives addressed in this thesis can be summarized as follow:

1. Implementation of the BALB/c asthmatic animal model to investigate:
    - Ultrastructure of platelets and fibrin networks and comparison to human subjects
    - Weights changes in the animals during the experimental period
    - White blood cell counts in the blood and bronchial lavage as well as platelet distribution in the bronchial lavage
    - Ultrastructural changes and infiltration of inflammatory cells in the lungs

2. *In vitro* activation of mice peritoneal macrophages to investigate:
    - The ability of Modul8® to activate peritoneal macrophages
    - The production of inflammatory mediators by LPS-and Modul8® stimulated macrophages.

This thesis concludes with a summary of the results found in all the above-mentioned experiments as well as the possible application thereof.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

In this thesis the effect of the homeopathic product Modul8® will be discussed on several different parameters including weight changes, white blood cell counts in the blood and bronchial lavage, histology and ultrastructure of the lungs, ultrastructural changes in the morphology of platelets and fibrin networks and the ultrastructure of activated macrophages *in vitro*. The current chapter includes an overview on asthma in general as well as its etiology, immunology, pathophysiology, airway remodeling and treatment.

Typical treatment remedies for asthma are discussed as well as the role of complementary and alternative medicine in the treatment of asthma. Homeopathy is also frequently used in the treatment of asthma and other allergic diseases, and therefore this chapter concludes with the role of homeopathy in the treatment of asthma.

Modul8® is a modified version of the known homeopathic product Canova® (which is a potent immunomodulator and, studied extensively) and although differences exist in the composition of Canova® and Modul8®, the principles used in both these products are the same. Since no research has been done on the anti-inflammatory and immunomodulatory role of the new product, Modul8®, this chapter reviews properties of Canova®. The rationale behind using mice as experimental animals and more specifically choosing the BALB/c murine asthmatic animal model is also reviewed in this chapter.

2.1.1 Asthma in general

Asthma is considered to be a serious global health problem that has increased over the past decades and still are increasing and affecting hundreds of million people worldwide. People who are at highest risk for this disease include children, young adults and racial and ethnic minorities living in urban areas (Steinbacher and Glick, 2001). The term “asthma” is derived from a Greek word “panos” which means panting and was first used over 2000 years ago to describe shortness of breath (Rosenblatt, 1976). The Greek physician Hippocrates (460–377 BC) first described asthma and the first etiological link with bronchospasm was made by Galen (130–201 AD), who also described the association between upper and lower airways (Diamant *et al*., 2007).

Ancient medical pioneers such as Hippocrates, Galen and Bernardino Ramazziniin, amongst others, have used this term to describe the airway condition that causes wheezing, chest tightness and an obstruction in the airways (Rosner, 1981; Marketos and Ballas, 1982).

The National Heart Lung and Blood Institute (NHLBI) states a working definition of asthma as follows: “Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular, mast cells, eosinophils, T lymphocytes, macrophages, neutrophils and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or early in the morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. The inflammation also causes an associated increase in the existing bronchial hyper-responsiveness to a variety of stimuli” (NHLBI, 1997).

Many years later, the term asthma is still described as a chronic inflammatory disease of the airways which involves the activation and infiltration of a large number of immune and inflammatory cells within the larger and smaller airways (Kornerup and Page, 2007). The clinical presentation of asthma includes wheezing, chest tightness, coughing and dyspnoea and the severity of the asthma symptoms may vary among patients and within individual patient episodes (Sollecito and Tino, 2001).

2.1.2 Etiology

Asthma is broadly classified into two main categories namely allergic or extrinsic asthma and idiosyncratic or intrinsic asthma (Sollecito and Tino, 2001). The first type of asthma mentioned extrinsic or allergic asthma, is the most common type and is associated with a history of atopy which includes eczema and seasonal allergies. This type of asthma typically begins in childhood (Tasota, 1996). This process involves increased serum IgE levels as well as a positive skin test for allergens. This type of asthma is associated with IgE mediated mast cell degranulation and a subsequent inflammatory response (Little *et al.*, 1997; McFadden, 1998).

Allergic asthma is also associated with reversible obstruction of airway, bronchospasm, infiltration of inflammatory cells into lung tissues, airway hyper-responsiveness (AHR), mucus overproduction and over-expression of Th2-mediated cytokines (Kon and Kay, 1999; Renauld, 2001).

Idiosyncratic or intrinsic asthma is not associated with allergy or increased IgE levels, and is more common in the elderly and middle-aged populations (Tasota, 1996). Bronchoconstriction and AHR are due to stimulation of airway parasympathetic nerves by certain allergens causing release of acetylcholine to sustain this process (Travis *et al.*, 2002). An asthmatic attack is often induced by specific triggers which

can be caused by pharmacological agents such as aspirin and beta-blockers, food preservatives, upper respiratory infections, exercise, cold, dry air and stress to mention only a few (Little *et al*., 1997; McFadden, 1998).

Some researchers include occupational asthma and exercise-induced asthma in the classification system. Occupational asthma is caused by the exposure to biological enzymes, plastic resins, dusts, wood and metal particles in the workplace. This is often considered as a temporary form of asthma, due to the fact that the airway inflammation and bronchial hyper-responsiveness observed after the inhalation of a specific agent can be removed by removal of the causative agent (NHLBI 1997; Venables and Chan-Yeung, 1997), however, the asthmatic condition can exist even after the removal of the causative agent (Venables and Chan-Yeung, 1997).

In 1999, Jeffery described and categorized exercise-induced asthma by the narrowing of the airways after triggering by vigorous activity. The author explained that patients with exercise-induced asthma have sensitive airways, especially to changes in temperature and humidity (Jeffery, 1999). In 2004, Robison revealed that one of the major mechanisms for exercise-induced asthma is the activation of the mast cells by osmotic stimuli through the IgE receptors (Robinson, 2004).

Another classification system for chronic asthma also exists and is based on the severity of the disease according to the frequency of acute asthmatic attacks. According to this system, asthma is divided into mild intermittent, mild persistent, moderate persistent and severe persistent (Sollecito and Tino, 2001). The symptoms associated with severe persistent asthma (continual symptoms), include the following: limited physical activity, frequent exacerbations and frequent night-time symptoms. The preferred treatment for this type of asthma includes anti-inflammatory, high-dose inhaled corticosteroids, systemic corticosteroids and long-

acting bronchodilators. When daily symptoms occur even with the daily use of inhaled short-acting bronchodilators, or when exacerbations occur more than, or two times per week, and affects the activity of the individual, it is classified as moderate persistent. Typically, nocturnal symptoms in this type of asthma will occur more than once per week. The preferred treatment for moderate persistent asthma includes anti-inflammatory, medium-dose inhaled corticosteroids, low-or medium dose inhaled corticosteroids and a long-acting β_2-agonist bronchodilator treatments.

Mild persistent asthma is classified according to the occurrence of symptoms less than two times per week and less than once per day. Exacerbations may also affect activity and night-time symptoms occur more than twice per month. Anti-inflammatory, low-dose inhaled corticosteroids, products like Cromolyn or Edocromil are the preferred treatment options for this type of asthma.

The final type of asthma, mild intermittent, is characterized by the following symptoms: symptoms occur less than, or twice per week, asymptomatic stages and normal peak expiratory flow are present between exacerbations which are brief and night-time symptoms occur less than, or twice per month. For mild intermittent asthma, no specific treatment is needed (Sollecito and Tino, 2001).

2.1.3 Immunology

Asthma is an inflammatory process in which many inflammatory cells participate and mediate a complex mixture of mediators. Cytokines in particular play an important role as mediators of chronic inflammation (Barnes, 1996). The inflammatory process in asthma involves a wide range of immune cells and immune mediators or cytokines that interact with one another in a specific way. According to Sollecito and Tino (2001), the most important cellular elements involved in asthma include mast cells, eosinophils, macrophages, epithelial cells and activated T-lymphocytes that are

associated with the Th2 response. These cells release certain mediators or cytokines, or are influenced by various mediators, cytokines and adhesion molecules (Sollecito and Tino, 2001). The role of some of these cells and mediators will be discussed individually in the following paragraphs.

2.1.3.1 Mast cells

The role that mast cells play in allergic diseases has extensively been studied over many years by a number of different researchers. Triggering of mast cells lead to the elicitation of the immediate phase of an allergic response that cause acute local responses such as edema formation, tissue swelling and bronchoconstriction. Mast cells also have an effect on the late phase responses of an allergic reaction by the release of chemotactic and pro-inflammatory mediators (Metz *et al*., 2007; Vliagoftis and Befus, 2005).

Antigens cross-link with the IgE on mast cells and the mast cells then release mediators such as histamine, cysteinyl leukotrienes and prostaglandin D_2 (PGD_2) (Galli *et al*., 2005) and according to Robison in 2004, airway hyperresponsiveness and increased number of mast cells in the airway smooth muscle (ASM) may be linked to one another (Robinson, 2004). Mast cells play an important role in initiating the acute responses to allergen and also to other indirect stimuli such as exercise and hyperventilation (Barnes, 1996).

In 2001, Page *et al*. also reported on the role of mast cells in the inflammatory process involved in asthma. The mast cells respond to non-IgE stimuli in the airways such as osmotic changes in the airway associated with exercise, hypertonicity and hypotonicity. Other mediators released from mast cells include prostaglandins, leukotrienes, cytokines such as IL1, IL2, IL4, IL10 and IL13, growth factors such as

platelet derived growth factor (PDGF) and transforming growth factor b (TGF), and lastly proteases such as tryptase (Page *et al*., 2001).

In 2007, Bradding and Brightling revealed that mast cells migrate into the airway epithelium, airway mucus glands and the airway smooth muscle in human asthmatics, while virtually no mast cells are found in these regions in normal individuals. These authors also showed that the number of mast cells infiltrated into the ASM bundles; correlate significantly with the bronchial hyper-responsiveness associated with asthmatics, indicating the importance of mast cells in the pathophysiology of asthma (Bradding and Brightling, 2007).

2.1.3.2 Macrophages

Macrophages are derived from blood monocytes and they move into the airways where they may be activated by allergens via low-affinity IgE receptors (Lee and Lane, 1992). These cells produce many different products and cytokines that orchestrate the inflammatory response in asthma and therefore macrophages have the ability to initiate a particular type of inflammatory response via the release of certain cytokines (Spiteri *et al*., 1994). According to Spiteri *et al*. (1994) macrophages may both increase and decrease inflammation, depending on the stimulus and therefore play an important anti-inflammatory role, preventing development of allergic inflammation (Spiteri *et al*., 1994). Another characteristic of macrophages is the ability to act as antigen-presenting cells (APC) by processing allergen for presentation to T-lymphocytes, a characteristic that is less effective in alveolar macrophages than in macrophages from sites such as the peritoneum (Holt, 1993).

2.1.3.3 Dendritic cells

Dendritic cells are specialized macrophage-like cells in the airway epithelium which are very effective APC. Dendritic cells sample allergens from the airway surface and

migrate to regional lymph nodes. They interact with regulatory T cells and stimulate the production of Th2 cells from naïve T cells (Kuipers and Lambrecht, 2004). Dendritic cells may therefore play an important role in the initiation of allergen-induced responses in asthma.

2.1.3.4 Eosinophils

One of the major features of asthmatic airways is the eosinophilic infiltration into the airways. As early as 1916 the term "*chronic eosionophilic bronchitis*" was used to describe asthma (Barnes, 1996). Inhalation of allergen causes a marked increase of eosinophils on the broncho-alveolar lavage fluid in the late stage reaction that indicates that there is a close relationship between eosinophil counts in the blood and bronchial lavage and airway hyper-responsiveness (Barnes, 1996; Gleich, 1990). In 2004, Kay and co-workers also stated that eosinophil numbers are always increased in the airways and these cells are known to release basic proteins and growth factors that may damage airway epithelial cells and thereby cause airway remodeling (Kay *et al*., 2004).

Eosinophilic infiltration is orchestrated by a number of cytokines that are released from the T- lymphocytes that are usually present in increased numbers in the airways. These cytokines include IL-4, IL-5, IL-9, and IL-13 (Larche *et al*., 2003; Akbari *et al*., 2006). In a study done by Oberholzer *et al*. (2008) the authors used the BALB/c murine asthmatic animal model to investigate the white blood cell count in the blood and bronchial lavage of untreated asthmatic animals and asthmatic animals treated with a plant material. The authors found an increase in the number of all white blood cells, but a significant increase in the number of eosinophils in the blood as well as bronchial lavage (Oberholzer *et al*., 2008).

2.1.3.5 Neutrophils

Neutrophils are immune cells that play key roles in both the acute and chronic inflammatory response (Brown *et al*., 2008). The exact pathophysiological role of neutrophils in asthma is not very clear; however neutrophils are usually increased in the sputum of patients with severe asthma, and it is stated by some researchers that this might be due to the effect of glucocorticosteroid therapy (Wenzel, 2003).

2.1.3.6 T-lymphocytes

T-lymphocytes play a very important role in the inflammatory response in asthma by releasing specific patterns of cytokines. This results in the recruitment and survival of eosinophils and in the maintenance of mast cells in the airways (Kay, 1991a). T-lymphocytes are usually present in increased numbers in the airways and they release a number of different cytokines such as IL-4, IL-5, IL-9, and IL-13 that orchestrate eosinophilic inflammation and IgE production by B-lymphocytes (Larche *et al*., 2003; Akbari *et al*., 2006).

2.1.3.7 Inflammatory mediators

Over the past years, a number of different mediators, which may have a variety of different effects on the airways, resulting in different pathological features, have been implicated in asthma. The role of histamine, prostaglandins, leukotrienes, cytokines and platelet activating factor (PAF) in the inflammatory response in asthma will be discussed in the following paragraphs.

- **Histamine**

Histamine is released from mast cells and contract ASM, increase microvascular leakage, increase airway mucus secretion and attract other inflammatory cells (Barnes, 1996). Histamine therefore contributes to bronchoconstriction and the

inflammatory response in asthma by causing inflammation directly (Andriopoulou *et al*., 1999) and indirectly (Marone *et al*., 1999).

- **Prostaglandins**

Prostaglandins are produced from arachidonic acid and are potent biologically active lipid molecules (Hata and Breyer, 2004; Funk, 2001). Cyclooxygenases (COXs)1 are the rate-limiting enzymes for prostaglandin production and depending on subsequent isomerases and oxidoreductases distal to COX catalysis, various bioactive prostaglandins can be produced. The pattern of prostaglandin production is determined in a stimulus and cell-specific fashion and alteration of the species of prostaglandins that are produced in tissue, plays an important role in pathophysiological events of many diseases, including asthma (Peebles *et al*., 2002; Wolff *et al*., 1998; La¨niguez *et al*., 1998; Burleigh *et al*., 2002).

Prostaglandin D_2 (PGD_2) has been shown to have strong pro-inflammatory and bronchoconstrictive action in human and animal models of asthma (Hardy *et al*., 1984) whereas prostaglandin E_2 (PGE_2), which is also detected in broncho-alveolar lavage fluid of asthmatic patients, has bronchoprotective properties and acts as an endogenous anti-inflammatory factor (Melillo *et al*., 1994; Pavord *et al*., 1993).

Uller *et al*. (2007) also reported on the role of PGD_2 and stated that mast cell derived PGD_2 may contribute to eosinophilic inflammation and mucus production in allergic asthma. The authors also reported that chemo-attractant receptor homologous molecule expressed on TH-2 cells (CRTH2), which is a high affinity receptor for PGD_2, mediates the trafficking of mast cells, eosinophils and Th2 cells to inflammatory sites (Uller *et al*., 2007).

- **Leukotrienes**

Leukotrienes originate from the polyunsaturated fatty acid arachidonic acid. Two classes of leukotrienes can be distinguished based on the respective synthetic pathways and their biological activities. Leukotriene B_4 (LTB_4) is a dihydroxylated derivative of arachidonic acid and has been shown to be a very potent chemotactic mediator for neutrophils and eosinophils. Leukotrienes C_4, D_4, and E_4 are synthesized from arachidonic acid by attachment of the tripeptide glutathione (yielding LTC_4) and subsequent removal of glutamic acid and glycine residues (yielding LTD_4 and LTE_4, respectively). These leukotrienes are known as cysteinyl leukotrienes. The amount of evidence available on the role of cysteinyl leukotrienes in airway inflammation is numerous (Capra *et al*., 2007).

Cysteinyl leukotrienes are potent spasmogenic mediators and affect inflammatory cell recruitment, mucus production, airways remodeling, and pulmonary vascular leakage (Ogawa and Calhoun, 2006). Some authors have reported on the inhibition of the formation and action of cysteinyl leukotrienes and have proven amelioration in inflammatory symptoms associated with asthma and other inflammatory diseases (Engels and Nijkamp, 1998).

- **Cytokines**

Cytokines play an important role in orchestrating the inflammatory response associated in chronic inflammation. Many different inflammatory cells are capable of releasing cytokines. These include macrophages, mast cells, eosinophils and lymphocytes. These cells are capable of synthesizing and releasing these cytokines and structural cells such as epithelial and endothelial cells may also release a number of cytokines and therefore participate in the inflammatory response (Barnes, 1994).

Cytokines act as the main drivers of the inflammatory response in asthma and may also determine the severity thereof. The cytokines that are specifically important in asthma are the lymphokines that are secreted by the T-lymphocytes: Interleukin (**IL**)-**3** plays an important role in the survival of the mast cells in tissues, **IL-4** is critical in stimulating B-lymphocytes to produce IgE and for expression of vascular cell adhesion molecules (VCAM-1) on endothelial cells, **IL-5** plays an important role in the differentiation, survival and priming of eosinophils (Hamid *et al*., 1991).

Other cytokines that are released from cells such as macrophages and epithelial cells and that are important in amplifying the inflammatory response include **IL-1**, **IL-6**, tumour necrosis factor-α (**TNF-α**) and granulocyte macrophage-colony stimulating factor (**GM-CSF)**.

TNF-α is a pro-inflammatory cytokine that is known to play a critical role in the pathogenesis of many inflammatory diseases, including asthma (Russo and Polosa, 2005). TNF-α is an important mediator in asthma and is found in increased amounts in the airways of asthmatics. One of the biological functions of TNF-α includes the modulation of growth differentiation and proliferation of a variety of cell types and is also a well-known inducer of the inflammatory response, as well as a regulator of immunity. Other pro-inflammatory cytokines that mediates the inflammatory properties of TNF-α includes IL-1, IL-2, IL-4, IL-6, IL-10, IL-12, IFN-*γ* (interferon-*γ*) and TGF-*β* (transforming growth factor- *β*) (Aggarwal, 2003).

- **Platelet activating factor (PAF)**

This mediator also plays an important role in asthma as it mimics many of the features of asthma including airway hyperresponsiveness (Barnes *et al*., 1988). PAF is a bronchoconstricting mediator secreted by activated platelets together with histamine, serotonin (5-HT) and arachidonic acid (Page, 1989). PAF is a potent

activator of platelets and neutrophils and can induce systemic anaphylaxis when given intravenously.

Ultrastructural studies have shown PAF, in addition to causing neutrophil and eosinophil recruitment into the lungs, induces intravascular platelet aggregation and degranulation in alveolar capillaries and stimulates platelet diapedesis to the alveolar lumen. The authors of this study revealed that these features are absent in guinea pigs pre-treated with prostacyclin or aspirin, and that the bronchoconstrictive response remains unchanged. With these results the authors suggested that platelet aggregation in the lungs is not essential for bronchoconstriction and that platelets release airway-constricting substances via a cyclo-oxygenasein dependent mechanism (Lellouch-Tubiana *et al*., 1985).

Other interesting results found by authors as early as 1988 and 1990 are that platelet depletion, as well as administration of prostacyclin (PGI_2) and PAF antagonists in animal models of asthma, significantly inhibited the infiltration of eosinophils (Coyle *et al*., 1990; Lellouch-Tubiana *et al*., 1988).

- **Nitric oxide (NO)**

Nitric oxide is a potent vasodilator that is produced by several cells in the airways predominantly from the action of inducible nitric oxide synthase (NOS) (Ricciardolo *et al*., 2004; Barnes, 1995). iNOS is an inducible form of the enzyme that is expressed in epithelial cells of asthmatic patients and can be induced by cytokines in airway epithelial cells (Hamid *et al*., 1993; Robbins *et al*., 1994). An increased concentration of exhaled NO is present in asthmatic patients (Kharitonov *et al*., 1994) and since NO itself is a potent vasodilator it may increase plasma exudation in the airways of asthmatic patients and may also amplify the Th2 lymphocyte mediated response (Barnes and Liew, 1995).

2.1.4. Pathology and airway remodeling

In a review article on airway remodeling in asthma, Sumi and Hamid (2007) defined airway remodeling as changes in the composition, content, and organization of the cellular and molecular constituents of the airway wall. Airway remodeling is one of the characteristics of asthma, and has important functional implications. The structural changes associated with airway remodeling in asthma include epithelial detachment, subepithelial fibrosis, increased ASM mass, decreased distance between epithelium and ASM cells, goblet cell hyperplasia, and mucus gland hyperplasia, proliferation of blood vessels and airway edema and changes in the cartilage. Each of these changes can contribute to airway hyperreactivity (AHR), and may lead to irreversible airflow obstruction as the disease progresses. Remodeling is thought to be characteristic of asthma because these structural changes can be observed in asthmatics from early onset of the disease (Sumi and Hamid, 2007).

Epithelial detachment is a characteristic feature of airway remodeling and may in part be a consequence of ongoing inflammation (Trautmann *et al*., 2002; White, 1997).

Subepithelial fibrosis is another important feature of airway remodeling in asthma and may range between simple thickening and extensive fibrosis (Sumi and Hamid, 2007). Roche *et al*. (1989) investigated the ultrastructure of subepithelial fibrosis and revealed that the basement membrane appear normal on electron microscopic level and beneath this layer there is a loose array of collagen fibrils in normal individuals whereas in asthmatic patients this layer is replaced by a dense network of collagen fibrils. This type of fibrosis is due to the deposition of extracellular matrix particularly collagen type I and III, fibronectin as well as proteoglycans (Roche *et al*., 1989).

Activated fibroblasts cause the increased extracellular matrix and are due to the up regulation of a number of cytokines and growth factors such as tumor growth factor

beta (TGF-β) and IL-11, and they are produced by structural as well as inflamed cells, particularly eosinophils (Sumi and Hamid, 2007).

Another common observation in asthmatic airways is the increase in airway smooth muscle mass. There is a number of factors that may cause an increase in airway smooth muscle cell number, such as increased rates of division (Johnson *et al*., 2001), decreased rates of apoptosis (Martin and Ramos-Barbon, 2003) and migration of mesenchymal cells to ASM bundles (although the latter is only speculated) (Schmidt *et al*., 2003; Beqaj *et al*., 2002; Hashimoto *et al*., 2001; Vancheri *et al*., 2005; Hirst *et al*., 2004; Johnson *et al*., 2004).

Stimulation of ASM cells by mitogenic or inflammatory stimuli may be one reason why ASM cells proliferate faster in asthmatic patients, compared to non-asthmatic patients. Mitogenic stimuli that have been identified include TGF-β, EGF, and IGF, PDGF isoforms, FGF-2, β-hexosaminidases, β-glucuronidase, α-thrombin, tryptase, sphingosine 1- phosphate, endothelin-1, substance P, phenylephrine, serotonin, thromboxane A_2, leukotriene D_4, mechanical stress and reactive oxygen species (ROS) (Roth *et al*., 2004). Another explanation may be the fact that profile of the extracellular matrix protein components are altered in asthmatic cells and that this altered profile may have the potential to influence the proliferation of these cells (Ebina *et al*., 1993).

Evidence exists that smooth muscle cells in asthma can act as inflammatory cells, because of the ability to produce chemokines and cytokines (Vermeer *et al*., 2003).

Increased size of the mucosal glands and increased numbers of goblet cells in the airway epithelium is also features of airway remodeling in asthma that leads to excessive mucus production which eventually lead to airflow obstruction. Mucus

hypersecretion may be induced by IL-9 and IL-13 (Wills-Karp and Chiaramonte, 2003; Kuperman *et al*., 2002; Li and Wilson, 1997; Tanaka *et al*., 2003).

Vascular change and airway edema also forms part of the airway remodeling in asthma and changes in the airway wall can contribute to airway wall edema (Sumi and Hamid, 2007). Bronchoscopic biopsies of patients with mild asthma (Hoshino *et al*., 2001) have showed increased airway vascularity as well as in the bronchial mucosa of stable and newly diagnosed asthma patients (Haraguchi *et al*., 1999).

Another feature of airway remodeling in asthma is changes in the airway cartilage, which is responsible for the stiffness and integrity of the airways. Structural and biochemical changes in the cartilage of the airways will therefore have an influence on airway function (Sumi and Hamid, 2007). A decrease in the cartilage volume and fibronectin deposition has been observed in asthmatic airways, and these alterations may result on more powerful bronchoconstriction from ASM load reduction (Sumi and Hamid, 2007; Black, 2004).

Airway remodeling in established asthma is poorly responsive to inhaled corticosteroids, administration of β_2-agonists, antileukotrienes, and theophylline, which are the currently used therapies for asthma (Sumi and Hamid, 2007). The therapeutic implications in asthma will be discussed in section 2.1.6.

2.1.5 Genetics and asthma

A number of studies have been done on the genetic aspect involved in asthma and these studies confirmed that age and sex play a role in the pathogenesis and prevalence of asthma (Lux *et al*., 2009). The genetic influences on the pathogenesis of asthma include genetic disposition, which seems to be the primary determinant of asthmatic disease and are defined by clinical symptoms and functional tests. Melén

et al. (2004) stated that the influence of parental allergy and male sex on the development of wheezing and sensitization during childhood may possibly be determined by sex-specific hereditary components consistent with x-linked genes (Melén *et al*., 2004).

2.1.6 Treatment of asthma

In earlier years, anti-asthmatic treatment involved the relief of symptoms or modification thereof (Hofbauer, 1931; Belcher, 1961). Nowadays there are a number of different asthma medications available that are used in many different indications. Some of these medications include inhaled β_2 -agonists (short-acting and long-lasting); oral β_2 -agonists; inhaled corticosteroids (ICS); systemic corticosteroids; mast cell stabilizers; methylxanthines; anticholinergics and leukotriene modifiers (Sollecito and Tino, 2001). The implications of some of the mentioned mediations will be discussed in the following paragraphs.

- **Corticosteroids**

ICS are recognized as standard therapy for pediatric and adult asthma (GINA, 2005; National Asthma Education and Prevention program, 2007) and are currently the most effective anti-inflammatory medication for the treatment of asthma (Sumi and Hamid, 2007). ICS are effective in most cases of asthma and some studies have shown that ICS are effective in preventing or reducing the decline of airway function in asthmatic patients over time (Selroos *et al*., 1995).

The exact effect of corticosteroids on epithelial cells is still controversial, although it is thought that they may be beneficial by stimulating a decrease in mucus production. In an *in vitro* study done by Stewart *et al*. (1995) Dexamethasone was shown to increase 3H-thymidine incorporation in asthmatic bronchial fibroblasts, without having

any significant effect on normal fibroblast proliferation. With these findings the authors suggested that the corticosteroids could influence some aspects of the bronchial fibroblast phenotype (Stewart *et al*., 1995).

In another study done in 2001, corticosteroids were shown to decrease smooth muscle cell proliferation (Lazaar and Panettieri, 2001), whereas other studies have shown that corticosteroids have a non-immunosuppressive effect on the ASM because no effect on the activation of nuclear factor-B transcription factor could be observed (Johnson *et al*., 2000).

In a retrospective biopsy study done by Lundgren *et al*. (1998) partial restoration of the epithelium could be observed after the use of corticosteroids. These results showed that treating asthmatics with ICS for long terms decreased inflammation and partially improved epithelial damage.

Goblet cell metaplasia and mucus hypersecretion are some of the important features of asthma and although corticosteroids and bronchodilators are not primarily targeted to act on goblet cell activity, they do seem to have direct and indirect suppressive effects on mucus production (Rogers and Barnes, 2006). In research done by using animal models, corticosteroids have shown to be effective in reducing goblet cell metaplasia (Leung *et al*., 2005; Kumar *et al*., 2003).

Although pharmacotherapy is generally effective in most of the patients and higher doses of ICS were previously recommended, recent evidence suggests that the addition of a long-acting β_2-agonist (LABA) is a better option for most. However, higher dosages of ICS are not problem-free and have been associated with a dose-related increase in risk of corticosteroid-related adverse outcomes (Cumming *et al*., 1997; Garbe *et al*., 1997; Greenfield and Samaras, 2006; Hubbard *et al*., 2002).

Korsgaard and Ledet (2009) also reported on the potential side effects in patients treated with LABA and ICS, which are widely used in patients with obstructive lung disease. The authors determined the occurrence of potential side effects to ICS and LABA in an open post-marketing observational study. The authors investigated a number of potential side effects of both ICS and LABA. The pharmacological predictable side-effects of treatment with ICS include occurrence of sore throat, skin bruises and oral thrush, while the pharmacological predictable potential side effects to treatment with inhalations of LABA were, unwillingly spontaneous muscle twisting, muscle cramps in hands, toes and foot and/or in the calves in the morning. They also found occurrence of hand tremors and heart palpitations in their study.

The authors concluded that four out of five adult patients treated with ICS and LABA seemed to experience some degree of side effects and they emphasized the importance of reducing the treatment to the minimum dose needed in each specific case of asthma in patients (Korsgaard and Ledet, 2009).

In 2009, Xu *et al*. did a study on cortisol suppression as a surrogate marker for ICS-induced growth retardation in children. The authors revealed that exposure to ICS results in adrenal suppression and growth inhibition in asthmatic children (Xu *et al*., 2009).

In another study in 2002 the authors stated that there is increasing evidence that the use of ICS is associated with a dose-related reduction in bone mineral density, and more specifically an association between the use of ICS and hip fractures (Hubbard *et al*., 2002).

Although the clinical affectivity of ICS has been established, the prolonged treatment with high doses of ICS can neither fully reverse all chronic aspects of the airway

inflammation nor cure the disease (Dworski and Sheller, 1992; Ward and Walters, 2005).

- **Anticholinergics**

In the late 1970s Ipratropium, which is a synthetic anticholinergic was developed for the treatment of asthma. Tiotropium was also developed later on and both these drugs both antagonize the effect of acetylcholine at the muscarinic M_1 and M_3 receptor. The role of these drugs in asthma is still limited but evidence shows that these anticholinergics may benefit patients with genetically determined adverse responses to β_2-agonists in up to 20% of the asthma population (Wechsler *et al*., 2006). It was also reported that during acute exarcerbation when response to short acting β_2 agonists (SABAs) is poor, addition of an anticholinergic may provide a faster-onset relief (Gross, 2006; Teale *et al*., 1992).

- **β_2 Agonists**

The use of adrenal substances in the treatment of asthma dates back to 1900 and during the 1940's epinephrine or more known as adrenaline became the standard bronchodilator for the treatment of acute asthma (Solis-Cohen, 1900; Sears and Lotvall, 2005). A major breakthrough came in 1960 with the discovery of the adrenergic receptor subsets, yielding α and β receptors which are further divided into the β_1 receptors which are located in the heart and intestinal smooth muscle and the β_2 receptors which are found in the bronchial and uterus smooth muscle (Lands *et al*., 1967; Ahlquist, 1948).

The first agonist interacting with the β adrenergic receptors was Isoprenaline, while the first agonists with a higher specificity for the β_2 adrenergic receptors were Salbutamol and Terbutaline (Bergman *et al*., 1969; Brittain *et al*., 1968) and today Salbutamol is the most widely used fast-acting reliever for asthma (GINA, 2005).

Other short-acting β_2 agonists include Carbuterol, Clenbuterol and Fenoterol and they have duration of action up to 6h (Engelhardt, 1976; O'Donnell, 1970). In the 1980s, LABAs such as Salmeterol were developed with an action of up to 12h (Johnson, 1995). Some researchers provided evidence that treatment with both SABAs and LABAs — in or without combination with ICS — is associated with potential masking of the airway inflammation (McIvor *et al*., 1998; Sears *et al*., 1990). Therapy with LABAs in combination with appropriate doses of corticosteroids, in the more severe cases, is recommended (Hasford and Virchow, 2006).

- **Antileukotrienes**

Leukotrienes have broncho-active properties and seem to mimic many features of asthma such as airway hyperresponsiveness; airway inflammation and airway remodeling (Diamant and Sampson, 1999) and research have shown that potent anti-leukotrienes effectively reduce several features of asthma in both adults and children (Bjermer and Diamant, 2004; Lane, 1998). Leukotriene receptor antagonists (LTRAs) such as Pranlukast, Zafirlukast and Montelukast also form part of anti-asthma therapy (Lane, 1998). The LTRAs combine anti-inflammatory, mainly anti-eosinophilic activity, with mild, bronchodilator properties, based on antagonism of cysteinyl leukotrienes (CysLTs) at the CysLT1- receptor within the airways and on inflammatory cells (Diamant and Sampson, 1999; Bjermer and Diamant, 2004; Lane, 1998; Ind, 1996).

2.1.7 Alternative and complimentary medicine

Alternative medicine is the term used to describe medical approaches that differ from conventional medicine and according to Passalacqua *et al*. (2006) complementary and alternative medicine (CAM) is the preferred term to use for this type of treatment (Passalacqua *et al*., 2006). Numerous CAM techniques are known and they include physical techniques such as acupuncture, chiropractic and massage therapy,

systematic medicines such as the Chinese traditional medicines, behavioral techniques such as dissociated diets and phytotherapy such as aromatherapy and herbal medicines. Allergy and allergic diseases such as asthma are frequently treated with CAMs, where homeopathy, acupuncture, herbal medicines, and yoga are the most used techniques. (Passalacqua *et al*., 2006).

Numerous studies have been done over many years investigating the role of alternative medicine, especially herbal products, on the allergic process in asthma. A few studies done on herbal products and asthma will be highlighted in the following paragraphs.

In 2009, Park and co-workers investigated the effect of Quercetin on the regulation of Th1/Th2 balance in a murine model of asthma. Many medicinal plants owe much of their activity to the high Quercetin content in the plant. Quercetin possesses potent anti-inflammatory properties and the authors revealed that Quercetin reduced the increased levels of IL-4, Th2 cytokine production in OVA-sensitized and –challenged mice. They also revealed that it increased IFN-γ and Th1 cytokine production in Quercetin administrated mice. It was also found that the administration of Quercetin before the last airway OVA challenge resulted in a significant inhibition of all asthmatic reactions and with these finding the authors suggested that Quercetin may play a critical role in the amelioration of the pathogenetic process of asthma in mice and also these findings provide new insight into the immunopharmacological role of Quercetin in terms of its effects in a murine model of asthma (Park *et al*., 2009).

In another study in 2008 the authors investigate an herbal medicine, *Sam So Eum* (SSE) as a remedy of allergen-induced asthma in a mouse model and the authors discussed the mechanism of restoring the immuno-modulating cytokines such as IL-10 and IFN- γ. The authors revealed that the SSE-treated mice showed reduced

levels of airway responsiveness to Methacholine, and these levels were initially elevated by the induction of asthma compared to the control group. The authors suggested the possibility for using SSE as treatment for patients with asthma and stated that its therapeutic efficacy may be involved in the restoration of the IL-10 and IFN- γ levels (Cho *et al*., 2008).

The inhibitory effect of *Duchesnea chrysantha* (an herb with anti-oxidative, anti-inflammatory and immune-enhancing properties) extracts on OVA-induced lung inflammation in an asthmatic mouse model was investigated by authors in 2008. The results revealed that this herb significantly inhibited leukocytosis and eosinophilia on the bronchoalveolar lavage fluid and also significantly reduced the elevated level of infiltration of inflammatory cells and mucus secretion in the airways. With these results the authors concluded by suggesting the possibility that this herb can exert suppressive effects on asthma and suggested that it might be a useful agent in the treatment of allergic airway diseases (Yang *et al*., 2008).

In another study by Lee and Kim (2008), the immunomodulatory effect of *Juglans sinensis*, *Psoralea corylifolia* and Cheong-a-hwan extract and cyclosporine A on Th1 (IFN-γ) /Th2 (IL-4) cytokine balance and eosinophil accumulation in a murine model of asthma were investigated. The results obtained from this study revealed that *J. sinensis*, *P. corylifolia* and Cheong-a-hwan have profound inhibitory effects on the accumulation of eosinophills into airways and blood. The up regulation of the production of OVA-specific Th1 cytokine (IFN-g) and down regulation of OVA-specific Th2 cytokine (IL-4) in culture supernatant of spleen cells could also be observed. The authors also suggested that *J. sinensis*, *P. corylifolia* and Cheong-a-hwan extracts may potentially be a novel therapeutic agent for asthma by modulation of the relationship between Th1/Th2 cytokine balances (Lee and Kim, 2008).

Artemisia iwayomogi, is a perennial herb that is found around Korea and has been used as a traditional anti-inflammatory medicine in liver diseases. This plant was used by authors in 2008 to investigate the effects of actin interacting protein (AIP1) which is the water soluble carbohydrate fraction from the mentioned herb, on OVA-induced allergic asthma in BALB/c mice and to possibly determine the mechanisms of its anti-allergic action.

It was found that AIP1 significantly reduced pulmonary eosinophilia and Th2 cytokine expression in the lungs as well as serum IgE levels. Results from the flow cytometric analysis of lung-infiltrating cells revealed a decrease in surface levels of CD11c and MHC II in CD11c+MHC II+ cells and potent dendritic cells in the animals treated with AIP1. Also, expression of TNF-α was down-regulated by treatment with AIP1, and with these results the authors suggested that it could account for the suppression of pulmonary eosinophilia and Th2-type cytokine production by AIP1 (Lee *et al*., 2008).

In 2007, Pretorius *et al.* successfully used the BALB/c murine asthmatic animal model to investigate the effect of the phytomedicine *Euphorbia hirta* on the ultrastructure of platelets and fibrin networks. The authors found that this phytomedicine does not impact on the fragility of the fibrin but it prevents the minor fibers form forming a net-like layer over the major fibers as can be seen in the untreated asthmatic animals. Platelet morphology in the *E. hirta*-treated animals appear similar to that of the control animals, and the authors therefore suggested that ultrastructural studies may lead to a better understanding of the disease and possible treatment regimes (Pretorius *et al*., 2007a).

Numerous studies have been done on asthma by using herbal products, of which only a few are mentioned above. Homeopathy is also one of the fields of complementary and alternative medicine and is frequently used in the treatment of

asthma and other allergic diseases. Therefore, the principles of homeopathy as well as the role of homeopathic immunomodulators will be discussed in the following paragraphs.

2.1.8 Homeopathy

The term homeopathy is derived from the Greek words *homeo* which means similar and *pathos* meaning suffering or disease. Homeopathy is an alternative medical system and one of the key concepts includes stimulation of the body's defense mechanisms and processes as to prevent or treat illness. Most homeopathic substances are derived from natural substances from plants, animals and minerals and these substances are diluted in a series of steps during the preparation of the homeopathic product (National Centre for Complementary Alternative Medicine).

Jütte and Riley (2005) define a homeopathic product or medicine as a product that has been prepared in accordance with a homeopathic manufacturing procedure as defined by the European Pharmacopoeia or recognized national homeopathic pharmacopoeias (Jütte and Riley, 2005).

It is also very important to distinguish between herbal products or phytotherapy and homeopathic products or homeopathy. If a medicine is prepared by an herbalist on the basis of the available literature on the use of herbs it is known as phytotherapy rather than homeopathy and when a medicine is prescribed on the basis of a patient's symptoms in accordance with the law of similars, it is said to be homeopathy and not phytotherapy. Therefore, Jütte and Riley, (2005) stated that whether a medicinal product is homeopathic or not, is determined, from a pharmaceutical perspective, by its production in accordance with a recognized homeopathic pharmacopoeia and whether a medicinal product is homeopathic or

phytotherapeutic, from a clinical perspective, is determined by the rationale behind its use (Jütte and Riley, 2005).

An important aspect of homeopathy is the so called "*Law of Similars*" or also called "*like cures like*". What this means is that a substance produces symptoms of illness in a well person when administered in large doses where if the same substances are administered in minute quantities, it will cure the disease in a sick person. Hahnemann, the founder of homeopathy, stated that this is because nature will not allow two similar diseases to exist in the body at the same time (Gold *et al*., 2008). This theory might, however, sound very unscientific to some traditional researchers as well as members of the medical fraternity. Also, there are still many debates on the plausibility, theoretical principles, clinical and basic research evidence, ethical considerations and other issues surrounding homeopathy. However, there is also evidence indicating the biological activity of homeopathic products that are not seen with the placebo.

In animal studies on experimentally induced paw edema, arsenic poisoning, chemically induced liver cancer, changes in normal physiology such as EEG sleep stages, and many other research have repeatedly demonstrated the biological effect of homeopathic products (Gold *et al*., 2008).

Homeopathic remedies exhibit biological activities across many *in vivo* and *in vitro* animal studies. The product that is focused on in the current thesis is such a homeopathic product. Therefore, the known properties of Modul8® as well as the effects of a homeopathic immunomodulator Canova®, from which Modul8® is a modified version containing some of the same components and as well as many others, will be discussed in the following paragraphs. No research has previously been done on Modul8® and therefore no literature is available on this product.

Therefore the immunomodulatory properties of the known homeopathic product Canova® will be discussed later in this chapter to highlight the effects of a homeopathic product on the immune system.

- **Modul8®**

Modul8® is a homeopathic product of natural origin that was developed in a laboratory in Argentina and today is produced, according to the Hanemannian homeopathic method, as a homeopathic remedy. The final product is an aqueous, odorless and colorless solution that contains: *Aconitum napellus* (**D20),** *Arsenicum album* (**D18),** *Asafoetida* (**D20),** *Calcarea carbonica* (**D16),** *Conium maculatum* (**D17),** *Ipecacuanha* (**D13),** Phosphorus (**D20),** *Rhus toxicodendron* (**D17),** Silicea (**D20),** Sulfur (**D24),** *Thuja occidentalis* (**D19),** Alcohol (**0.2% v/v)** and Purified water (**44.050 ml.)**.

The composition per 50 ml bottle is expressed as declared potencies (D), which indicates an index of 10-fold dilution. No signs of toxicity or mutagenic effects have been reported. The properties and common uses of the most abundant components in Modul8® will be mentioned in the following paragraphs.

Aconitum napellus is commonly known as monkshood. A non-toxic, diluted extract of aconite is used in homeopathy to treat symptoms similar to that of poison. Anodyne, diuretic and diaphoretic are some of the medicinal actions of this plant. (www.botanical.com).

Arsenicum album is used in the treatment of acute ailments, chronic diseases and acute colds, bronchitis and fever and is also used in the treatment of asthma (Cummings and Ullman, 1997; Kent, 1996).

Asafoetida is also known as *Ferula* which is a genus of many species and is a perennial herb that is widely used in India as flavoring agent in food and it is also used in traditional medicine for many different conditions including indigestion, whooping cough, inflammation, epilepsy, pain, cholera, infertility and convulsions. It was reported that it possesses antioxidant and anti-carcinogenic activity in Swiss albino mice (Saleem *et al*., 2001).

Calcarea carbonica is a component that is derived from the middle layer of shells and in chemical terms is impure calcium carbonate ($CaCO_3$) and is commonly used to treat acne, arthritis, vaginal discharges in women, night terrors in children and ringworm on the scalp (Cummings and Ullman, 1991).

***Conium maculatum* L**. is an umbelliferous weed known worldwide for its toxicity to many domestic animals and humans. It produces eight different alkaloids of which Coniine and γ-coniceine are the most abundant and are known to have antifungal and antimicrobial properties (Al-Barwani and Eltayeb, 2004).

Ipecacuanha is part of the family *Rubiaceae* and is a perennial medicinal plant of which the roots are the sources for an expectorant, emetic and amoebacide and this plant is cultivated for the alkaloids, emetine and cephaeline (Rout *et al*., 2000).

Rhus toxicodendron is a traditional homeopathic name for a plant called *Toxicodendron pubescens.* This plant is used in various inflammatory conditions and in 2007 it was reported that *Rhus toxicodendron* in homeopathic dilution interfered with inflammatory mediators including histamine and other prostaglandins indicating that it may act through inflammatory mediators in the immune response when the authors investigated carrageenan-induced paw oedema in rats (Santos *et al*., 2007).

Thuya occidentalis is a popular homeopathic remedy used to treat warts. It has also been indicated for dandruff, skin problems, torn muscles and joints and chronic urinary infections (www.botanical.com).

- **Homeopathic products as immunomodulators**

Modul8® is thought to have immunomodulatory properties and immunomodulators are defined as agents that enhance the immunity of an individual to favor a particular immunological response. Researchers have found that immunomodulators stimulate host defenses against several pathological states (de Oliveira *et al*., 2006).

- **Effects of an immunomodulator similar to Modul8® on cellular level**

Canova® is a complex homeopathic immunomodulator that contains many of the substances found in Modul8®. Canova was developed in the Canova laboratory in Argentina and today it is produced as a homeopathic medicine in Brazil (Seligmann *et al*., 2003), according to the Hanemannian homeopathic method. The final product is an aqueous, odorless and colorless solution that contains *Thuya occidentalis (Cupresaceae), Bryonia alba (Curcubitaceae), Aconitum napelius (Ranunculaceae), Arsenicum album (arsenic trioxide)* and *Lachesis muta (Veripidae)* (Lopes *et al*., 2006).

It is known to be neither toxic nor mutagenic, is available as drops, and is used by researchers in a wide concentration range from 4% to 50%. The composition per 50 ml bottle is expressed as declared potencies (D), which indicates an index of 10-fold dilution. *Aconitum napelius*: D 11, *Arsenicum album*: D 19, *Bryonia alba*: D 18, *Lachesis muta*: D 18, *Thuya occidentalis* D 19, and 49.965 ml water and 0.035ml alcohol.

In 2002, Piemonte and Buchi analyzed the production of IL-2, IFN-γ and TNF-α as well as the ultrastructure of peritoneal macrophages treated with a homeopathic medicament Canova®. The authors revealed that 86% of the macrophages treated with Canova® were activated compared to the 15% of the control group. Activation was determined by certain characteristics such as enlarged nucleus, more euchromatin than heterochromatin, spreading and projections. Conduction of the ELISA revealed no difference in IL-2 and IFN-γ between the treated and control group whereas Canova® showed to decrease TNF- α production compared to the control group.

In another study in 2006, Abud and co-workers investigated the activation of bone marrow cells treated with Canova® *in vitro* (Abud *et al*., 2006). As previous studies have found that Canova® induces up-regulation in a number of leukocytes and increase the number of TCD_4 lymphocytes as well as NK cells in peripheral blood, (Sato *et al*., 2005), the authors aimed to determine whether Canova® can promote the differentiation, proliferation and survival of mouse bone marrow cells *in vitro* as it is the major site for blood cell formation. This study showed an increase in the number of adherent, larger and activated cells in the Canova®-treated group. The authors revealed that most of the adherent cells found in their experiment were macrophages ($CD11b^+$) and Canova® is known to activate macrophages *in vivo* and *in vitro* (Piemonte and Buchi, 2002; de Oliveira *et al*., 2006; Lopes *et al*., 2006; Pereira *et al*., 2005).

The authors concluded by stating that these findings are very important because Canova® is a non-toxic medication (Seligmann *et al*., 2003) and therefore can possibly be used in *in vitro* cell therapy for many diseases that affect bone marrow cells (Abud *et al*., 2006).

In 2006, Lopes *et al*. investigated the cellular aspects of macrophage activation by Canova®. The authors aimed to study the activation of mice peritoneal macrophages when exposed to *in vitro* and *in vivo* Canova® treatment as well as morphological parameters and acid phosphatase activity by using light- and electron microscopy.

The results obtained revealed a greater spreading ability in the Canova®-treated macrophages, a higher phagocytic activity of non-infective microorganisms as well as a tendency to lower the phagocytic activity of the infective microorganisms *T. cruzi* trypomastigotes and *Leishmania amazonensis*, comparing to the control cells. Analysis of the acid phosphatase activity showed that Canova® treatment stimulates an increase of the endosomal/lysosomal system. With their results, the authors concluded that Canova® is an effective stimulator of macrophage activity (Lopes *et al*., 2006).

These findings correlate with those found by Pereira *et al.* in 2005 who investigated the immunomodulatory effect of Canova® on experimental *Leishmania amazonensis* infection. The *in vitro* results showed that at 40% Canova® the infection index was reduced and NO production was induced in the elicited macrophages. The *in vivo* tests also revealed reduced infection as well as decreased parasite load in the regional popliteal lymph nodes and in the spleen. From the results obtained by the authors they suggested that Canova® modulates experimental infection, control infection progression and limit dissemination (Pereira *et al*., 2005).

Canova® was also tested using human monocytes. Canova® has been shown to activate macrophages *in vitro* and *in vivo*, with resultant enhanced spreading of the cells and formation of microvillus extensions.

Since monocytes are the precursor cells of macrophages and dendritic cells, the objective of the study was to investigate the effects of Canova® on the differentiation of human blood monocytes *in vitro*. Monocytes were isolated, grown in culture and exposed to 10% and 20% Canova® without the addition of cytokines. After 48 hours, monocytes were prepared for analysis by scanning electron microscopy (SEM), while cells kept in culture for 7 days and exposed to Canova® on days 1, 3 and 4 were analyzed by flow cytometry for alterations in the levels of expression of various markers.

SEM analysis revealed monocytes exposed to 10% Canova® with morphology similar to that of macrophages. Various cytoplasmic projections (pseudopodia) were observed. Flow cytometric analysis indicated high cell viability and up-regulation of CD80, compatible with differentiation into either macrophages or dendritic cells. Exposure to Canova® per se causes activation of monocytes with differentiation into large macrophage-like cells of indeterminate phenotype, which has increased expression of CD80. Like cytokines, Canova® induces differentiation of monocytes, and this may underpin the immunomodulatory activity of the product (Smit *et al*., 2008).

- **Homeopathy in cancer therapy**

Sato and co-workers also investigated Canova® for its potential in cancer therapy in 2005. They studied the effect of Canova® in normal and sarcoma 180-bearing mice. A comparison between the Canova®-treated and the control groups revealed a delay in the development and reduction in size of the tumor. They also found an increase in the infiltration by lymphoid cells, granulation tissue and fibrosis surrounding the tumor in the Canova®-treated group. Determination of the white blood cell count showed an increase in the total number of leukocytes and lymphocytes. These results all reflect

an enhanced immune response of the host after treatment with Canova® (Sato *et al*., 2005).

- **Homeopathy and HIV**

In 2008, Pretorius *et al*. investigated the ultrastructural changes in platelet aggregates of HIV patients. The ultrastructure of platelet aggregates of patients with HIV have shown membrane blebbing and ruptured platelet, which is indicative of apoptosis. With these observations the authors suggested that HIV patients may develop thrombocytopenia as a result of peripheral platelet destruction. The differences in platelet morphology from control, Canova®-treated HIV patients and non-treated HIV patients were investigated by using scanning electron microscopy.

The authors revealed slight morphological changes when comparing the control fibrin networks with Canova®-treated HIV and untreated HIV patients, which suggests that the HIV does not have an impact on the fragility of fibrin networks. However in the HIV patients, bleb-like bulges on the membrane of platelets as well as membrane breakages on the aggregate could be observed, where membrane blebbing was less pronounced in the Canova®-treated patients. From these observations, the authors suggested that Canova® protects the membranes of platelets and that blebbing does not appear in such great proportions as was found in the untreated HIV group. The authors concluded by stating that this study supports previous research done on Canova® where it was found that Canova® protects the immune system, by keeping the ultrastructure intact and also preventing the cyto-destructive effects of HIV (Pretorius *et al*., 2008).

- **Clinical trials using a homeopathic immunomodulator**

A number of clinical trials have also been done, focusing mainly on HIV and cancer research. Table 2.1 indicates the different clinical trials done on Canova® as well as the findings on the studies done.

Table 2.1: Clinical trials done on HIV and cancer using an immunomodulator as treatment remedy

Year	Title	Authors	Findings
2006	Patients' recovery with HIV/AIDS in Botswana / Africa, with the use of the homeopathic Medicine Canova®.	Bernardi, R.P. *et al*.	Decrease pain; Increase appetite; Recovered capability to perform tasks; Decreased absenteeism
2005	HIV/AIDS+ patients treated with the homeopathic Medicine Canova®:Prospective Observational study in Laboratory, Clinical and of Quality of Life Indexes.	Stroparo E. *et al*.	Increase appetite and energy; Decrease pain and depression; Decrease opportunistic infections; Increase total number of lymphocytes, CD4 and erythrocytes; Increase in life quality
2004	Quality of life and handling of Cancer or Aids with Immunomodulator Canova®	Castanheira, P. *et al*.	Improvement of live quality in cancer and HIV patients
2003	Evaluation of Canova® Immunomodulator's use in patients with cancer.	Castanheira, P. *et al*.	Decrease pain complaints; Improvement of appetite and cognitive function; Decrease of tumor volumes;
2002	A Multicentric Clinical Survey of HIV/AIDS patients submitted to Canova® Immunomodulator treatment associated with Antiretrovirus Medicaments	Berbert, A.A. *et al*.	Weight gaining; Decrease viral load; Improvement of life quality
2002	Clinical study work for evaluation of Immunomodulator Canova® in the therapeuticas of oncologic patient considered OTP – Out of Therapeutic Possibility	Cabral, M.P. *et al*.	Weight gain; Improved appetite; Reduction of nausea and vomits; Increase willingness for daily activities; Increase in some

			lymphocytes and monocytes
2002	Immunomodulator Canova® – Aids and quality of life.	Brito, D. J. M	Improvement of general health; around 10% weight gain and appetite improvement; decrease insomnia; decrease cramps; better perspectives; accentuated decrease of infections; Increase in total leucocytes and CD4; decrease viral load
2001	Randomized Placebo-Controlled Clinical Trial to appraise the effectiveness and safety of Immunomodulator Canova® in the therapeutics of patients who have HIV/AIDS on anti-retroviral use.	Sasaki, M.G.M. *et al*.	Reduction of viral load; Decrease occurrence of opportunistic diseases; Decrease antiretroviral toxicity; Improvement of life quality

2.1.9 Animal models in asthma

Mice are used all over the world as experimental animals in many different studies. Using mice as experimental animals offers a number of advantages. Firstly, the mouse genome is extensively characterized and exhibits a high degree of homology with its human counterpart and in 2002 it was reported that approximately 99% of genes are shared between mice and humans and around 90% of the two genomes can be portioned into regions of conserved synteny (Waterston *et al*., 2002).

Another advantage of using mice is the fact that a wide range of developmental, biochemical, and physiologic features are shared between humans and mice and this resemblance that exists, especially on immunological level, holds meaning that many human functions and dysfunctions can be modeled efficiently in mice and that these models deliver important an relevant insights into human pathology (Willis-Owen and Valdar, 2009).

In 2002, Pennisi reported that mice are an established experimental tool and have been used in the laboratory since 1664 (Pennisi, 2002). Another advantage also includes the fact that mice are quick to breed and economical to house, since they have a short gestation period of just 19-21 days, an early puberty with an average of 42 days to reach fertility, and a short estrus cycle of 4-5 days and tend to produce relatively large litters usually 4-12 pups. Taking all this characteristics into account, it is said that mice has become the foremost mammalian system in which to model human disease (Willis-Owen and Valdar, 2009).

In conclusion, it is clear from the literature that many studies have been done on asthma by using different substances and investigating different parameters aiming to better understand the mechanisms involved in this disease. The present study therefore aims to contribute to the global pool of knowledge and research that exist

up to date by investigating the possible role of a homeopathic product in the treatment of asthma.

2.2 Aims and objectives of the current study

The general aims and objectives of this study include the following:

1. Implementing the asthmatic BALB/c mouse model
2. To use the BALB/c mouse model to investigate the ultrastructure of platelets and fibrin networks by using scanning electron microscopy
3. To compare the ultrastructure of platelets and fibrin networks of the asthmatic BALB/c mice to that of human control and asthmatic subjects
4. To statistically evaluate the changes in weight of each individual mouse in the different experimental groups over the 43- day experimental period
5. To use the BALB/c mouse model to determine the effect of Modul8® on white blood cell counts in blood as well as bronchial lavage
6. To investigate the ultrastructure of the LPS-activated peritoneal macrophages *in vitro* by using scanning electron microscopy and to analyse the production of TNF-α and nitric oxide (NO) in LPS-activated peritoneal mouse macrophages exposed to Modul8®
7. To use light and transmission electron microscopy to investigate the histological and ultrastructural changes in the lungs including inflammatory cell infiltration

2.3 Hypothesis

In asthma, platelets, fibrin networks and lung tissue are altered morphologically. Also, white blood cell counts are increased in the blood as well as in the bronchial lavage and the levels of TNF-α are changed. Therefore the hypothesis of this study is that Modul8® will stabilize the platelets, fibrin networks as well as lung tissue in asthmatic BALB/c mice, and that the ultrastructure of the mentioned tissues will appear similar to that of the control animals. We also hypothesise that the white blood cell counts in asthmatic animals will decrease and the levels of TNF-α will be stabilized due to Modul8® administration and that Modul8® will activate macrophages *in vitro*. It is also known that the experimental procedures influence the weights of the animals over the experimental period. We hypothesise that Modul8® will positively influence the weight changes by stabilizing it.

CHAPTER 3

IMPLEMENTATION OF THE BALB/c ASTHMATIC MOUSE MODEL

3.1 Introduction

Research that has been done by using animal models has provided important information over the years on many aspects of the pathophysiology of human diseases. Well-performed animal studies can be used to determine potential benefits of proposed therapeutic interventions and the results obtained from many experimental animal models on many different diseases have also served as the basis for many clinical trials (O'Neil *et al*., 1999). Although there is no animal model that exactly reproduces the pathology of human asthma, researchers in 2003 stated that they play an important role in the development of novel therapies and in the understanding of the pathogenesis of the disease (Isenberg-Feig *et al*., 2003).

A wide range of different animal models is available and choosing the appropriate model for a specific study is very important and sometimes difficult. Mice and rats and also guinea pigs are some of the most popular animal models used (Zosky and Sly, 2007). Murine asthmatic animal models have contributed significantly to the knowledge and understanding of airway disease, such as in delineating the role of T lymphocytes in the orchestration of inflammation and airway hyperresponsiveness and the induction of respiratory tolerance (Willis-Owen and Valdar, 2009).

According to Epstein (2006), antigen-induced mouse allergic asthma is a useful model for testing novel therapeutics (Epstein, 2006) and that these models offer excellent tools for studying the effects of allergens *in vivo* in their natural target organs (Epstein, 2004).

In vivo animal models have been used successfully during the past few years to study diseases like asthma (Epstein, 2004).

Since a correlation exists between the allergic response in sensitive mice and that of human asthmatics, asthmatic animal models have been developed and used to study the immune and inflammatory mechanisms of the disease. These models have also been used to investigate the efficiency of new drugs in the treatment of asthma. There are a number of corresponding features, with regards to airway remodeling, in humans and mice. This includes mucus cell metaplasia, shedding of the epithelium, thickening of the basement membrane and edema (Blyth *et a*l., 1996; Hogan *et al*., 1996).

However, there are important differences in the airway development and morphology in humans and mice and thereby preventing the direct extrapolation of results between species. Differences in the number of branches, the pattern of branching, lobation and submucosal gland distribution exist (Metzger *et al*., 2008).

The murine model in particular, is used successfully because mice allow for a variety of *in vivo* immunological applications (Bice *et al*., 2000), however it is important to take into account that mouse models are not exact replicas of human asthma but they help to understand some of the basic mechanisms involved in the production of this disease (Roberto *et al*., 2005).

The murine asthmatic BALB/c mouse model was used as experimental model in this study, and therefore this chapter includes the materials and methods used on implementation of the BALB/c asthmatic mouse model. The different experimental procedures that were conducted on specific days are indicated in Table 3.1. This model with its experimental procedures was also used before by researchers to

investigate the role of alternative products on asthmatic animals (Oberholzer *et al*., 2008). Experimental procedures, including sensitization, nebulization and treatment are stated as well as the equipment, materials and methods used during each of the abovementioned procedures.

3.2 Implementing the asthmatic BALB/c mouse animal model

6-week-old (female) BALB/c mice (each of average weight 20g), maintained in the University of Pretoria Biomedical Research Centre and provided OVA-free food (Balanced EpolT mice cubes and pellets, obtained from EPOL- a division of Rainbow Farms PTY LTD, SA) and water *ad libitum*, were used. Polycarbonate type III cages were obtained from Techniplast. The animals were kept in a barrier unit with a temperature range of 20-24°C and a relative humidity of 40-60% with a 12h daylight and 12h night-time.

6 mice were housed per cage and autoclaved pinewood shavings were used as embedding material. White facial tissue paper was also added for enrichment. All experimental protocols complied with the requirements of the University of Pretoria's Animal Use and Care Committee and ethical clearance was obtained from the University Animal Ethics Committee (UPBRC). Ethical clearance number: (151/2006).

Animals were monitored daily by the lab animal technologist for clinical signs and abnormalities. No clinical signs of respiration, stridor or abnormal food intake could be observed. Also, no abnormal behaviour could be seen in any of the animals.

Mice were randomly divided into the following groups:

- 6 control mice
- 6 asthmatic mice receiving no treatment
- 6 asthmatic mice exposed to physiologically comparable levels of Modul8® (10%) (referred to as the Modul8® group) and
- 6 asthmatic mice exposed to hydrocortisone (referred to as the hydrocortisone group) at a concentration of 100mg/kg body weight.

Table 3.1: The different experimental procedures conducted on specific days

DAY: 0, 5	Sensitization
DAY: 13, 14, 15	Nebulisation
DAY: 15, 16, 17, 18	Treatment
DAY: 22, 23, 24, 25	Treatment
DAY: 30, 31, 32	Nebulisation
DAY: 36, 37, 38, 39	Treatment
DAY: 42, 43	Termination

3.3 Sensitization

Sensitization of mice involved the intraperitoneal injection of 25mg ovalbumin (OVA) (Grade V; Sigma Aldrich) and 2mg $Al(OH)_3$ dissolved in 0.5ml of 0.9% saline solution. All mice except those in the control group were sensitized.

3.4 Nebulization

The mice were nebulized with 1% OVA dissolved in PBS. An inhalation exposure system (IES) (Glas-COL® Inhalation Exposure System, Model 099C A4212, Terre Haute, Indiana) was used for the nebulization. The protocol used for the nebulization

is standard and the times used are recommended by the manufacturer of the IES and programmed on the machine.

Nebulization involved placing the 6 mice from each experimental group except the control group, inside a stainless steel sire mesh basket, which is divided into five equal compartments. Each compartment therefore held the six animals from the same experimental group. One complete cycle in the IES included a preheat cycle of 15 minutes followed by the nebulization with the OVA for 60 minutes, a cloud decay cycle for 15 minutes and a decontamination cycle also 15 minutes. Mice were nebulized twice daily.

3.5 Treatment

Treatment involved the administration of Modul8® and hydrocortisone to asthmatic animals. Solu-Cortef™ 100mg hydrocortisone [Pfizer Laboratories (Pty) Limited], dissolved in sterile PBS was injected intra-peritoneally, while Modul8® [Natel Healthcare SA (PTY) Ltd] was administered orally on the specific treatment days as indicated in Table 3.1.

For humans it is generally advised to take 10 drops of Modul8® 3 times per day. And since the average of 10 drops equals approximately 600µl, it can be said that humans take 600 µl three times per day. This will equal an amount of 1800 µl per day (1.8ml/day). When the average weight of a human adult is also considered (between 60-70kg: average 65kg): It can therefore be assumed that a normal person that weighs 65kg will take in 1800ul = 1800µl / 65kg. This will imply that the intake is: 28µl/kg. The average weight of a mouse is around 20g (0.02kg). And since it was established that the intake per day is 28µl/kg, it can be calculated that each mouse should receive 0.56µl of the Modul8® per day in order to keep the same values as the

human. Since only one daily administration of the test compound was needed, oral gavage was used as it allows for administration of a controlled amount of compound.

The adult dosage of 100mg/kg body weight hydrocortisone was converted to the average mass of a mouse, which was taken at 20g. The hydrocortisone was administered via intraperitoneal injection, as was indicated on the product itself and since it is one of the most common routes of administration in mice.

3.6 Conclusion

The BALB/c murine asthmatic animal model was successfully implemented in this study over the 43 –day experimental period. No animals died during the course of the study nor did any of the animals suffer excessive weight loss. The results presented in the chapters to follow, indicate that this model was successfully implemented and that the animals were indeed asthmatic. It is also reported that there is a correlation between the allergic response in sensitive mice and that of human asthmatics.

Therefore the results found in this study indicate that the BALB/c asthmatic mouse model can successfully be used to study a disease like asthma and furthermore that this OVA-induced allergic mouse model is useful in testing alternative medication on asthma.