CHAPTER 4

INVESTIGATING THE EFFECT OF MODUL8® ON THE ULTRASTRUCTURE OF PLATELETS AND FIBRIN NETWORKS OF EXPERIMENTAL ASTHMATIC BALB/c MICE

4.1 Introduction

Platelets and fibrin play an important physiological role in allergic processes and immunological mechanisms including those associated with asthma where platelets participate by acting as inflammatory cells as they are releasing mediators, spasmogens and interacting with other inflammatory cell types. Platelets are activated by a number of stimuli and this activation may be due to, amongst others, inflammatory processes (Camera *et al*., 1999; Butenas and Mann, 2002; Lazarus *et al*., 2003).

An important role for platelets in various inflammatory diseases has been reported on including atherosclerosis, rheumatoid arthritis, eczema, allergic rhinitis and asthma (Kornerup and Page, 2007). It was also found that platelets play an important role in asthma as they have been found to actively participate in most of the main features in asthma such as bronchial hyperresponsiveness, bronchoconstriction, airway inflammation and airway remodeling. Platelet-release products as well as the expression of adhesion molecules on the surface of the platelet and the ability to undergo chemotaxis are all involved in these features in the inflammatory process in asthma (Kornerup and Page, 2007).

Platelets also play an important role in bronchoconstriction and several animal studies have been done to investigate this. Activated platelets secrete various mediators that contribute to the bronchoconstriction such as histamine, serotonin (5-

HT), platelet activating factor (PAF) and arachidonic acid metabolites (Page, 1989). In a study done on guinea pigs, the intravenous administration of PAF resulted in acute bronchoconstriction and recruitment of macrophages, eosinophils and platelets into the airways (Vargaftig and Braquet, 1987).

PAF is a potent activator of platelets and neutrophils and can induce systemic anaphylaxis when given intravenously and ultrastructural studies have also shown that in addition to causing neutrophil and eosinophil recruitment into the lungs, PAF also induces intravascular platelet aggregation and degranulation in alveolar capillaries and stimulates platelet diapedesis to the alveolar lumen, features which are absent in guinea pigs pre-treated with prostacyclin or aspirin (Lellouch-Tubiana *et al*., 1985).

Platelets also play a role in the recruitment of inflammatory cells into the airways of asthmatic individuals. It was previously reported that platelet depletion, as well as administration of prostacyclin (PGI_2) and PAF antagonists in asthmatic animal models, significantly inhibits eosinophil infiltration into the lungs (Coyle *et al*., 1990; Lellouch-Tubiana *et al*., 1988). It was also shown in an OVA-induced murine model of allergic lung inflammation that eosinophil recruitment into the airways is inhibited in thrombocytopenic animals (Pitchford *et al*., 2003), suggesting that platelets are essential for leukocyte recruitment in allergic models of asthma.

Together with the role of platelets in bronchoconstriction and the recruitment of inflammatory cells into the airways, is the role of platelets in the process of airway remodeling in asthma. Platelets secrete various mediators including free radicals and cationic proteins that have been shown to contribute to the increase in vascular permeability of the airway epithelium and the stimulation of mucus secretion

(Kornerup and Page, 2007). Platelets therefore represent an interesting new target for the study of inflammatory diseases whose pathogenesis remains unclear.

In the current chapter the BALB/c mouse model was used to investigate the possible effect of Modul8® on the ultrastructure of platelets and fibrin networks by using scanning electron microscopy. Hydrocortisone is used as positive control in the study as it is the most commonly used medicine in the treatment of asthma (methodology of model described in Chapter 3).

4.2.1 Preparation of fibrin clots

Blood was collected on the day of termination via orbital puncture. 11 µl of citrate was added for every 100 µl of blood drawn. Blood was then centrifuged at 1250 rpm for 2 minutes to obtain platelet rich plasma (PRP).

Human thrombin (provided by The South African National Blood Services) was used to prepare the fibrin clots. The thrombin is 20 U/ml and is made up in biological buffer containing 0.2% human serum albumin. When thrombin is added to PRP, fibrinogen is converted to fibrin and intracellular platelet components e.g. transforming growth factor, platelet derived growth factor and fibroblastic growth factor are released into the coagulum.

10 µl of mouse PRP was mixed with 10 µl of human thrombin. The PRP and thrombin mix was immediately transferred with a pipette tip to a 0.2 µm millipore membrane to form the coagulum (fibrin clot) on the membrane. This millipore membrane was placed in a Petri dish on filter paper dampened with PBS to create a humid environment and placed at 37ºC for 10 minutes. A washing process followed where the millipore membranes with the coagula were placed in PBS and

magnetically stirred for 1hr. This was done to remove any blood proteins trapped within the fibrin network (Pretorius *et al*., 2006).

4.2.2 Preparation of washed fibrin clot for SEM

Washed fibrin clots were fixed in 2.5% glutaraldehyde in Dulbecco's Phosphate buffered saline (DPBS) buffer with a pH of 7.4 for 1hr. Each fibrin clot was rinsed three times in phosphate buffer for 5 minutes before being fixed for 1hr with 1% Osmium tetraoxide (OsO_4.) The samples were rinsed three times with DPBS for 5 minutes and dehydrated serially in 30%, 50%, 70%, and 90% and three times with 100% ethanol.

The SEM procedures were completed by drying of the material with hexamethyldisilazane (HMDS) (Araujo *et al*., 2003), mounting, coating with ruthenium tetraoxide (SPI Supplies, West Chester USA) and examination of the tissue with a ZEISS ULTRA plus FEG scanning electron microscope.

4.3 Results

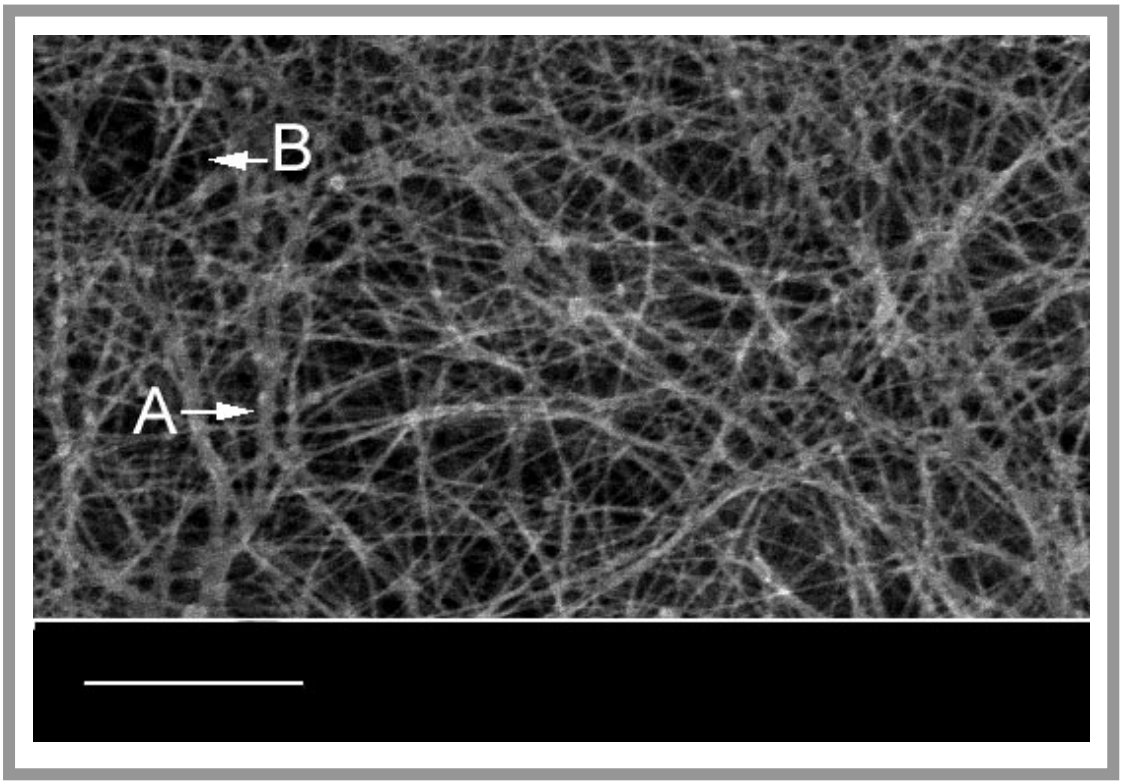

Figure 4.1a: Control fibrin network of BALB/c mice with thick, major fibers as well as thin, minor fibers. Label A = thick, major fibers; Label B = thin, minor fibers. (Scale bar = 200nm)

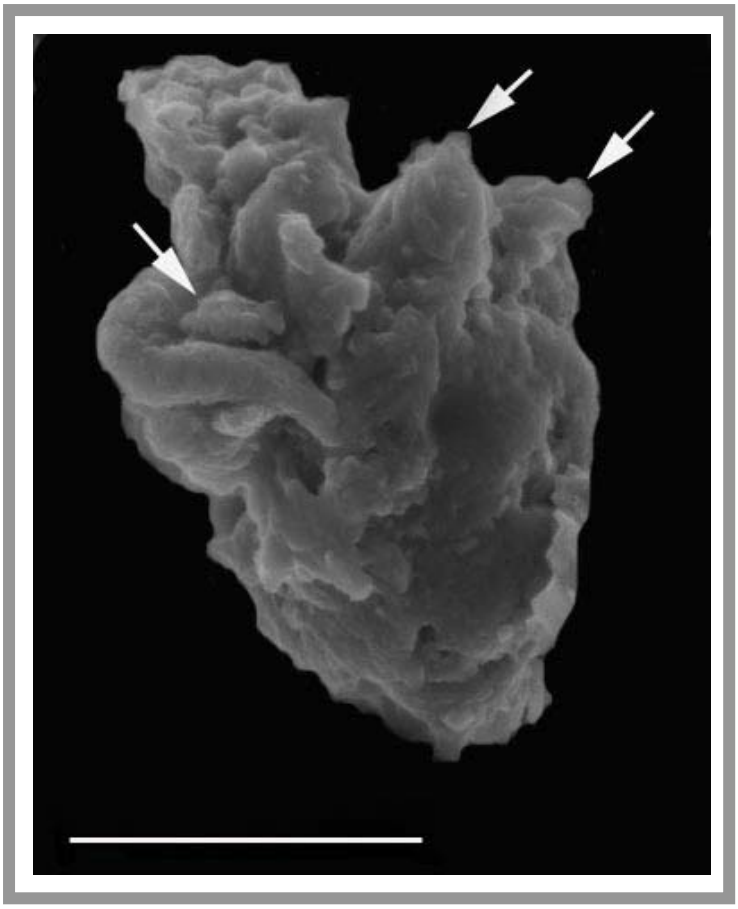

Figure 4.1b: Control platelet aggregate of BALB/c mice; white arrows – pseudopodia. (Scale bar = 1µm)

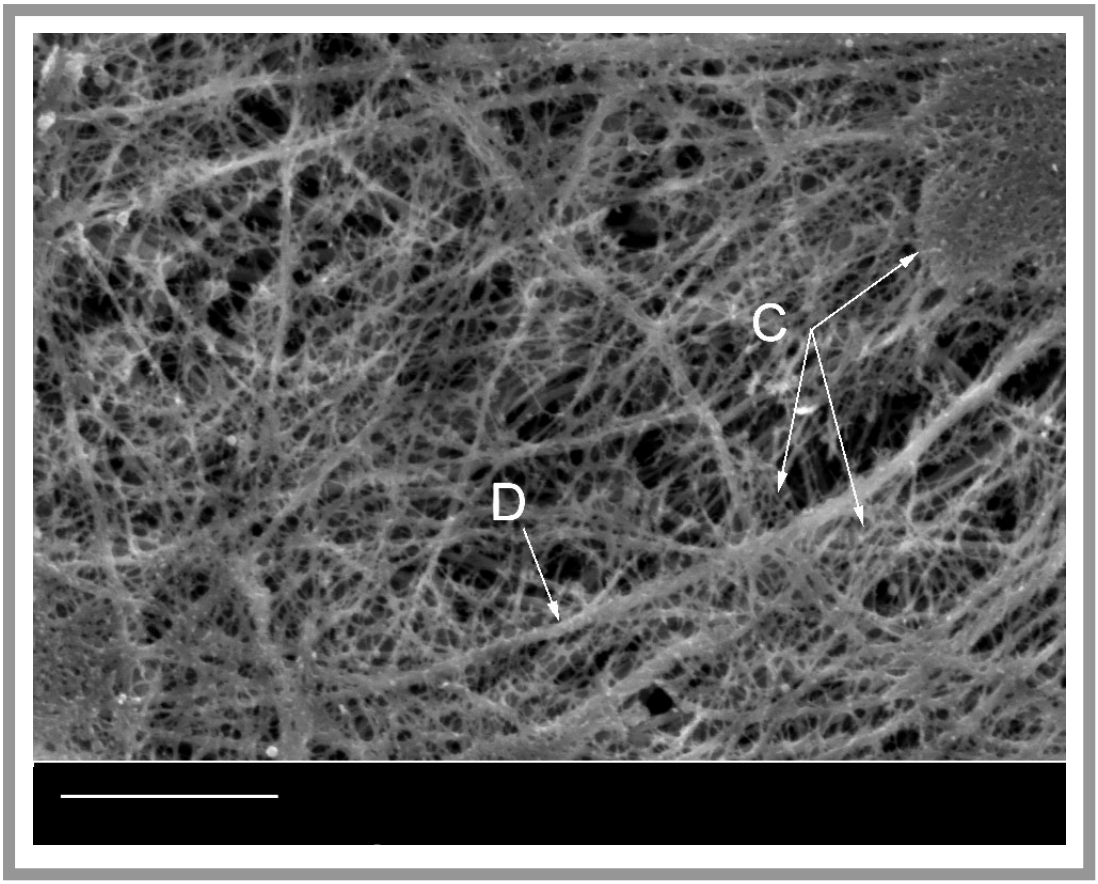

Figure 4.1c: Fibrin network of asthmatic BALB/c mice, forming flimsy fibrin network. **Label C** = matted, thin minor fibers; **Label D** = thick, major fibers.
(Scale bar = 200nm)

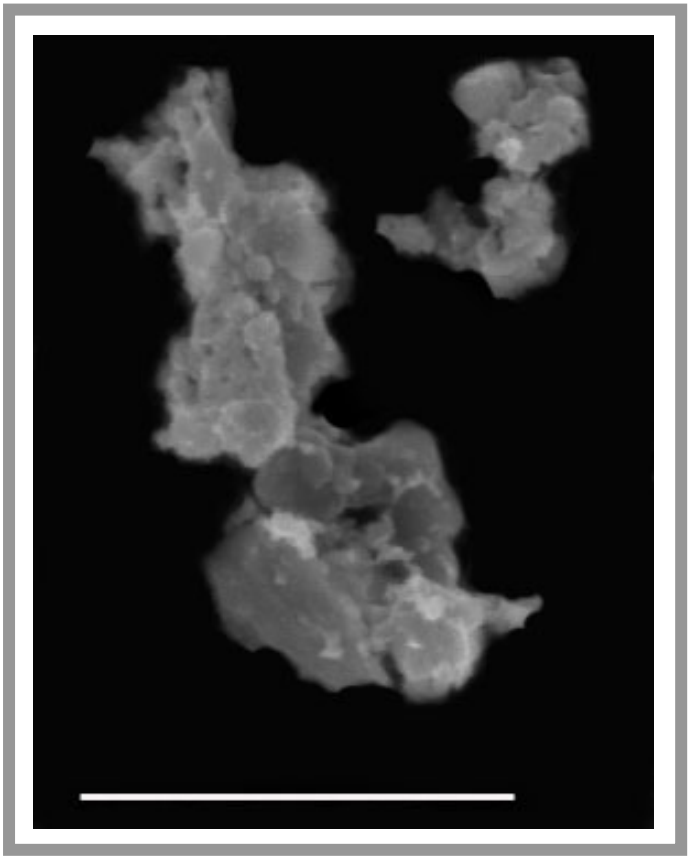

Figure 4.1d: Platelet aggregate of asthmatic BALB/c mice.
(Scale bar = 1µm)

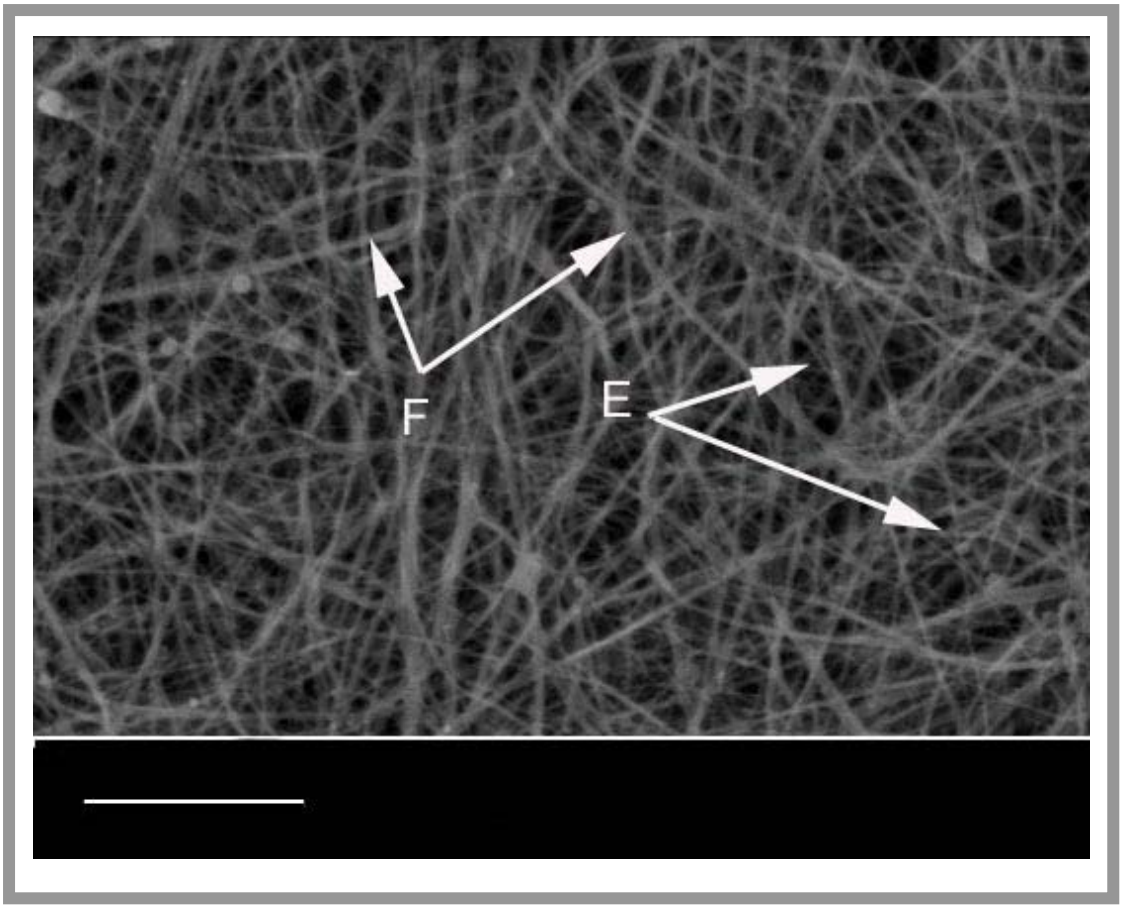

Figure 4.1e: Fibrin network from asthmatic BALB/c mice treated with Modul8®. **Label E** = Thin, minor fibers; **Label F** = Thick, major fibers
(Scale bar = 200nm)

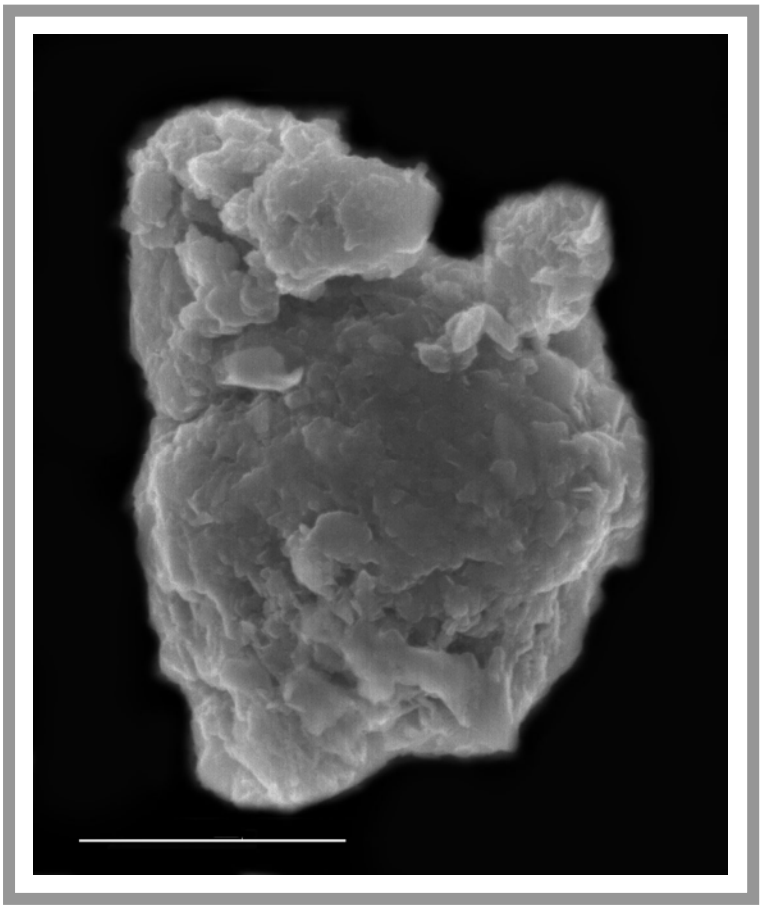

Figure 4.1f: Platelet aggregate from asthmatic BALB/c mice treated with Modul8®. (Scale bar = 1µm)

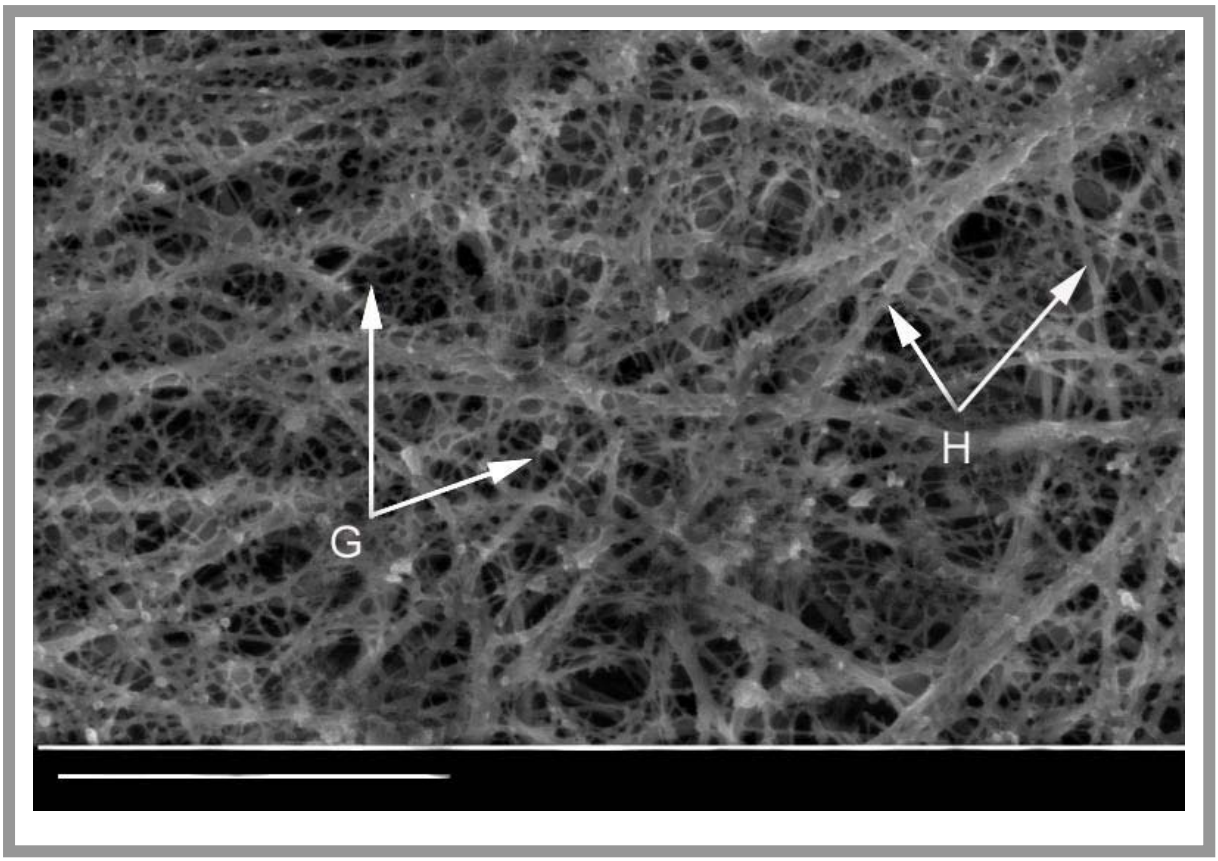

Figure 4.1g: Fibrin network from asthmatic BALB/c mice treated with hydrocortisone. **Label G** = thin, matted minor fibers; **Label H** = thick major fibers (Scale bar = 1µm)

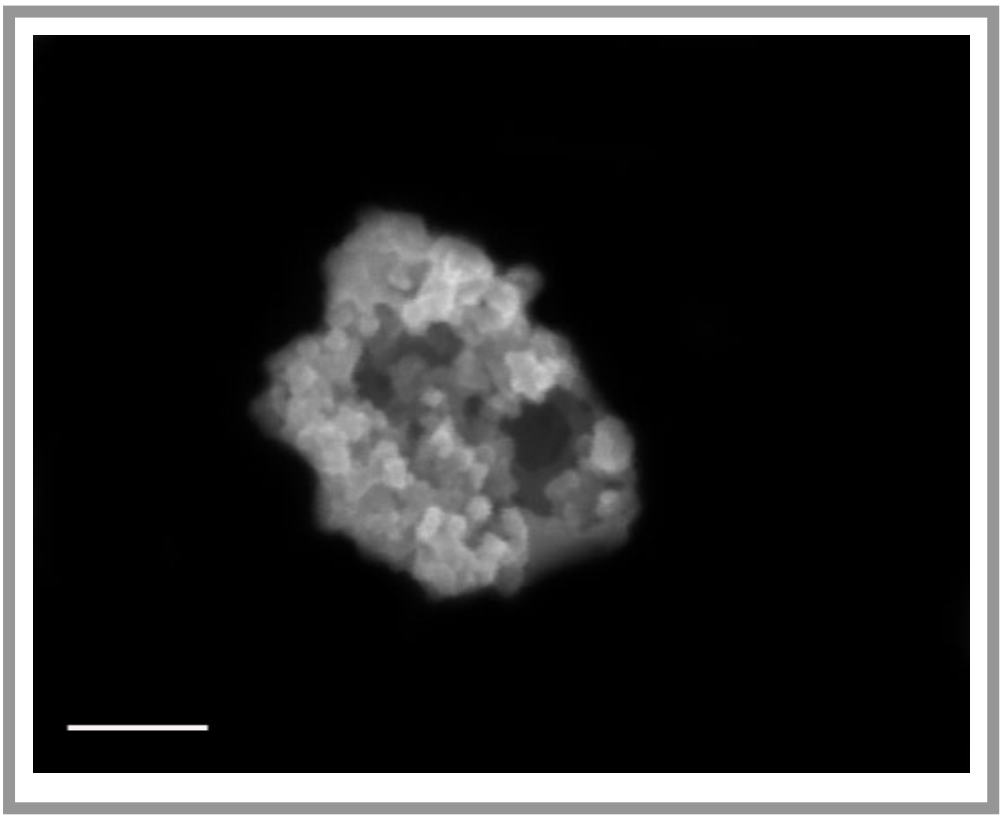

Figure 4.1h: Platelet aggregate from asthmatic BALB/c mice treated with hydrocortisone. (Scale bar = 200nm)

Figure 4.1 a-h shows the differences in the ultrastructure of platelets and fibrin networks in the different experimental groups. Figure 4.1a and b shows control fibrin networks and a platelet aggregate. Thick major fibers are indicated with Label A and Label B shows fine, minor fibers (Figure 4.1a). Figure 4.1b shows a round compact platelet aggregate with pseudopodia (white arrows).

Figure 4.1c shows fibrin networks of asthmatic animals where the minor fibers (Label C) form a thick, matted layer over the major fibers (Label D). Figure 4.1d shows a platelet aggregate of untreated asthmatic animals, where the aggregates are loosely arranged.

Figure 4.1e and f shows the fibrin network and platelet aggregate of Modul8® -treated animals showing thin minor fibers (Label E) and thick, major fibers (Label F).

Figure 4.1 g and h are fibrin networks and platelet aggregate micrographs of the asthmatic animals treated with hydrocortisone. Label G and H indicate respectively the matted layer of thin fibers covering the thick, major fibers.

It was seen that Modul8® stabilized both the fibrin networks and platelet aggregates and SEM analysis revealed the morphology to be the same as that of the control animals. Platelets and fibrin networks play an important role in asthma and therefore it is an important observation that Modul8® positively influenced the ultrastructure of these structures.

4.4 Discussion

Figure 4.1 shows scanning electron micrographs of the ultrastructure of platelets and fibrin networks in the different experimental groups. Previously it was reported that asthma has an effect on the ultrastructure of platelets and fibrin networks. Pretorius *et al.* in 2007a reported on the ultrastructure of platelets and fibrin networks of asthmatic mice compared to that of control mice, hydrocortisone-treated mice and asthmatic mice treated with the plant *Euphorbia hirta*. The results indicated that control mice possess major, thick fibers and minor thin fibers and tight round platelet aggregates could be observed showing typical pseudopodia formation. The minor fibers from the asthmatic animals formed a net covering the major fibers and the platelets appeared as loosely arranged granular aggregates compared to the tight round appearance of the control mice. Hydrocortisone seemed to influence the fibrin networks by making it more fragile and platelet morphology changed form a tight platelet aggregate to a more granular aggregate, almost similar to that of the asthmatic animals (Pretorius *et al*., 2007a).

This was also found in the current study as shown in Figure 4.1 g and h where the minor fibers (Label G) of the fibrin network of the hydrocortisone-treated animals formed a thin, matted layer over the major fibers (Label H) and the platelet aggregate also changed from a tight aggregate as seen in the control- and Modul8®-treated animals to a granular, loosely arranged aggregate as can be seen in the asthmatic animals.

The current research showed that Modul8® stabilized fibrin networks and platelet aggregates. Fibrin networks of Modul8®-treated animals (Figure 4.1e) appeared similar to that of the controls (Figure 4.1a) while the fibrin networks of the untreated asthmatic animals (Figure 4.1c) as well as that of the hydrocortisone-treated group appeared fragile and the thick fibers had a matted covering formed by the thin, minor

fibers. Modul8® seems to also stabilize platelet aggregates (Figure 4.1f) and these aggregates looked similar to the control animals (Figure 4.1b). Platelet aggregates of the untreated asthmatic animals (Figure 4.1d) as well as that of the hydrocortisone-treated asthmatic animals (Figure 4.1h) appeared granular without the tight round appearance as can be seen in the control platelet aggregates. The ultrastructure of the platelets and fibrin networks of the Modul8® treated group are similar to that of the control group where thick major and thin minor fibers can clearly be distinguished and a tight mass of platelet aggregate could be observed.

4.5 Conclusion

From this study it is clear that platelet aggregate and fibrin network morphology are changed in the asthmatic animals. It is also well known that platelets and fibrin play a fundamental role in asthma since platelets act as inflammatory cells in the immune response. Modul8® seemed to stabilize platelet and fibrin morphology to a profile similar to that of the control animals. However, the question arose whether results from the BALB/c asthmatic animal study are indeed comparable to that of human asthmatics. In chapter 5 the control and asthma platelets and fibrin networks from the BALB/c asthmatic animal model will be compared to that of human control and asthmatic subjects.