CHAPTER SEVEN

Inflammatory Cell Infiltration and Structural Changes in the Airways

7.1. Introduction

Asthmatic inflammation usually affects the entire respiratory tract, from the nose down to the most distal (and therefore much smaller) respiratory passages as well as the alveolar tissue (Martin, 2002; Togias, 2003), causing a wide range of structural changes. Some of the common changes include thickening of the reticular basement membrane (Roche et al., 1989; Wilson and Li, 1997), thickening of the smooth muscle layer as well as infiltration of different cell types. Thickening of the basement membrane could be due to deposition of collagen types I, III and V (Roche et al., 1989).

Cell infiltration into the airway has also been considered a remarkable feature of asthma that causes structural changes. Some studies have indicated that the predominant cells in bronchial asthma were eosinophils and lymphocytes (Hoshi et al., 1995) and yet other studies have shown that CD4 T-lymphocytes, eosinophils and mast cells were the predominant cells in asthma (Saetta and Turato, 2001). Other studies reported the presence of eosinophils, neutrophils and macrophages in lung tissues (McKay et al., 2004). Infiltration of T-cells, macrophages and eosinophils into the proximal and distal lung tissues has also been reported in rare cases of sudden asthma death (dying within one hour of onset of symptoms) (Faul et al., 1997) and in soybean dust induced asthma (Synek et al., 1996).

The distribution pattern of eosinophilic infiltration was found to differ between the central and peripheral airways, suggesting important functional consequences (Saetta and Turato, 2001). A comprehensive review by Epstein (2006) stated that peril-bronchial and peril-vascular infiltrates were evident in all asthma models but the cellular composition, severity of overall inflammation and location of infiltrates

was protocol-dependent. The study also showed that although the protocol-dependent differences in asthma histopathology are poorly understood, and could provide new insights into the heterogeneity of disease.

Many previous studies have shown that the entire length of the airways is involved in asthma (Huber and Koessler 1922; Dunnill 1960; Dunnill et al., 1969; Kuwano et al., 1993). Using surgically resected lung tissue (Taha et al., 1999), trans-bronchial biopsies (Kraft et al., 1999) and autopsy lung specimens (Christodoulopoulos et al., 2000), more severe inflammatory and structural changes were observed in small airways (Faul et al., 1997) and in peripheral airways and lung parenchyma (Kraft et al., 1996) of 'asthmatic' persons when compared to the larger airways. Regional variations were also observed in inflammatory cell distribution within 'asthmatic' airways (Haley et al., 1998).

The types of inflammatory cells present in the airway walls and spaces could indicate the pattern of asthmatic progression. The present study examines the effects of HC and extracts of the medicinal herb *E. hirta* on the structural changes and cellular disposition and characteristics in especially the distal respiratory passages using the Balb/c mouse asthma model.

7.2. Mixed inflammatory infiltrate in the lung parenchyma

A mixture of inflammatory cells, notably eosinophils, lymphocytes, mast cells, basophils, macrophages, fibroblasts and platelets usually infiltrates the subepithelial layers of respiratory passages. Results from previous studies are given in the following sections.

7.2.1. Eosinophils

Tissue eosinophilia is a characteristic of asthma but it is not necessarily specific to asthma (Lacoste et al., 1993). The classic descriptions of asthma pathology include the presence of eosinophils (and neutrophils) in both the lamina propria and the airway lumen of patients with asthma (Kaliner et al., 1976). Biopsy specimens from 'mild asthma' patients have confirmed the presence of eosinophils in the mucosa, often beneath the basement membrane as well as in the epithelium (Djukanovic et al., 1990).

Eosinophils also appear to be important cells of airway remodelling as they release growth factors (Ohno et al 1992, Walz et al 1993), elastase (Lungarella et al., 1992) and metalloproteases (Ohno et al., 1997) that are all involved in the process of tissue remodelling and pulmonary fibrosis (Schlick et al., 1993), mostly through fibroblast stimulation (Pincus et al., 1987). Recruited eosinophils become activated during segmental allergen challenge and then release products that cause contraction of the human bronchial smooth muscle and increased vascular permeability (Collins et al., 1993; Rabe et al., 1994; Shaver et al 1997).

7.2.2. Lymphocytes

T-cells possibly play a role in controlling the chronic inflammation of allergic and non-allergic asthma via the release of Th2-cytokines (Ying et al., 1995, Robinson et al., 1992; Del Prete et al., 1993). After allergen challenge, the population of activated T-cells appears to increase in 'asthmatic' subjects (Robinson et al., 1993; Bentley et al., 1993) with only a few B-cells present in the bronchi.

7.2.3. Mast cells

Mast cells are found in the bronchi of normal subjects and “asthmatics” alike (Pesci et al., 1993; Koshino et al., 1995) that are often degranulated in the airways of “asthmatics” in both their stable phase and after allergen challenge (Laitinen 1985; Beasley et al., 1989). Mast cells appear to be critical “trigger” cells during episodes of acute asthma (Broide et al., 1991). These cells may be involved in airway remodelling because they appear to play an important role in pulmonary fibrosis (Jordana et al., 1993; Chanez et al., 1993) especially because they are potential sources of products that stimulate migration and proliferation of fibroblasts (Ruoss et al., 1991; Nagata et al., 1992). Research has also shown that mast cell lines can release components of basement membranes such as laminin and collagen IV (Thompson et al., 1991) as well as angiogenic growth factors (Meininger and Zetter, 1992). Mast cell products have also been reported to have anti-inflammatory properties (Green et al., 1993; Tyrell et al., 1995).

7.2.4. Basophils

The role of basophils in the pathogenesis of asthma appears poorly defined (Denburg, 1998). There has been some controversy over the extent to which basophils participate in the inflammatory processes of asthma (Walls et al., 2001) even though these cells are known to be responsible for the mediator release observed during the asthmatic response (Lichtenstein and Bochner, 1991). After anti-IgE challenge, most human blood basophils release histamine and other inflammatory mediators without degranulating (Youssef et al., 2007). Though often regarded as circulating counterparts of the mast cell, many basophils infiltrate the tissues and fluids that bathe mucosal surfaces (Walls, 2001).

7.2.5. Macrophages

A study by Calhoun et al (1992) showed that activation of alveolar macrophages occurred immediately after antigen challenge, indicating a major role for macrophages in asthma. Alveolar macrophages have been reported to exhibit enhanced capacity to release pro-inflammatory cytokines (John et al., 1998). In addition, macrophage numbers increased in the airways and may be activated by allergens through low-affinity IgE receptors to release inflammatory mediators and cytokines that amplify the inflammatory response (Peters-Golden, 2004).

Phagocytic cells such as polymorphonuclear leukocytes and macrophages, respond to a variety of membrane stimulants via the production and extracellular release of a number of reactive oxygen reduction products (Santos et al., 2004). Alveolar macrophages may also contribute to inflammation via the release of reactive oxygen species such as superoxide (SO) anion, hydroxyl radicals, hydrogen peroxide, singlet oxygen and hypohalous acids, which may cause cellular injury and alterations in airway functions (Freeman and Crapo, 1982; Barnes 1990). These reactive groups may be involved in the regulation of airway remodelling through secretion of growth-promoting factors for fibroblasts, cytokines as well as growth factors possibly involved in fibrosis (Kovacs and DiPietro, 1994).

7.2.6. Polymorphonuclear neutrophils

The role of neutrophils in stable asthma remains unclear but neutrophil numbers were noted to have increased after an allergen challenge in the airways in the different forms of asthma during the late-phase reaction (Koh et al., 1993; Montefort et al., 1994). In one study, both bronchoalveolar lavage (BAL) and lung

tissue digestion were used to determine rat lung inflammatory cell contents following a 4-hour exposure to 2-ppm ozone. Immediately following the exposure, the neutrophil content of the lung tissue significantly increased and by three hours post-exposure, reached a value fourfold higher than air-exposed controls. Although lavage-recovered neutrophils were elevated 24 hour afterwards, tissue neutrophil numbers had returned to control values. This transient elevation of tissue neutrophils directly correlated with an elevation and subsequent decline of airway hyperresponsiveness (DeLorme et al., 2002).

7.2.7. Fibroblasts

Fibroblasts are often found in connective tissue and are responsible for the production of collagen, reticular and elastic fibres as well as for the synthesis of proteoglycans and glycoproteins of the amorphous intercellular substance (Sheppard and Harrison, 1992). Although these cells are regarded as fixed cells of connective tissue origin, they retain the capacity for growth and proliferation and are pluripotent cells (precursors for various other cell types), including smooth muscle cells (Gizycki et al., 1997).

7.2.8. Myofibroblasts

Myofibroblasts may potentially contribute to the regulation of bronchial inflammation via the release of cytokines (Zhang et al., 1996) and to tissue remodelling via the release of extracellular matrix (ECM) components such as elastin, fibronectin and laminin (Leslie et al., 1992). During the late-phase reaction after allergen challenge, myofibroblasts increase from the normal of 2% of cells to approximately 15% within a day (Gizycki et al., 1997). In asthma, myofibroblasts are increased in numbers beneath the reticular basement membrane and there is

an association between their numbers and the thickness of reticular basement membrane (Brewster et al., 1993; Gabbrielli et al., 1993).

7.2.9. Dendritic cells

Dendritic cells are receiving increasing scientific and clinical interest due to their key role in anti-cancer host responses and potential use as biological adjuvants in tumour vaccines, as well as their involvement in the immunobiology of tolerance and autoimmunity (Satthaporn and Eremin, 2001). Dendritic cells are a major source of many cytokines, namely, interferon-alpha (IFN-a), IL-1, IL-6, IL-7, IL-12 and IL-15 and also produce macrophage inflammatory protein (MIP1g), all of which are important in the elicitation of a primary immune response (Macatonia et al., 1995; Cella et al., 1996; Mohamadzadeh et al., 1996).

In humans, there is a network of dendritic cells within the epithelium of the conducting airways (Jahnsen et al., 2001). These cells may be critically important to the induction of immune responses within the airways as they are specialized in antigen processing and presentation. In animals, the dendritic cell population in the airway epithelium is renewed every 48 to 72 h (Hance, 1993) and it appears that the number of dendritic cells can be altered after exposure to both topical and systemic corticosteroids (Moser et al., 1995).

Dendritic cell numbers are greater in the airways of asthmatics compared with those of control subjects (Tunon-De-Lara et al., 1993; Moller et al., 1996). Their role in asthma remains under investigation. Mouse models and culture systems of human cells have been used to investigate the implications of the dendritic cell-epithelial interaction for the pathogenesis of asthma (Lambrecht and Hammad, 2003).

7.2.10. Aim of study

The aim of this study was to determine the presence of inflammatory cells in the airway walls and spaces in especially the distal respiratory structures using the BALB/c mouse asthma model, and to compare the findings in the control (CT), asthmatic (AS), HC-treated and *E. hirta*-treated specimens. Differences in cellular characteristics as well as structural changes in the airways were also analyzed and compared. Although the amount of inflammatory cells in the airway walls was not determined, their possible relationship to the findings in peripheral blood cell counts in chapter five was analyzed.

7.3. Materials and methods

7.3.1. Materials

7.3.1.1. BALB/c Mice

A total of sixty (60) six-week-old male BALB/c mice (mean weight 20g) were used in this study.

7.3.1.2. Hydrocortisone (HC)

HC was used in this study as a pharmaceutical control for the extracts of *E. hirta* as used iin the cell culture study. One hundred milligrams (100 mg) of the sterile powder (Brand name Solu-Cortef®) which contains HC sodium succinate as the active ingredient was dissolved in 2 ml of bacteriostatic injection water, giving a concentration of 50mg/ml. In this study, a high dose (125mg/kg) and a low dose (100mg/kg) of the 50mg/ml HC preparation were used. Fresh solution was prepared for each injection.

7.3.1.3. *Euphorbia hirta*

Only the aqueous plant extract of *E. hirta* was used for the animal studies. The plant material was collected in the Gezina region of Pretoria South Africa. A herbarium specimen was prepared and compared to an authentic specimen in the HGJW Sweikerdt herbarium at the University of Pretoria. The aboveground parts of the plant were allowed to dry at room temperature for one week in the Cell Biology laboratory of the Department of Anatomy, University of Pretoria, and the material was grounded into a fine powder. Shortly after, 50 grams of the sample was extracted in 500ml of double distilled water after which it was filtered, and dried on a rotary evaporator at 40 °C. A stock aqueous solution of 50mg/ml plant extract was prepared and stored in a fridge until used.

The doses of 62,5mg/kg and 25mg/kg were prepared from the stock solution and administered orally to the mice. This implied that each animal (average weight 20g) received 0.01 ml of plant material. This dose was decided upon after studying literature that mentioned physiological doses suggested by herbalists. Typically, a teaspoon of the herb is added to a teacup volume of water and allowed to simmer for 20 minutes (Lindsey et al., 2002). Alternatively, an extract of the plant could be prepared and the recommended adult dose range of the fluid extract is 0.2-0.3ml, taken three times daily and of the infusion, 120-300mg three times daily (Skidmore-Roth, 2001).

7.3.1.4. Reagents and equipment

The reagents and equipments used for this study include ovalbumin (OVA) purchased from Sigma-Aldrich Co., Phosphate Buffered Saline (PBS), alum, formaldehyde, assorted clearing agents, glutaraldehyde, epoxy resin, 1% OsO_4,

cacodylate buffer, Agar 100 Resin, DPSS, KLAVA ultrasonic nebulizer, oral-pharyngeal canula, injection syringes and needles, light microscope (Olympus Japan binocular BH2 (BHTU) model) and transmission electron of microscope (Multi-purpose TEM (Philips 301), 100kV, eucentric goniometer stage, heating holder).

7.3.2. Light microscopy

For light microscopy, samples from the lungs were processed using basic histological techniques. Tissues were fixed in 10% formaldehyde for 24 hours and later dehydrated in increasing grades of ethanol, cleared in xylene and infiltrated with molten paraffin wax. Thereafter, the specimens were embedded in paraffin wax to form tissue blocks and five-micron thick sections were cut from the blocks and stained with Haematoxylin and Eosin (H and E) dyes. The stained sections were later observed with the light microscope (Olympus Japan binocular BH2 (BHTU) model).

7.3.3. Transmission electron microscopy (TEM)

All specimens were taken to the central Laboratory for Microscopy and Microanalysis at the University of Pretoria. Intrapulmonary tissues were cut into very small sizes and fixed in glutaraldehyde for one hour. The samples were then put in buffer (0.075M sodium potassium phosphate at pH = 7.4) for 15 minutes and later washed twice in same buffer. Thereafter, samples were placed in a secondary fixative (1% osmium tetra-oxide solution: OsO_4) for one hour. Following OsO_4 fixation, the tissues were rinsed again thrice in the buffer as described earlier and later dehydrated through 30%, 50%, 70%, 90%, and then three changes of 100% ethanol. The dehydrated samples were later embedded in resin

and ultra-thin sections prepared and stained with uranyl acetate for 15 minutes followed by 10 minutes staining with lead citrate. Later the stained specimens were allowed to dry for a few minutes before examination under the multi-purpose transmission electron microscope (Philips 301 100kV, eucentric goniometer stage, heating holder). Ultra-photomicrographs of the internal morphology of the intrapulmonary respiratory tract were also taken.

7.4. Results

7.4.1. Light microscopy

At an objective magnification of X10, the following photomicrographs were taken for the different study groups to show the possible effects of the two treatment agents on alveolar sizes, alveolar wall thickness, as well as the presence of other features as the presence of smooth muscle masses in the terminal bronchioles.

Results from the light microscopy studies show that the CT specimens had relatively thin and continuous (interconnected) alveolar walls with the alveoli being of regular shapes. In contrast, alveoli appear irregularly shaped in the asthma group, their walls very thick and discontinuous, with smooth muscle masses seen in the walls of distal airways. These findings appear to confirm the presence of asthma induced in mice of the AS group.

In the case of specimens of mice treated with the HHC, the alveoli were relatively compact, relatively small and of irregular shapes. Alveolar walls were also discontinuous and moderately thick. Smooth muscle thickenings of terminal airways were also seen. The LHC treatment specimens showed alveoli that were relatively thick and irregular in shape with walls that were discontinuous at some regions. Smooth muscle masses were also seen in the walls of distal airways.

These findings tend to indicate that at the level of light microscopy, both doses of HC did not ameliorate the effects of asthma on the lung microstructure in specimens of this group in terms of the characteristics earlier mentioned.

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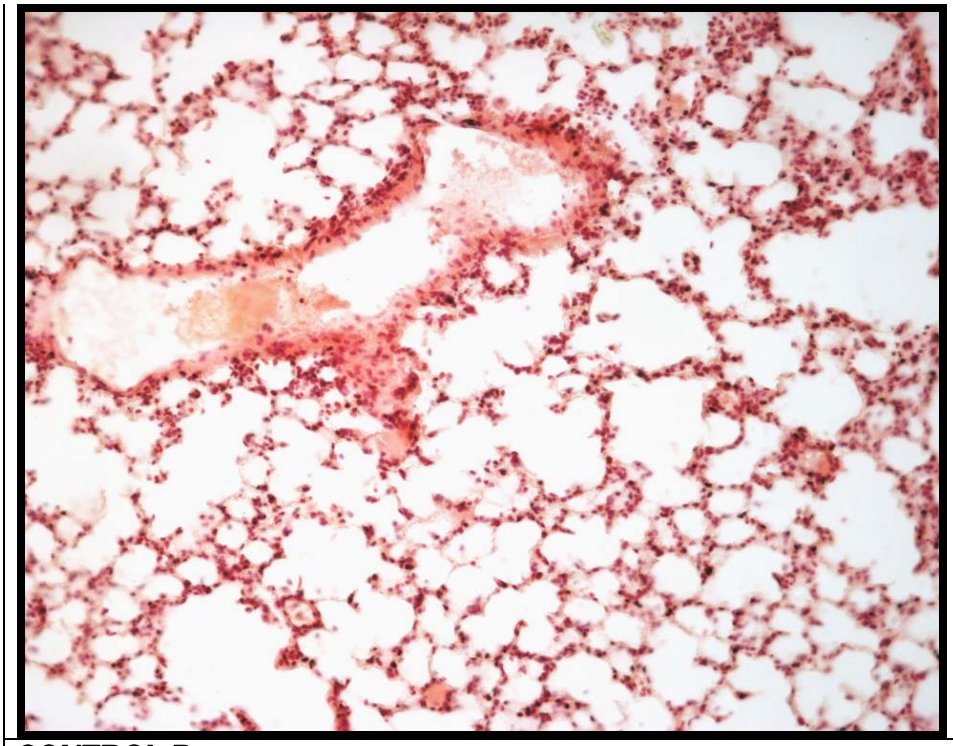

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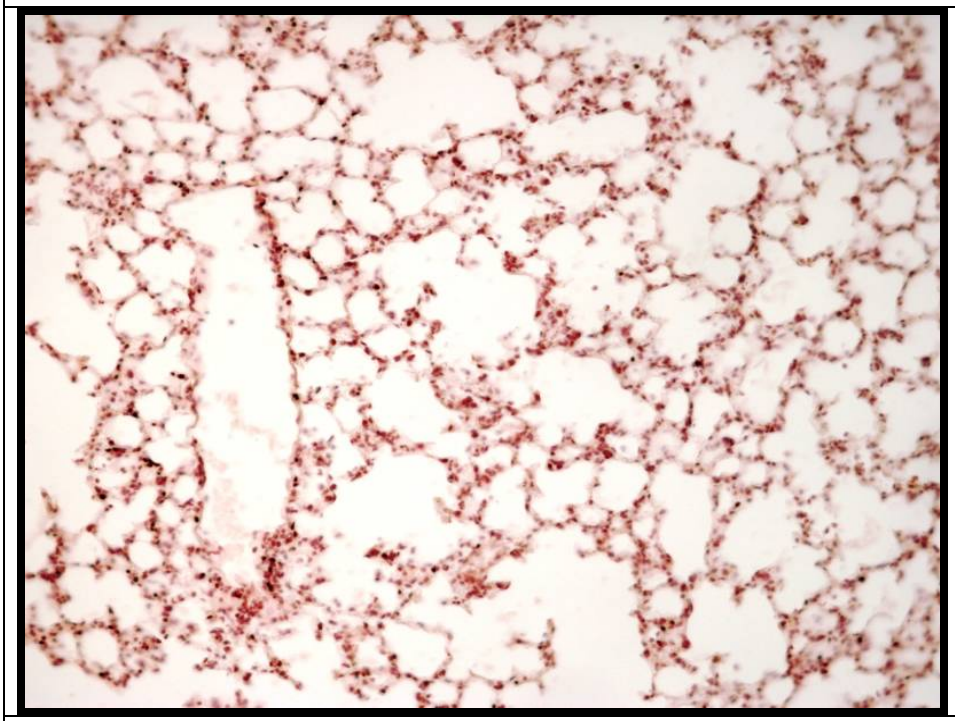
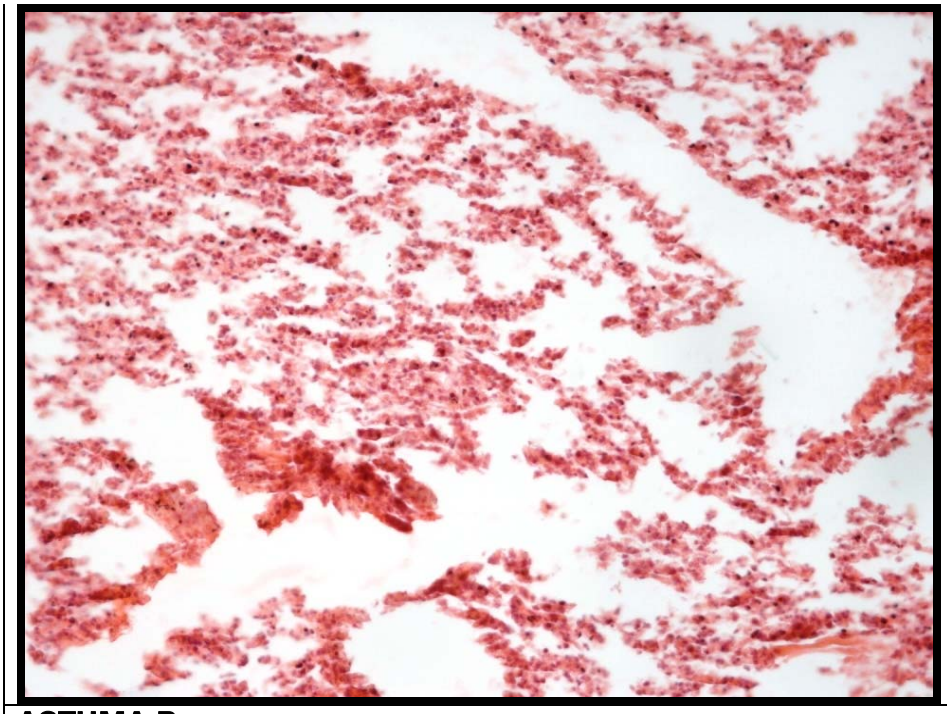


Figure 7.1: Histological cross sections of control mice lungs showing **A)** and **B)** thin and continuous alveolar walls; regular shaped alveoli. H and E staining. Magnification X 100

ASTHMA A:



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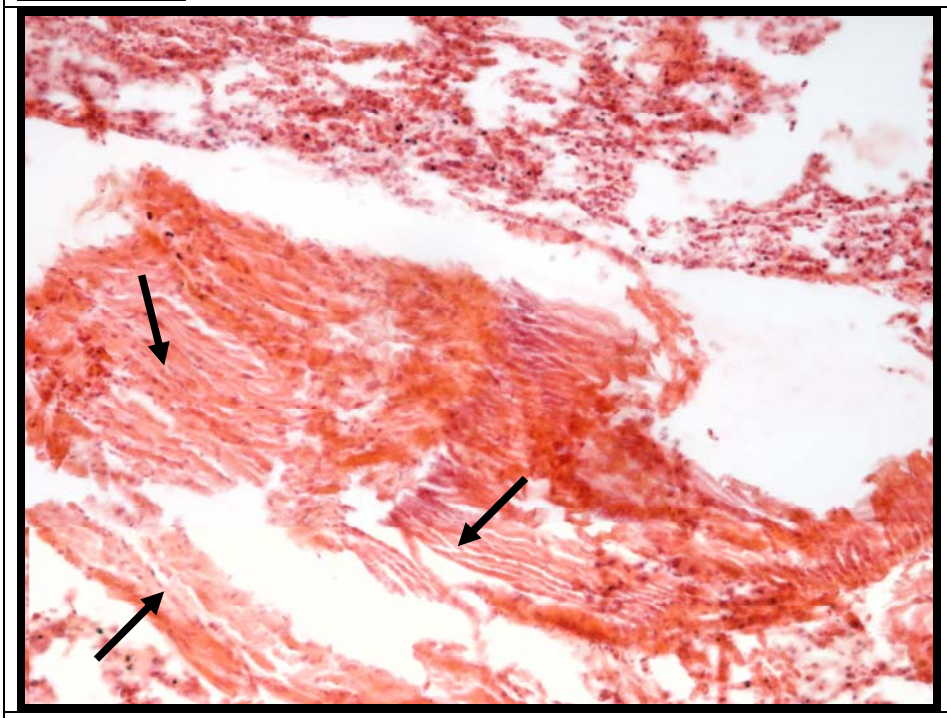
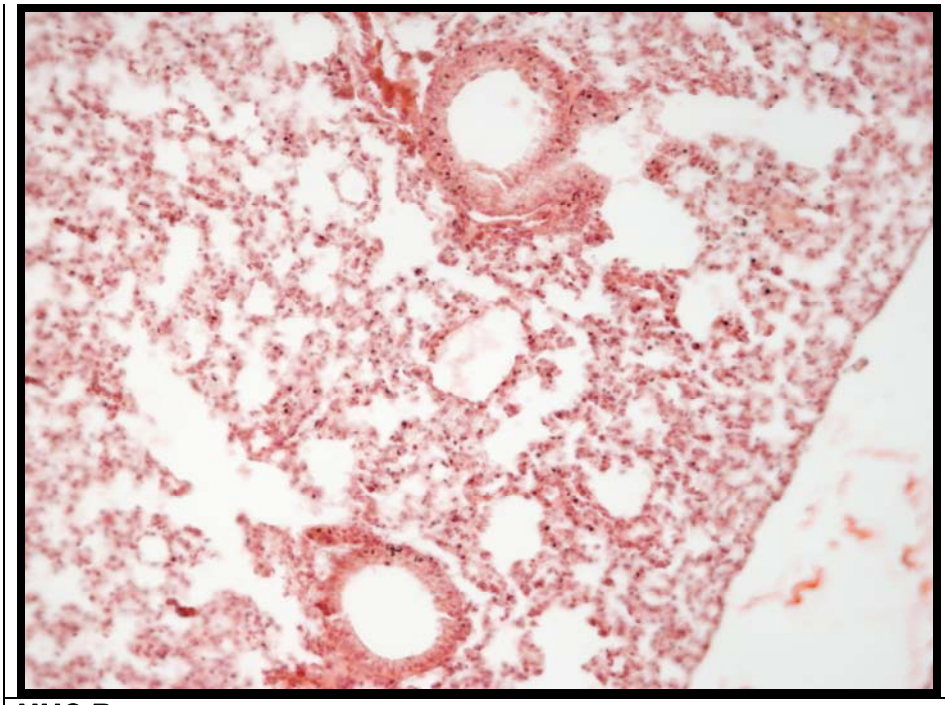


Figure 7.2: Histological cross sections of asthma mice lungs showing **A)** thick and discontinuous alveolar walls and **B)** thick smooth muscle mass in walls of distal airways indicated with arrows. H and E staining. Magnification X 100

HHC A:



HHC B:

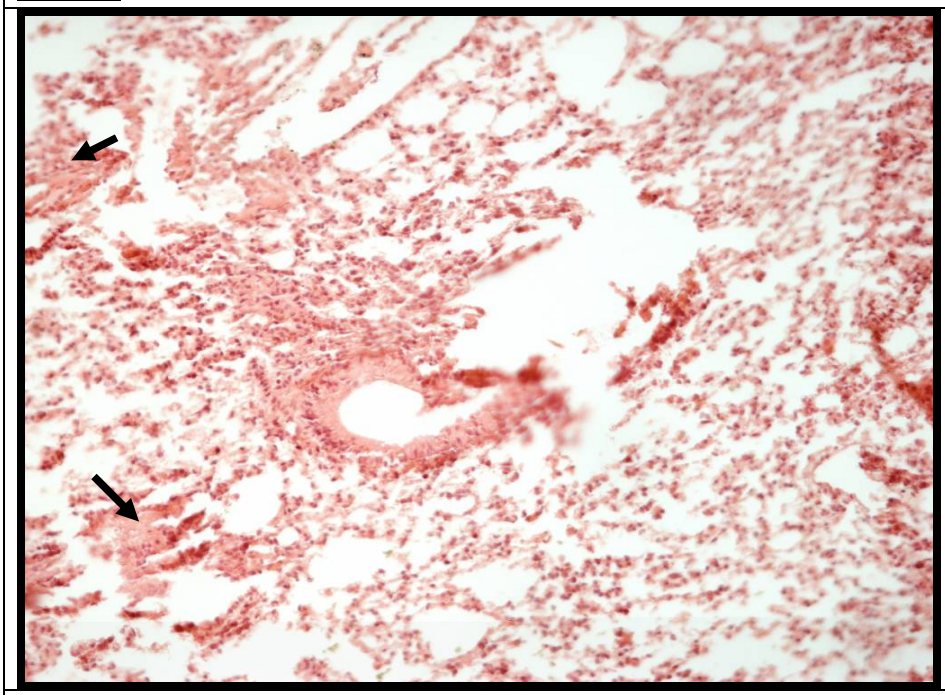
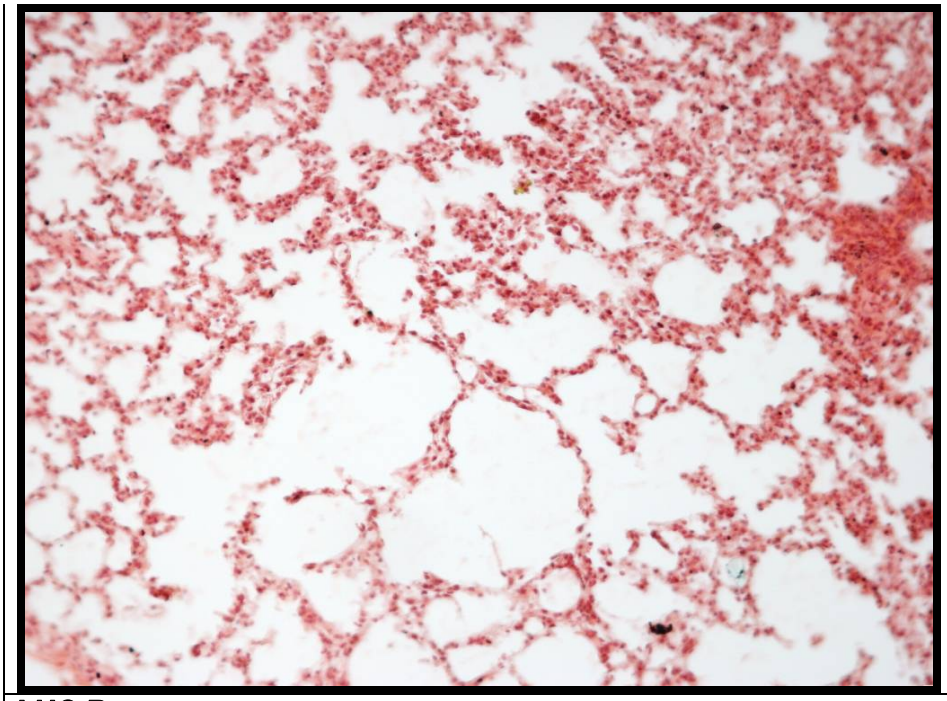


Figure 7.3: Histological cross sections of the lungs of mice treated with HHC showing **A)** and **B)** alveoli that are relatively small but numerous; alveolar walls moderately thick, alveoli compact and irregular in shape; alveolar walls discontinuous; smooth muscle thickenings of terminal airways seen, shown with arrows. H and E staining. Magnification X 100.

LHC A:



LHC B:

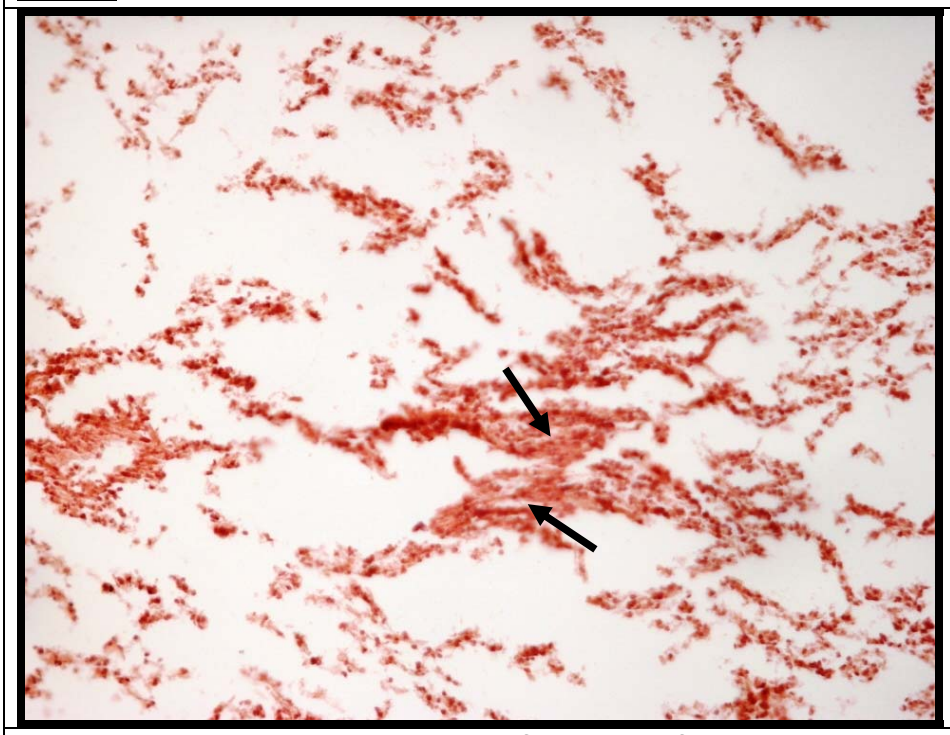
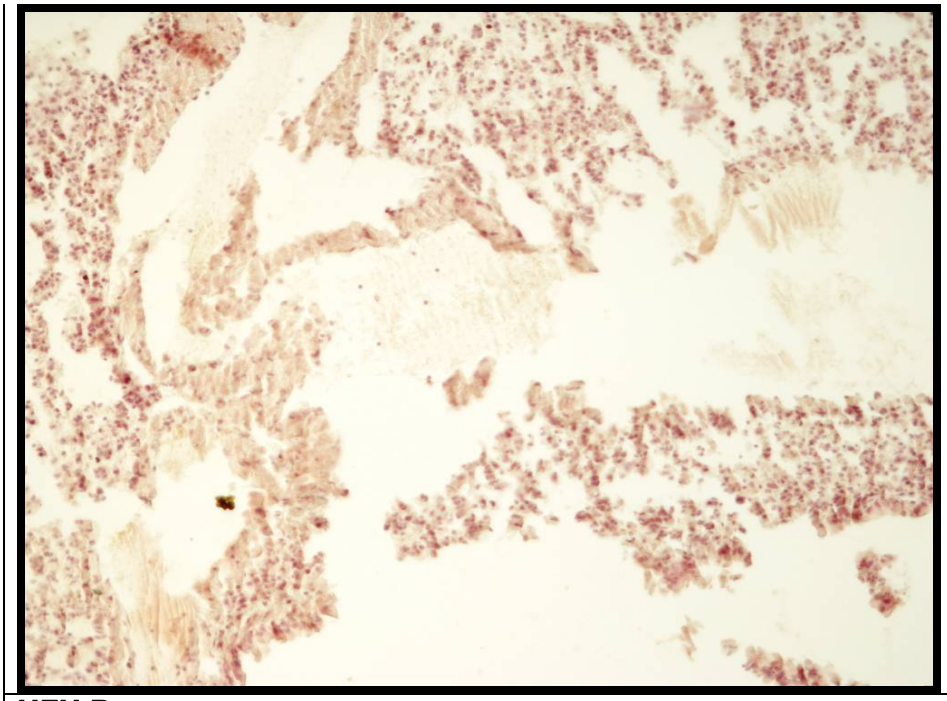


Figure 7.4: Histological cross sections of the lungs of mice treated with low HC showing **A)** and **B)** alveolar walls discontinuous and irregular; smooth muscle, masses seen in walls of distal airways) H and E staining. Magnification X 100

HEH A:



HEH B:

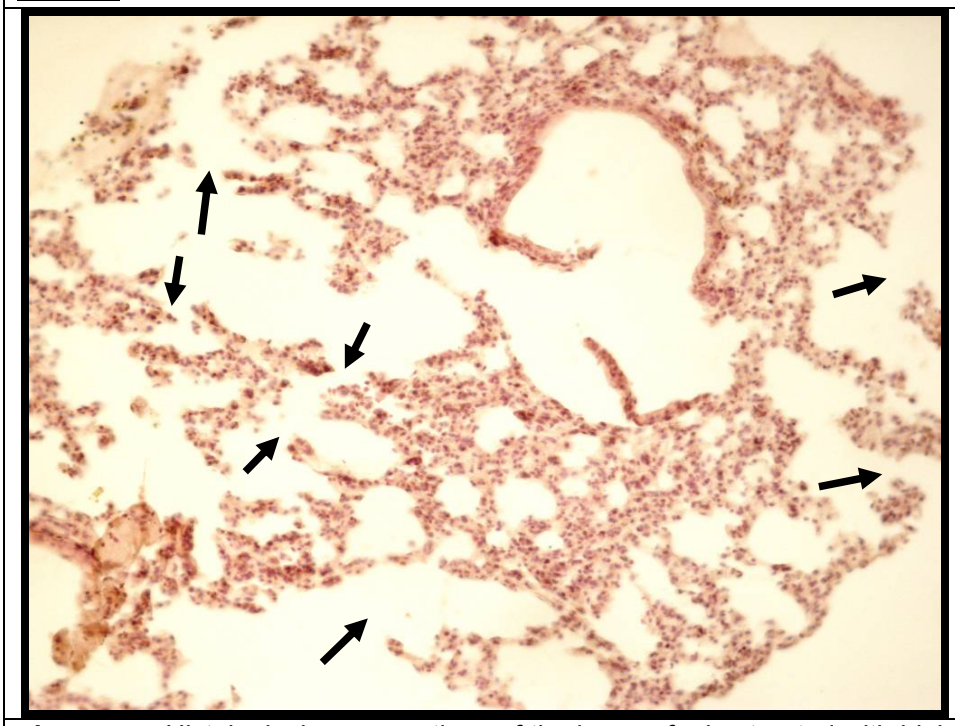
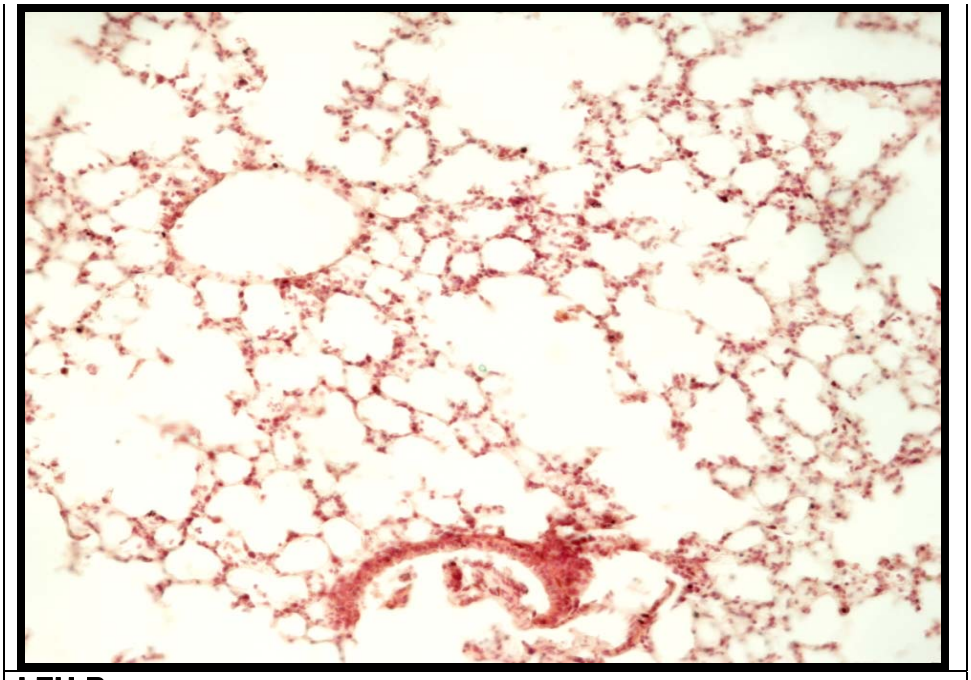


Figure 7.5: Histological cross sections of the lungs of mice treated with high EH showing **A)** and **B)** thick alveolar walls; alveoli very compact and irregular in shape; alveolar walls discontinuous (shown with arrows in B). H and E staining. Magnification X 100

LEH A:



LEH B:

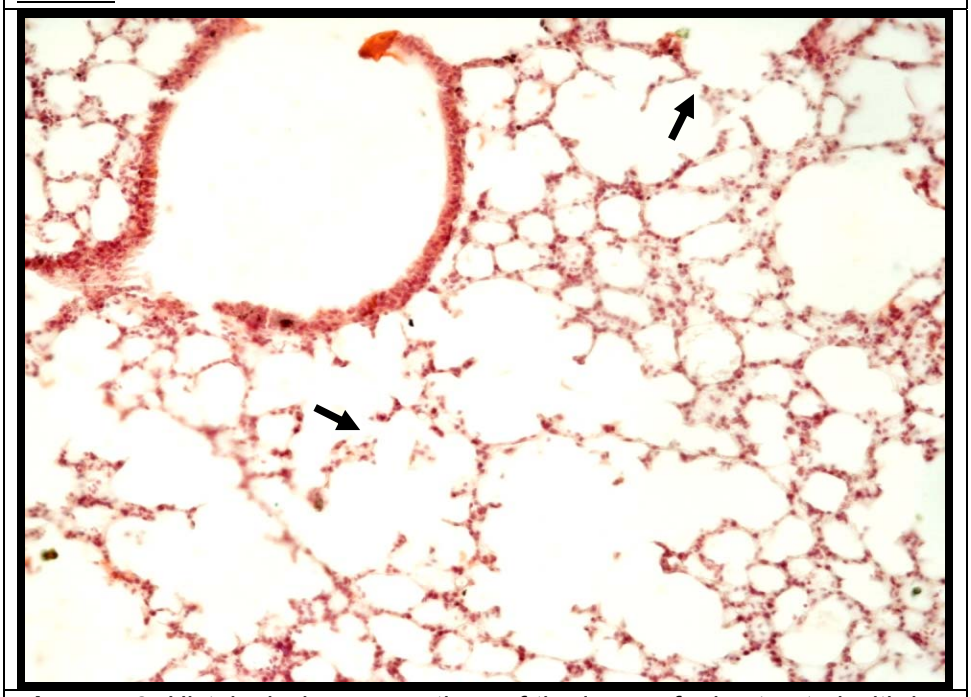


Figure 7.6: Histological cross sections of the lungs of mice treated with low EH showing **A)** thin alveolar walls; less compact and more patent alveoli; alveolar walls are only partially discontinuous (shown with arrows) and **B)** thinner alveolar walls; alveoli less compact and irregular in shape; alveolar walls only partially discontinuous (arrowed). H and E staining. Magnification X 100

The HEH treatment specimens showed compact, irregularly shaped alveoli having thick and discontinuous walls. In contrast, the low *E. hirta* specimens had thinner alveolar walls, less compact and more patent alveoli, with the alveolar walls only partially discontinuous. The features seen in the LEH specimens resemble those of the CT group specimens and therefore suggest that treatment with the LEH appears effective in ameliorating any histological alterations observed in the lung parenchyma in the presence of asthmatic conditions.

7.4.2. Transmission electron microscopy (TEM) analysis

Transmission electron micrographs revealed a variety of intra cellular and extracellular structures shown in the labelled figures below.

7.4.2.1. Cell types

Results from the photomicrographs below show that different cell types are present in distal respiratory passages and in lung parenchyma as follows:

Fibroblasts: were scanty in the CT specimens as well as in the low dose treatment specimens. However, these cells were as many in the “asthmatic” (AS) specimens as were in the high dose treatment groups.

Lymphocytes: were few in the specimens of the CT and LHC groups but were relatively scanty in specimens of the AS, HHC and HEH groups.

Monocytes: were absent in specimens from animals treated with the LHC.

Neutrophils: were generally not prominent but only very few were seen in CT group specimens.

Macrophages: were numerous in specimens of the asthmatic and LHC groups but only a few were present in specimens of the CT, HHC as well as in the HEH and LEH specimens.

Plasma cells: were scanty or absent in specimens of all groups except those from the low dose *E.hirta* treatment group. These cells were characterized by the elaborate presence of rough endoplasmic reticulum in their cytoplasm.

7.4.2.2. Other structures

Collagen fibres: were scanty in specimens from the CT and LHC groups. The remaining groups had varying amounts of collagen fibres.

Lamellar bodies: are usually associated with type 2 pneumocytes (great alveolar cells) and in this study, lamellar bodies were present in specimens of the low HC and low *E. hirta* doses.

Thick alveolar walls: were more prominent in specimens of the LHC group and less thick in the CT specimens.

Mitochondria: were prominent in specimens of the asthma and HHC groups.

Striations: were observed as special structures in some of the asthma group specimens, much similar to those observed in a typical striated muscle.

Mucous-secreting structures: were prominent in specimens of the LHC and LEH groups.

Smooth muscles: were relatively well developed in bronchiolar specimens of the asthma group compared to other treatment groups.

Filopodia: were seen in fibroblasts as cytoplasmic extensions only in specimens of the LHC and the HEH groups.

CONTROL

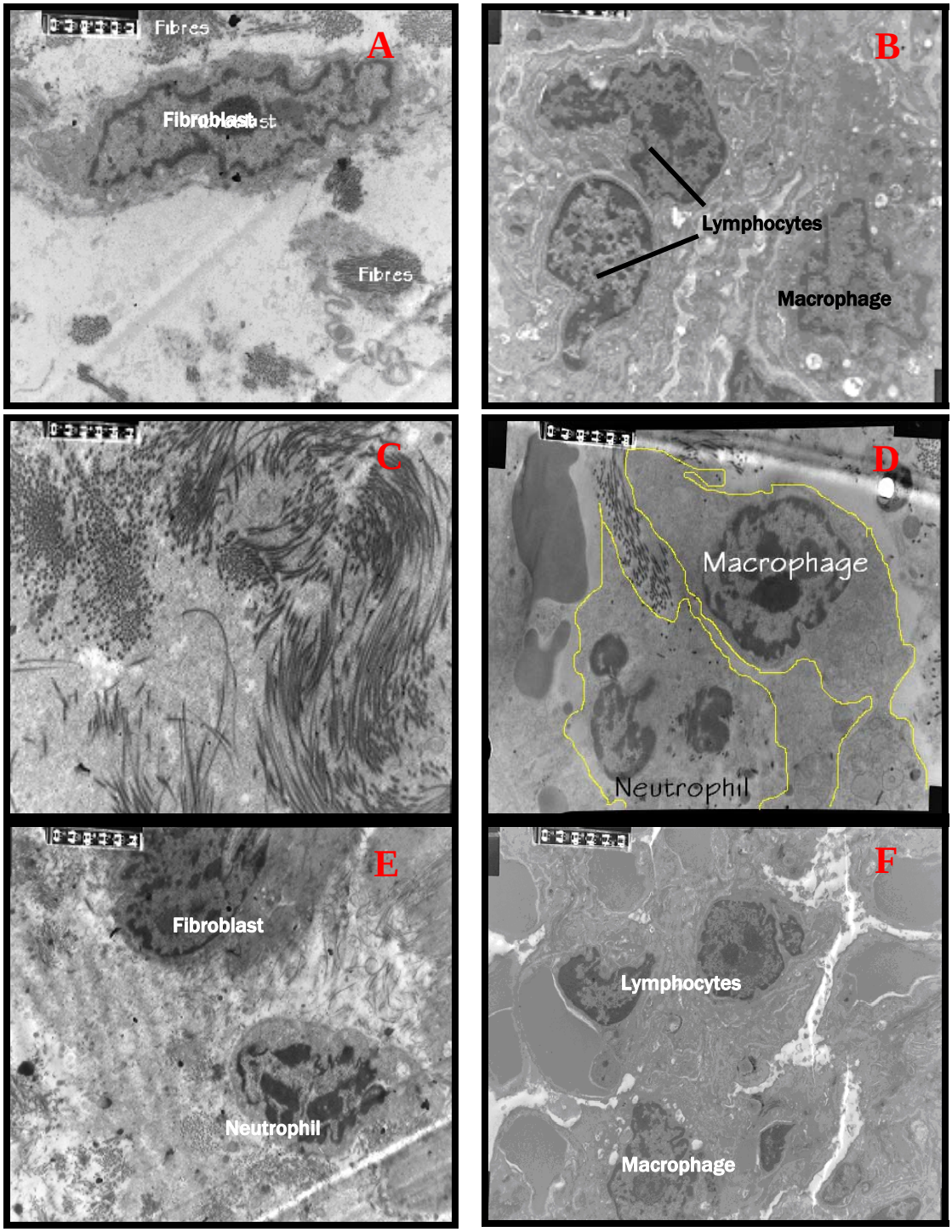


Figure 7.7: TEM micrograph of lung tissue from **control** mice showing **A** = Fibroblast (F), scanty collagen fibres [x7500]; **B** = Macrophage and lymphocytes in interstitium [x9800]; **C** = Collagen fibres [x13000]; **D** = Neutrophil, macrophage [x7500]; **E** = Fibroblast, neutrophils scanty fibres x5900; **F** = Lymphocytes and macrophage in lung interstitium [x4300].

ASTHMA

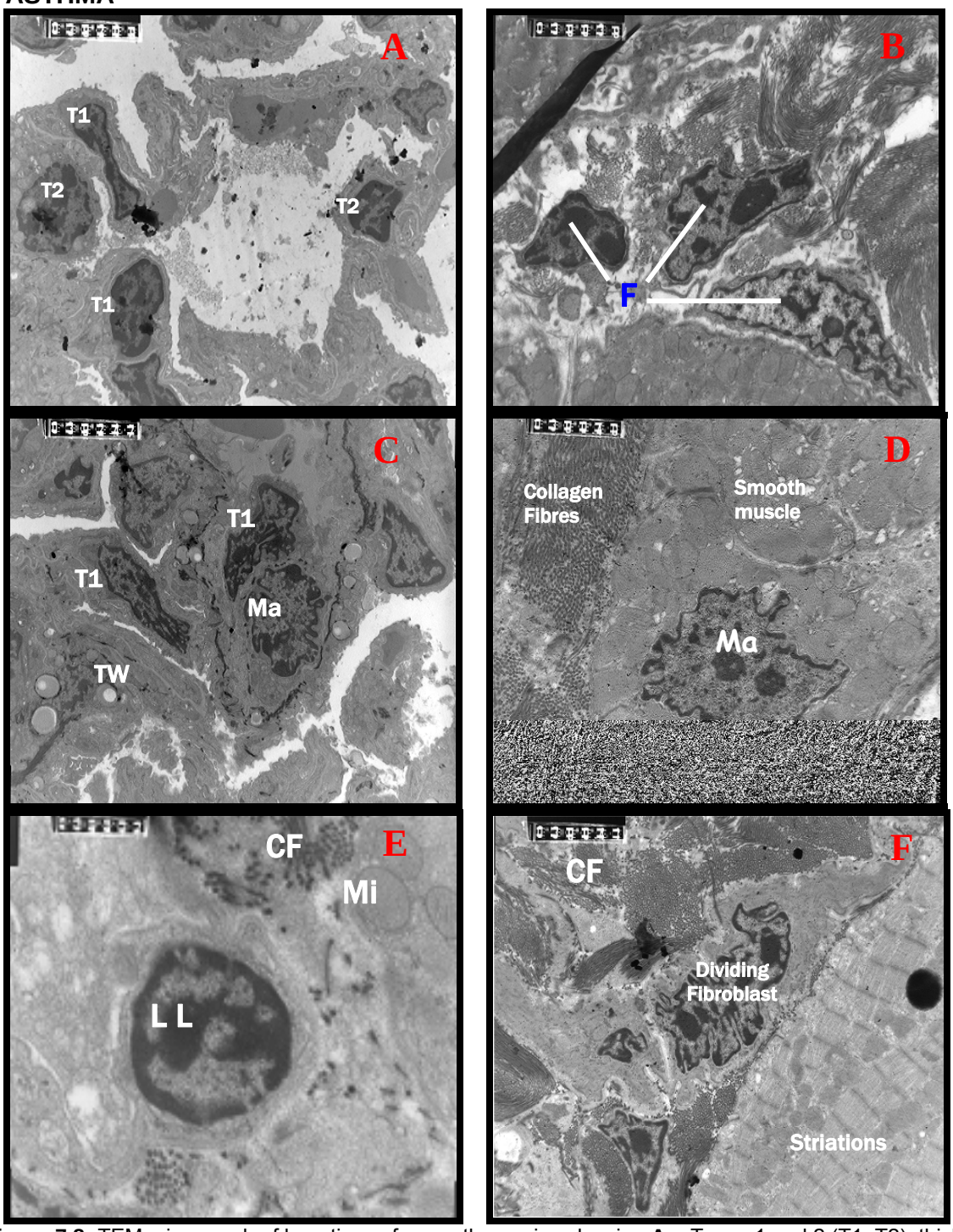


Figure 7.8: TEM micrograph of lung tissue from asthma mice showing **A** = Types 1 and 2 (T1; T2); thick walls [x3600]; **B** = Fibroblasts (F), many collagen fibres, smooth muscle [x5900]; **C** = Thick walls (TW), Type 1 cells (T1); macrophage (Ma) [x4300]; **D** = Macrophage (Ma), collagen fibres, smooth muscle [x9800]; **E** = Large Lymphocytes (LL); fibres (F), mitochondria (Mi) [x18000]; **F** = Many collagen fibres (CF), fibroblast, striations [x5900].

LHC

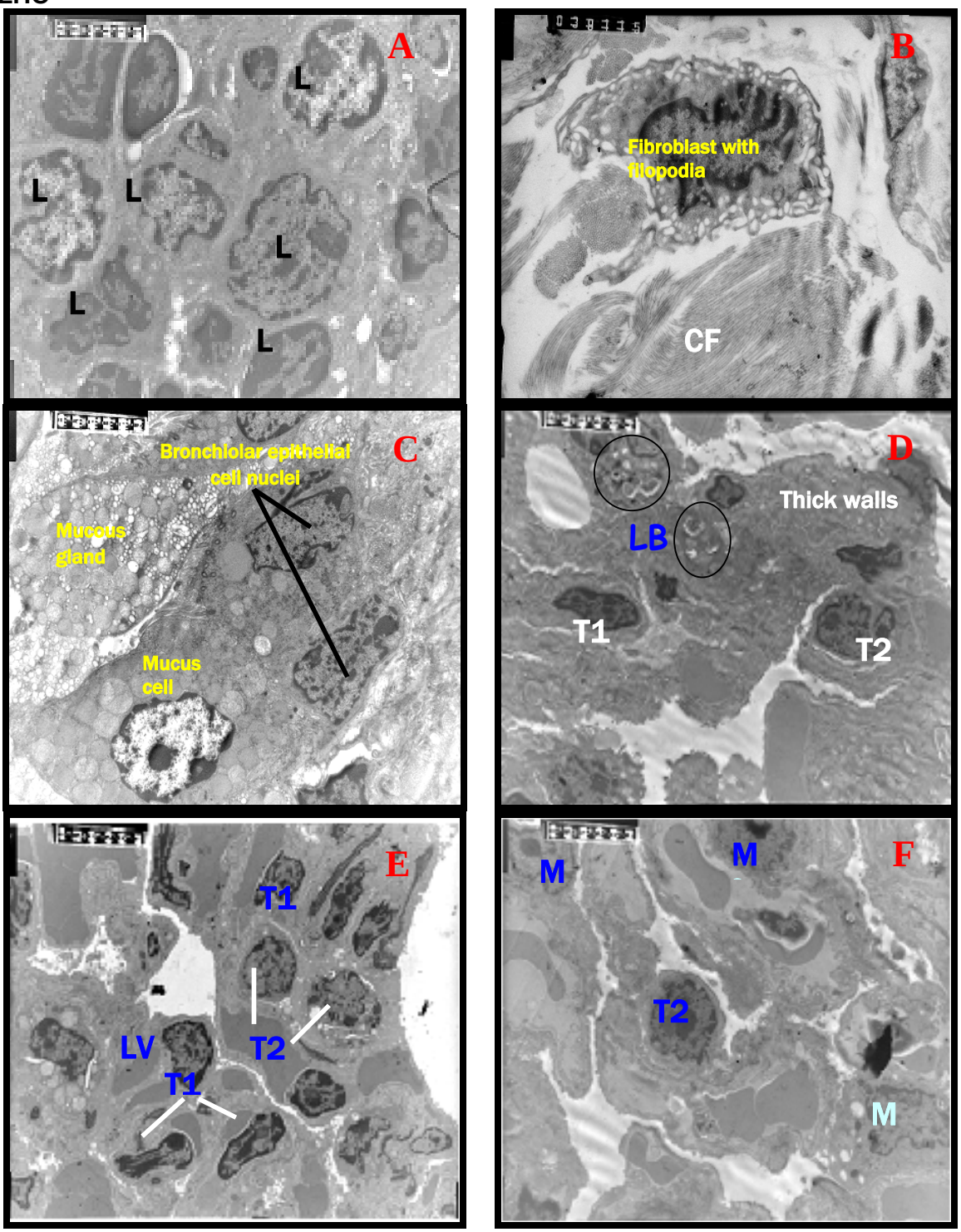


Figure 7.9: TEM micrograph of lung tissue from asthma mice treated with LHC showing **A =** Monocyte (Mo), lymphocytes (L) [X5900]; **B =** Fibroblast, collagen fibres (CF) [X7500]; **C =** Mucous secreting structures [X4300]; **D =** Thick walls, lamellar bodies LB, Types 1, 2 cells [X3600]; **E =** Lymphocyte with vesicles (LV), Types 1, 2 cells [X2800]; **F =** Thick walls, macrophages (M), T2 cells [x3600].

HHC

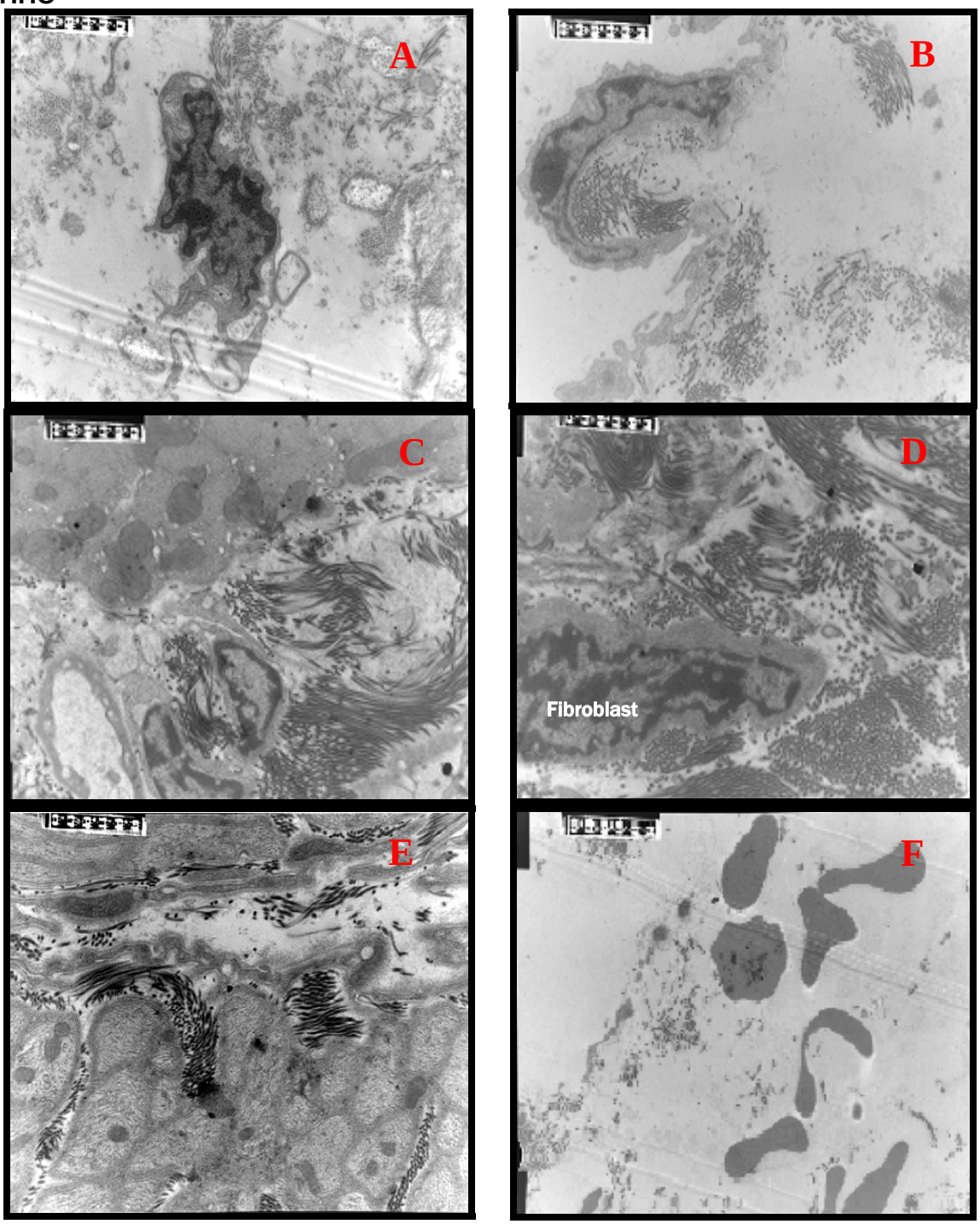


Figure 7.10: TEM micrograph of lung tissue from asthma mice treated with HHC showing **A =** Fibroblast and scanty collagen fibres [x9800], **B =** Fibroblast and scanty collagen fibres [x9800], **C =** Fibroblasts, few fibres, many mitochondria [x9800], **D =** Fibroblast (F), relatively more fibres (CF) [x9800], **E =** Unmyelinated nerves and collagen fibres [x13000], **F =** Very scanty collagen fibres, red blood cells [x4300]

HEH

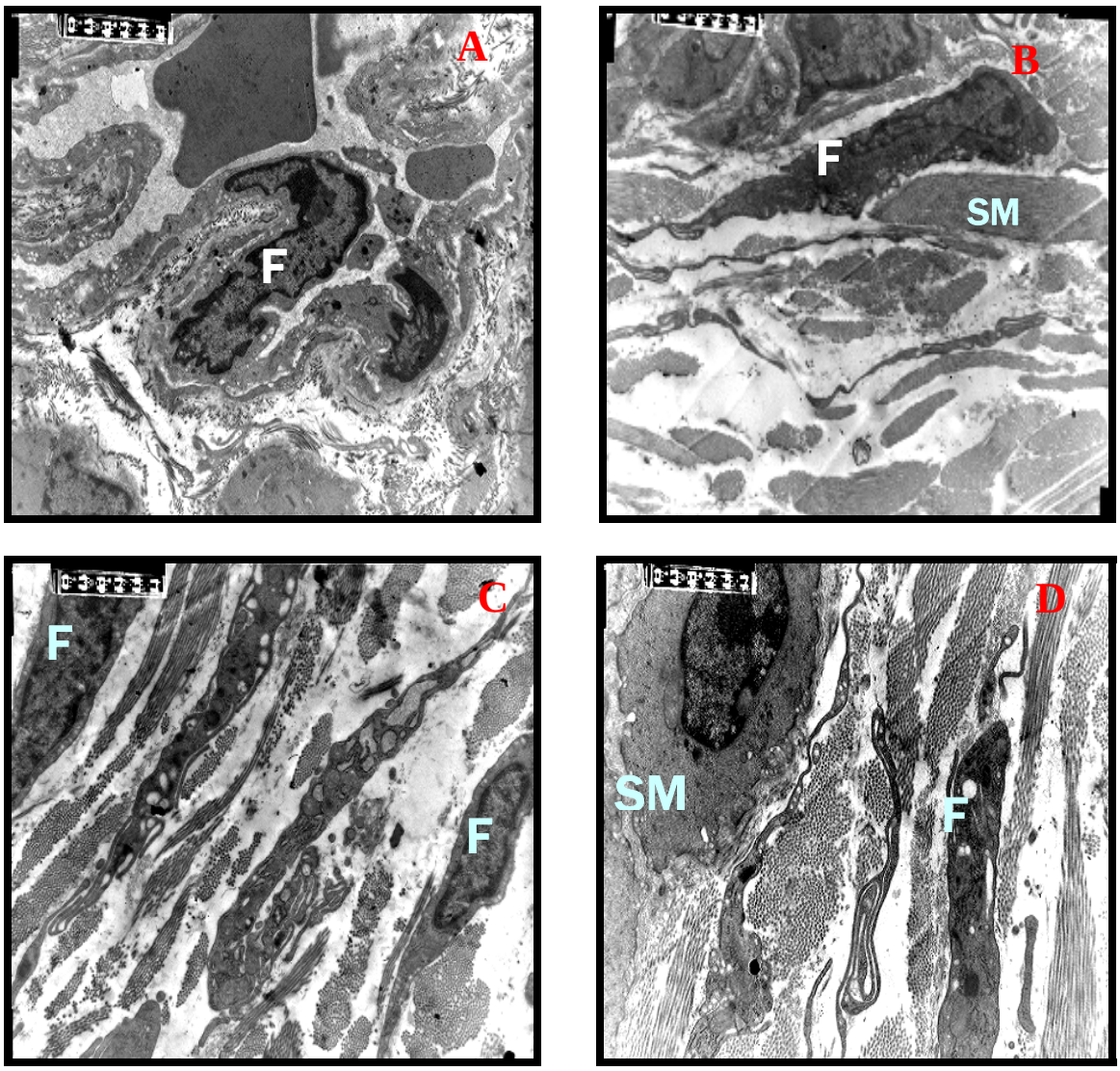


Figure 7.11: TEM micrograph of lung tissue from asthma mice treated with HEH showing **A** = Highly fibrous lung parenchyma, fibroblast [x5900]; **B** = Highly fibrous lung parenchyma, fibroblast [X5900]; **C** = Fibroblasts (F) and fibres [x7500]; **D** = Smooth muscle (SM) cell, many fibres [x9800]

LEH

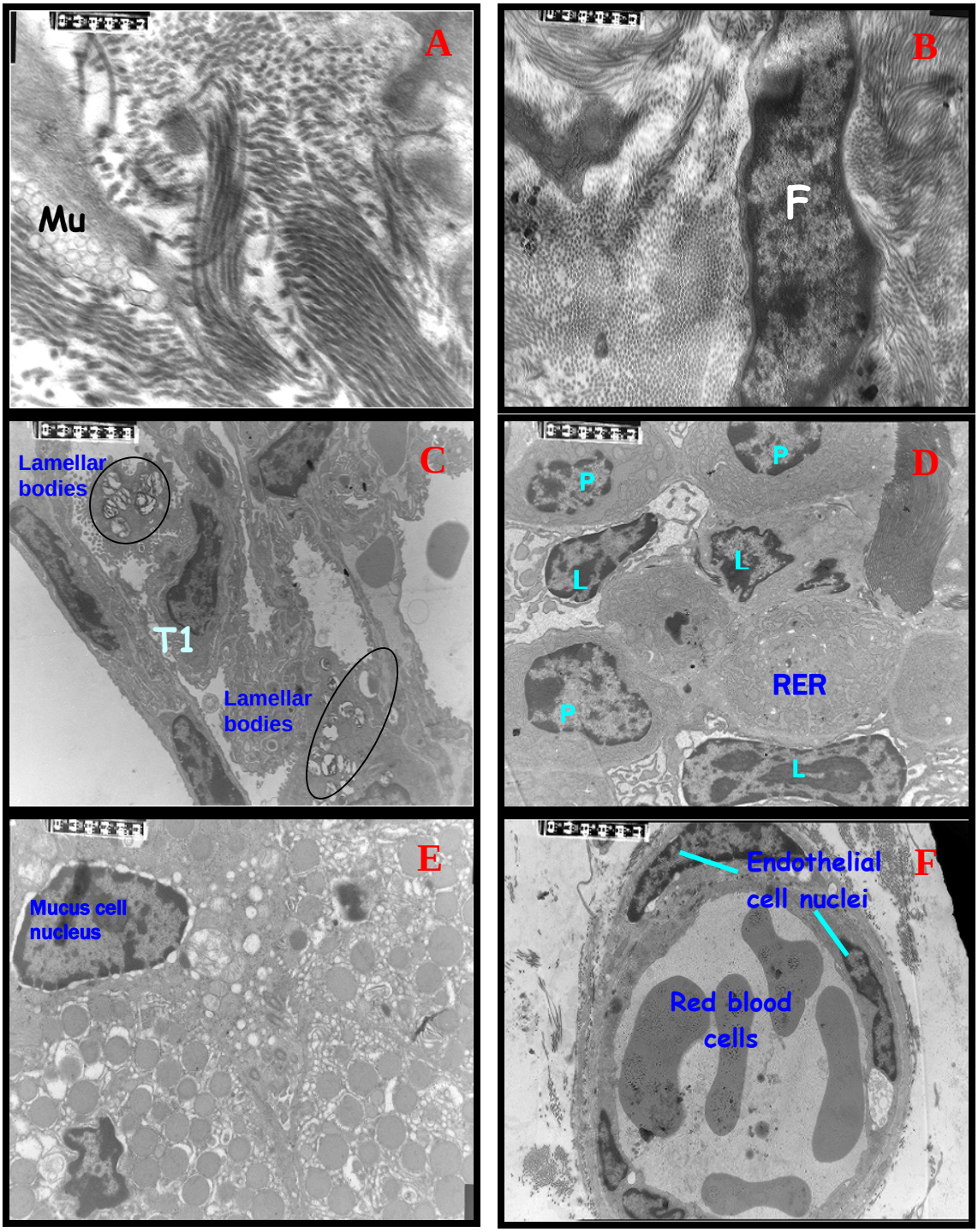


Figure 7.12: TEM micrograph of lung tissue from asthma mice treated with LEH showing **A =** Mucus gland (Mu) and many fibres [x3600]; **B =** Fibroblast (F), many fibres [x13000]; **C =** Alveolar walls, Type 1 (T1) cells, lamellar bodies [x4300]; **D =** Bronchioles: Plasma cell (P), lymphocyte (L), Rough ER [x4300]; **E =** Bronchial seromucous gland and cell [x7500]; **F =** Bronchial artery containing red blood cells [x5900]

7.5. Discussion

The airway wall consists of epithelial, interstitial, nervous and immunological components (Plopper 1996, Pinkerton 1997, Evans et al., 1999) all of which actively interact with one another (Kips et al., 2003). The common structural changes that characterize the asthmatic condition include an increase in epithelial goblet cells, mucous gland hyperplasia, subepithelial fibrosis (with deposition of collagen and fibronectin), smooth muscle hypertrophy and/or hyperplasia, formation of new vessels (Ordonez et al., 2001; Kips et al., 2003), among others.

Cell migration into the airway lumen and parenchyma is a common and very important feature of asthma and a wide range of cells are known to be involved. In prior experiments discussed in chapter five of this study, results from blood parameters have confirmed the presence of asthmatic conditions in the experimental mice used in this study.

7.5.1. Cellular structures

Fibroblasts

Results obtained from this study show that fibroblasts were scanty in both the LHC and LEH specimens respectively, almost as much as was seen in the CT specimens. This finding appears to indicate that treatment with the LHC and LEH could lower fibroblast proliferation. On the other hand, the HHC and HEH specimens had more fibroblasts but only as much as in the 'asthmatic' specimens. This finding may suggest that the high dose treatment with both HC and *E. hirta* has minimal or no effect on fibroblast proliferation since about same amount of fibroblasts was seen in the AS specimens.

It is not clear why the low dose treatments were more effective in reducing fibroblast proliferation in this study but previous studies have long shown that glucocorticoids inhibit fibroblast proliferation albeit through obscure molecular mechanisms (Kruse et al., 1978; Ponec et al., 1979; Ramalingam et al., 1997). The effects of many botanical extracts on fibroblast proliferation showed similar results. Van Wyk et al (1994) demonstrated that areca nut extract was toxic to cultured fibroblasts and inhibited their proliferation in a concentration-dependent manner. Valentiner et al (2003) also showed that six out of seventeen 'potentiated' plant extracts, had no effect on fibroblast proliferation while eleven of the plant extracts had a dose-dependent inhibitory effect.

Lymphocytes

These cells are among the most prominent cell types during asthma. The 'compartmental' distribution of these cells was studied in the Brown Norway rat asthma model and lymphocyte numbers were observed to have increased in the BALF and in the lung parenchyma (Schuster et al., 2000). The CD8 subtype of T-lymphocytes is also known to form a significant component of alveolar wall inflammatory infiltrate (Finkelstein et al., 1995; Saetta et al., 1999).

Lymphocyte migration to the airways and into airway tissue appears to occur via an indirect mechanism and the effects of the treatment agents used in this study on lymphocyte migration have been studied. It was found in one study, that in spite of their widespread effects during asthma, glucocorticoids were capable of reducing the effects of cytokine-induced recruitment and survival of inflammatory cells in general, of which the lymphocytes are a part (Schwiebert, et al., 1996).

In this study, a few lymphocytes were seen in the lung parenchyma of the LHC dose specimens but in all other groups, including the AS group, there were much fewer lymphocytes. The implication of this finding for the high HC group may possibly be that HC doses above the low dose used in this study possibly inhibit cytokine-induced lymphocyte migration, resulting in less lymphocytes being attracted to the airways unlike as observed in the low HC specimens in which the effect on inhibition seemed minimal.

The exact mechanisms by which the *E. hirta* extract acts on lymphocytes are not clear but both the high and low doses of this extract appear to exert similar pattern of effects on lymphocyte migration to the airway interstitium probably via alteration of adhesion molecules. Previous studies have shown that Bromelain, a natural proteinase preparation derived from pineapple stem, selectively removed certain cell surface molecules that possibly affected lymphocyte migration and activation following *in vitro* treatment (Hale and Haynes, 1992).

Neutrophils

Neutrophils were not seen in specimens of all other groups (including the asthma group) but very few were found in the CT group specimens. Neutrophils normally have a high turnover and rapid clearance via apoptosis, followed by macrophage phagocytosis (Cox et al., 1995; DeLeo 2004). The few neutrophils present in the CT specimens could be naïve residual cells normally attracted to the airways prior to an infection or inflammation. In the AS and all other treatment groups, an initial neutrophil accumulation could have occurred, followed by either migration away or apoptotic elimination from airways. A previous study by (DeLorme et al., 2002) showed that airway tissue neutrophil numbers were capable of reverting to control

values after 24 hours airway exposure to ozone. This could explain the observation for the AS group but not for the different treatment interventions. It is unclear whether the transient elevation of tissue neutrophils in the airways and the accompanying changes apply only to ozone exposure or is of general application.

The mechanism through which the different treatment applications contributed to the absence of neutrophils in the airways is not clear, but glucocorticoid treatment is generally known to inhibit neutrophil apoptosis in the airways (Cox 1995) implying that neutrophil numbers were supposed to be much higher in the airway tissue of the HC treated specimens. It thus appears that in this study even treatment with either *E. hirta* or HC did not prevent the rapid elimination of neutrophils from the airways nor promote their retention in the airway and lung parenchyma.

Monocytes

In this study, monocytes were only seen in the LHC specimens but absent in all other specimens. This could be because in asthma, circulating monocytes are mobilized via differentiation into the macrophage pool that addresses any prevailing inflammatory conditions in the airways and even down to the alveoli (Landsman and Jung, 2007). Thus, there would be only few or no monocytes in especially the distal respiratory structures in all the other specimen groups. It is possible that unlike *E. hirta*, HC alters the monocyte-macrophage balance in a reverse dose-dependent manner.

Macrophages

There is much information in literature about the dynamics of macrophage involvement in non-asthmatic forms of inflammation than in asthmatic

inflammation. Macrophage infiltration into the airway and lung parenchyma has only been identified as a common feature in COPD and bronchitis (Saetta et al., 1993; Barnes, 2002) but little has been reported about their involvement in asthma. Results from this study showed that there were relatively more macrophages in specimens of the AS and LHC groups than in specimens of the CT, HHC as well as the HEH and LEH.

The presence of only a few macrophages in the CT specimens and relatively more of the cells in the AS group is understandable but in all the other treatment groups, it seems that only a few of the mobilized macrophages were able to penetrate the airway walls while the rest possibly remained within the airway spaces, including the alveolar spaces. The HHC and both doses of *E. hirta* plant extract appear to inhibit migration of the macrophages into the airway parenchyma while the LHC appeared to have little or no inhibitory effect on the ability of macrophages to penetrate the airway parenchyma.

Plasma cells

These cells are essential immunological cells in asthma and are known to be derived from B-lymphocytes. Under the influence of cytokines, plasma cells produce antigen specific IgE. Allergens induce T cells to activate B cells, which then develop into plasma cells that produce and release more of the IgE antibodies (Maddox and Schwartz, 2002). After their role in early immune reactions, plasma cells appear to migrate finally to the bone marrow where they survive without undergoing any further proliferation (Manz et al., 1997; Slifka et al., 1998).

In this study, plasma cells were easily identified by the elaborate presence of rough endoplasmic reticula in their cytoplasm. Plasma cell numbers were either very scanty or absent in all treatment specimens except those of the LEH group. The AS group also contained but only a few plasma cells in the airway and lung parenchyma, an indication that these cells are only prominent and active at the early stage of the inflammation process and not resident in the airways.

The presence of a few cells observed in the LEH group could indicate that this dose was inadequate to promote migration of plasma cells away from the airways but it is not clear whether this dose rather promoted the migration of the plasma cells into the airway interstitium. On the other hand, though plasma cells ultimately migrate away from the airways, treatment with the HEH as well as with both HC doses appears to be effective in promoting this migration, hence the fewness or absence of these cells in specimens of these treatment groups.

Pneumocytes

These alveolar cells are resident structural cells and therefore not expected to vary remarkably in number between the specimens of the respective treatment groups. In most species, there are generally more type-II cells than are type-I cells in the alveoli even though type-I cells cover 95% of the alveolar surface (Berthiaume et al., 2006). In the rat lungs for instance, type-II cells cover approximately 3.6% while type-I pneumocytes cover 96.4% of the entire alveolar surface (Travis and Tucker, 1986). In this study, there were more of the flat type-I cells than the cuboidal type-II cells in the alveolar parenchyma of all specimens indicating that the numerical composition of these resident cells was not distorted by the different experimental interventions.

7.5.2. Other structures

Collagen deposition

In the subepithelial "basement membrane" (SBM), collagen deposition is a known feature in the pathophysiology of asthma. Submucosal collagen deposition has also been studied especially because this airway region is larger and anatomically closer to smooth muscles than it is to the subepithelial "basement membrane" (Chu et al., 1998). The authors suggested from their findings that large airway collagen deposition might not be a key contributor to the symptoms and pathophysiology of asthma.

The present study explored the distal airways and the lung parenchyma. Collagen fibre deposition was minimal in the CT specimens as well as in specimens of the LHC groups. Specimens of the AS group had a high amount of fibre deposition compared to others. The remaining groups had varying amounts of collagen fibre deposition, albeit higher than observed in the CT specimens indicating that treatment with *E.hirta* extract as well as with the HHC did not cause significant reduction in collagen fibre deposition even after a possible increase at the onset of asthma prior to treatment. The LHC specimens had minimal collagen deposition possibly because the low dose was ineffective in reducing collagen deposition.

The exact mechanism involved in the reduction of collagen fibre deposition following HC or *E. hirta* treatment is not understood. Results from previous studies have been indeterminate regarding the effects of steroids on airway remodelling. Laitinen et al (1992) and Jeffery et al (1992) demonstrated that inhaled corticosteroids reduced airway inflammation, but could not reduce the thickness of the SBM in mild/moderate asthmatics. In contrast, other studies (Trigg et al., 1994;

Olivieri et al., 1997; Sont et al., 1997) showed reduction of the thickness of bronchial subepithelial basement membrane after a few months of inhaled corticosteroid treatment in mild/moderate asthmatics. Not much information is available on the effects of plant extracts on collagen deposition in airway parenchyma and no previous studies are available on the effects of *E. hirta*. However, extracts of *Ecklonia Cava* (a polyphenol/phlorotannin derived from Brown Algae) have been reported to cause reduction in airway epithelial hyperplasia by 75% as well as reduced collagen-causing fibrosis in lung interstitium by 20% (Levine, 2007).

Lamellar bodies

Alveolar type II cells synthesize, store and secrete pulmonary surfactant (King, 1974) and the final secretory product is segregated as concentric lamellar bodies, which are destined for secretion into the alveolar space to become part of the surfactant-lining layer (Chevalier and Collet, 1972). The presence of many lamellar bodies could therefore indicate increased surfactant secretion.

In this study, lamellar bodies in type II cells were more abundant only in the LHC and LEH specimens. A previous study showed cortisol-accelerated synthesis of pulmonary surfactant in the human fetal lung as early as the second trimester (Ekelund et al., 1975). Cortisol also caused a marked stimulation of synthesis and accumulation of lamellar body phosphatidylcholine in lung explants from both 21-day and 28-day fetal rabbits (Mendelson and Synder, 1985). Similarly, Asabe et al (2007) obtained results which suggested that maternal dexamethasone (a glucocorticoid) treatment accelerates the maturation of the surfactant system, especially the expression of lamellar bodies in type II pneumocytes, even in

hypoplastic lungs induced by oligohydramnios. It is not clear if the stimulatory effect of HC observed in this study was dose dependent.

There is no information available in literature on the effects of *E.hirta* extract administration on either the formation of lamellar bodies or surfactant production. However, the effects of other herbal products on the surfactant system have been reported. Amygdalin, which occurs naturally in a number of plant materials but usually commercially obtained from the kernel of *Prunus armeniaca L.* (apricot pits) was found to partially protect type II alveolar epithelial cells from the effects of hyperoxia-induced lung injury (Huaping et al., 2004). In another study, "Bakumondo-to" (a traditional Chinese medicinal prescription consisting of six herbs (*Ophiopogonis tuber, Pinelliae tuber, Zizyphi fructus, Glycyrrhizae radix, Ginseng radix, and Oryzae fructus*), was found to have characteristic secretory-enhancing effect on pulmonary surfactant and normalizes hypersecretion by the inhibition of superoxide (Miyata et al., 1999).

The absence of lamellar bodies in the asthmatic group specimens as well as specimens of both higher doses of the test agents used in this study is not understood. In the asthmatic group, it seems that induction of asthma in the mice did not influence a high type II cellular activity by way of lamellar body formation and surfactant secretion. This seeming absence of surfactant-producing cells possibly relates to the reasons the airways remain constricted and the alveoli collapsed during an asthmatic attack unlike what is seen in normal lungs. It is not clear why in this study there was a dose-related disparity in the effects of treatment on the presence of lamellar bodies. However, the absence of lamellar bodies in the high dose specimens could mean that treatment with these high

doses does not stimulate lamellar body formation and surfactant production in spite of other positive anti-asthmatic effects associated with these dose levels.

Alveolar wall thickness

Another feature observed in this study was the relative thinness of the alveolar walls in specimens of the CT group as well as the moderate thickness in all other groups with the thickest walls seen in specimens of the LHC group. This finding tends to suggest that all other treatment agents and doses had varying degrees of effect in reducing alveolar wall thickness except the low HC treatment, which appears to have a mild effect on collagen fibre deposition in the alveolar wall.

Mitochondria

An important role for mitochondria in the pathogenesis of inflammation and in the development of bronchial asthma has been reported (Heinzmann et al., 2003). In this study, increase in mitochondria number in the different cells studied showed that there were relatively higher numbers of mitochondria in the cells of the asthma and HHC specimens. Damage to mitochondria in asthma could affect clearance of any free radicals released in asthmatic inflammation and increased mitochondria numbers could point to the high-energy requirement of these cells. The number and activity of mitochondria in smooth muscle cells from patients with asthma was found to increase due to an altered calcium homeostasis that increases mitochondrial biogenesis (Trian et al., 2007). The reason why there were higher mitochondria numbers in the asthmatic group is not clear but much energy is apparently required in asthma by cells to respond to the inflammatory reactions associated with the sudden onset of experimental asthma and this will involve mitochondrial activity.

Results of experiments by (Lowe et al., 1954) have long indicated that the number of mitochondria per cell is significantly reduced in rat liver cells following parenteral administration of HC. It is therefore not understood why the HHC specimens had relatively higher numbers of mitochondria compared to specimens from other treatment groups. Perhaps organism specificity and activity levels are possible implicating factors since in this case HC was administered as a treatment for the asthma induced in the mice.

Striations

The presence of striated muscle cells in non-neoplastic lung parenchyma is an uncommon observation (Aterman and Patel, 1970; Chellam 1988) even though these cells have been previously detected in pulmonary anomalies. Studies with human specimens have shown many cases of proliferation of these cells in non-neoplastic lung tissue (Fraggetta et al., 2000) and the authors believed these findings are more common than usually reported.

Striations seen in this study were only present in specimens of the AS group and were much similar to those observed in a typical striated muscle. It is not clear why in this study, such rare finding occurred only in the AS group and therefore the association of this structure with asthma is uncertain. Although the exact origin of these cells is speculative, a wide morphogenetic error has been suggested (Drut et al., 1988; Fraggetta 2000). No previous studies on experimental asthma have shown similar results.

Mucus-secreting structures

Mucus plugs occur in airways of all sizes, from the second-generation airway to bronchioles (Roche et al., 1989). Although only relatively few mucous-secreting

structures were identified in the asthma group specimens, these structures were very prominent in specimens of the LHC and LEH groups respectively but absent in specimens of the respective high doses and in the controls. This finding tends to suggest that the low doses of the two treatment agents are ineffective in ameliorating or eliminating mucous cell proliferation and possibly mucus secretion. The presence of relatively few mucous-secreting structures in the AS group under the TEM may not imply that mucous secretion was not a feature of this model of asthma. It is possible that mucus secretion is not dependent solely on the number of cells but on the metabolic potency of the few mucous cells and glands present.

Smooth muscle cells

Smooth muscle cells surround the airways from the trachea down to the alveolar ducts and their constriction could be induced by a variety of stimuli. It is not understood why more smooth muscle is present in the airways of patients with asthma, although the commonly held paradigm suggests that mediators released during chronic inflammation induce airway smooth muscle (ASM) proliferation (Panettieri 1998; Hirst et al., 2000). The precise role of this muscle in the pathogenesis of asthma is not certain but it is known that ASM contraction certainly causes acute narrowing of the airway and airflow obstruction in asthma, and airways (Panettieri, 2004; Solway, 2007).

In this study, smooth muscle mass increased prominently in specimens of the AS group while specimens of other groups exhibited varying degrees of muscle hypertrophy that possibly relate to the respective treatment doses. This finding conforms to information from previous studies. Treatment with HC and the extract of *E. hirta* appear to be effective in preventing airway smooth muscle cell

proliferation. Glucocorticoids have been shown to inhibit the proliferation and migration of airway myocytes and suppress their expression of a number of proinflammatory cytokines (Bonacci et al., 2006; Solway, 2007). In one previous study with herbal products, the ethanolic extract of a Malagasy species *Euphorbia stenoclada* (ES) (Euphorbiaceae), traditionally used as a herbal remedy against asthma and acute bronchitis, was tested to evaluate possible anti-proliferative activity on human airway smooth muscle cells (HASMC) (Chaabi et al., 2007). This extract was found to completely abolish the interleukin-1β (IL-1β)-induced proliferation of HASMC. It is therefore possible that the absence of airway smooth muscle proliferation in the *E. hirta* group reflects similar effects as seen with the *Euphorbia stenoclada* treatment.

Filopodia

Filopodia are thin, dynamic cell extensions comprising tight bundles of long actin filaments covered with cell membrane. Filopodia allow cells to explore their environment (extracellular matrix and surfaces of other cells), to identify appropriate targets for adhesion, and then generate guidance cues and traction forces to move the cell body (Jacinto and Wolpert, 2001). The presence of these structures in cells may indicate a high cellular activity. In this study, only specimens of the LHC group had fibroblasts with many filopodia. It is not understood whether this low dose treatment promotes filopodia formation but its inability to prohibit filopodia formation appears certain. The absence of filopodia in the fibroblasts of the HHC dose specimens could indicate that this dose inhibits filopodia formation by fibroblast.

A previous study showed that Piperlactam S inhibited filopodia formation in macrophages among other effects (Chiou et al., 2003). Piperlactam S is an alkaloid isolated from *Piper kadsura*, an anti-inflammatory Chinese herbal medicine used for the treatment of asthma and rheumatic arthritis (Han et al., 1990). Since *E. hirta* extract also contains alkaloids, the absence of filopodia in the specimens of both the high and low doses of *E. hirta* extract in this study possibly denotes inhibition of filopodia formation by the extract via a mechanism similar to that described for Piperlactam S by Chiou et al (2003).

A summary of all the findings is given in *table 7.1.*

Table 7.1: Summary of the comparative effects of HC and *E hirta* extract treatments on inflammatory cell infiltration and structural changes in the airways as determined by TEM analysis.

Cellular structures						
	CT	**AS**	**LHC**	**HHC**	**LEH**	**HEH**
Fibroblasts	Very few	Many	Scanty	Few	Scanty	Few
Lymphocytes	Very few	Very few	Few	Very few	Very few	Very few
Neutrophils	Very few	Absent	Absent	Absent	Absent	Absent
Monocytes	Absent	Absent	Very few	Absent	Absent	Absent
Macrophages	Very few	Many	Few	Very few	Very few	Very few
Plasma cells	Scanty	Few	Scanty	Scanty	Very few	Scanty
Pneumocytes	More type-I cells than type-II cells in all specimens; treatment did not alter the cell ratio.					
Other structures						
	CT	**AS**	**LHC**	**HHC**	**LEH**	**HEH**
Collagen fibre deposition	Minimal	More than in CT	Few	More than in CT	More than in CT	More than in CT
Lamellar bodies	Absent	Absent	Many	Absent	Many	Absent
Alveolar wall thickness	Very thin	Very Thick	Thickest	Thick	Thick	Thick
Mitochondria	Few	Many	Few	Many	Few	Few
Striations	Absent	Present	Absent	Absent	Absent	Absent
Mucus-secreting structures	Absent	Few	Many	Absent	Absent	Many
Smooth muscle cells	Few	Many	Few	Few	Few	Few
Filopodia in fibroblasts	Absent	Absent	Present	Absent	Absent	Absent

7.6. Conclusion

The general effects of both treatment agents show that treatment with either *E. hirta* or HC did not prevent the rapid elimination of neutrophils from the airways nor promote their retention in the airway and lung parenchyma whereas lymphocyte migration into the lung parenchyma was found to be inhibited by both HC and *E. hirta* in a dose-dependent manner. In addition, HC treatment appeared to be more effective than *E. hirta* in promoting monocyte differentiation into macrophages, albeit in a dose dependent manner.

This study also showed that the numerical composition of alveolar pneumocytes was not distorted by the different experimental interventions and abnormal muscle striations were observed only in the AS specimens but all treatment agents and doses were found to have varying effects on alveolar wall thickness. Treatment with both HC and *E. hirta* also appeared to abolish smooth muscle hypertrophy.

There were relatively more mitochondria in specimens of the 'asthmatic' mice than others and treatment with the LHC appeared more effective in promoting lamellar body formation and plasma cell migration into the airway parenchyma but caused reduced fibroblast proliferation. Treatment with the HHC dose appeared to promote migration of plasma cells into the airway parenchyma but inhibited filopodia formation by fibroblasts as well as migration of macrophages into the airway parenchyma. The HHC treatment also appeared to inhibit mucous cell proliferation but did not reduce collagen fibre deposition. In addition, there were relatively more mitochondria in the HHC specimens than in others.

The LEH extract not only caused a reduction in fibroblast proliferation but also inhibited the migration of macrophages into the airway parenchyma as well as filopodia formation by fibroblasts. However, treatment with this dose of *E. hirta* promoted lamellar body formation whereas treatment with the HEH dose appeared promote plasma cell migration into the airway parenchyma but inhibits migration of macrophages into the airway parenchyma. This HEH dose also inhibits filopodia formation by fibroblasts as well as mucous cell proliferation but did not reduce collagen fibre deposition.

When compared to the white cell counts in *tables 5.1* and *5.2*, no correlations were seen in terms of abundance. Neutrophils, lymphocytes and monocytes were all more abundant in the asthma group than in other cells, extreme variations from this were observed in the peripheral blood cell counts.