CHARACTERIZATION OF THE SURFACE MACROPHAGES OF THE AVIAN LUNG WITH OBSERVATIONS ON A PHAGOCYTIC RESPIRATORY EPITHELIUM

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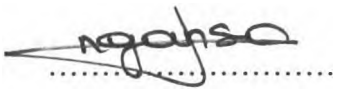
Johannesburg, 2001

I, L.N. Nganpiep declare that this dissertation is my own work. It is being submitted for the degree of Master of Sciences in Anatomical Sciences in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



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I dedicate this thesis to God almighty for giving me the strength and courage to achieve this work, to my father and mother Mr and Mrs Ngampiep Jean who provided love and sponsoring for my studies, without them I would not have traveled so far on the academic path.

PRESENTATION ARISING FROM THIS STUDY

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ABSTRACT

Due to paucity of free respiratory macrophages (FRMs), compared with mammals, birds have been alleged to be more susceptible to pulmonary infections and affliction. The goal of this study was to question or validate this speculation.

Twenty-two mature healthy chickens, 24 domestic ducks and 20 rats were used in various experiments. After pulmonary lavage, FRMs were stained with trypan blue for cell count and with neutral red and trypan blue to assess cell longevity. The cell dynamics was determined by counts made on serial lavages. The morphological attributes of the FRMs and that of the respiratory "phagocytic epithelium" were quantified stereologically.

The rat had a significantly greater number of macrophages and the surface density of the filopodia of the FRMs was higher than that of the birds. The volume density of the vesicular bodies of the macrophages in the three groups of animals was not significantly different. Putative cell flux onto the respiratory surface was observed in the birds and not in the mammal. The surface area of the "phagocytic epithelium" of the birds was very extensive.

Compared with mammals, in general, FRMs are fewer in birds. Despite this, the pulmonary defensive status in birds may not necessarily be compromised: functionally, avian FRMs appear to be more efficient. Moreover, their defense function is complimented particularly by the phagocytic activity of the epithelium immediate to the blood-gas barrier, the most vulnerable site of the lung-air sac system.

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1.0 INTRODUCTION

As observed, e.g. by Stevens and Wen-Zhe Ho (1990), modern study of mammalian phagocytes began in the nineteenth century when Ilya Metchnikoff recognized two principal phagocytes among the migratory cells of the blood: the microphages or polymorphonuclear granulocytes and the macrophages or phagocytic mononuclear cells. The former cells are normally the commonest in the blood stream while the latter circulate as monocytes. As reported by Brain, Sorokin and Godleski (1977), Ilya Metchnikoff demonstrated that macrophages remove endogenous cell debris, foreign bodies and defend organisms against bacterial invasion.

In parallel with the production of other hemoietic cells, macrophage production is continuous throughout life. The production of tissue macrophages is the end result of sequential events in which multipotential stem cells generate progenitor cells committed to granulocyte and monocyte production. Individual progenitor cells generate clones of cells that progressively lose the capacity for further division and produce maturing monocytes that are released to the circulation from the bone marrow and the spleen. Circulating monocytes seed into the tissues either to die or to form relatively long-lived tissue macrophages (e.g. Metcaff, 1982). Blusse, Mattie and van Furth (1983) found that at least 70% of pulmonary macrophage population comes from monocyte influx and at most 30% by local division of immature mononuclear phagocytes originating from the bone marrow. They reported that the turn over time of pulmonary macrophages is about 6 days.

Macrophages are named according to the tissue in which they are found. For instance, histiocytes are found in the connective tissue, Kupffer cells in the liver, alveolar macrophages in the lung, pleural and peritoneal macrophages in the serous cavity, type-a cells in the synovial lining, and osteoclasts in the bone tissue. All of these cell types belong to the same cell line called mononuclear phagocyte system (MPS) (e.g. van Furth, Goud, van der Meer *et al.*, 1982).

In the poultry industry, avian respiratory diseases are the greatest cause of mortality. As in other animals, many agents cause avian respiratory diseases. While studies on the biology of the mammalian alveolar macrophages abound, relatively little is known about the corresponding cells in the lung-air sac system of birds. In the mammalian lung, alveolar macrophages (AMs) are found mainly on the respiratory surface where they provide the first line of defense against inhaled foreign particles and pathogens (e.g. Brain *et al.*, 1977). Corresponding cells on the lung-air sac system of birds are referred to as free avian respiratory macrophages (FARMs). Paucity of FARMs has been reported on the lung-air sac system of birds (e.g. Toth and Siegel, 1986; Maina and Cowley, 1998) and even lack of them (e.g. Klika, Scheuermann, De Groodt *et al.*, 1996; Lorz and Lopez, 1997). Without concrete proof, this has been considered to validate the purported high susceptibility of the avian respiratory system to pathogens. Furthermore, as speculated by Maina and Cowley (1998) the unique morphology of the lung-air sac system of birds where some air sacs pneumatise certain bones and others lie close to the surface of the skin (e.g. Bezuidenhout, Groenewald and Soley, 1999), has been said to predispose birds to fast diffusion of air-borne diseases while intensifying the spread of harmful air

pollutants. The actual location of the FARMs in the lung-air sac system, the correlation between the small number of FARMs and the susceptibility of birds to respiratory diseases, and the structural and functional properties of FARMs have not been investigated before.

In this study, a mammal (rat- *Rattus rattus*) was used for comparison with with birds, i.e. the domestic fowl (*Gallus gallus* variant *domesticus*) and the domestic duck (*Cairina moschata*) to establish: (a) whether free (surface) macrophages are fewer in the avian respiratory system, and (b) to question whether the assertion that birds are more susceptible to respiratory diseases by virtue of their having fewer free macrophages is correct or not. The following experiments and analyses were performed:

- The numerical density of FARMs was determined after pulmonary lavage
- The longevity (robustness) of FARMs, i.e. the ability to survive after lavage was evaluated.
- The dynamics of the FARMs, i.e. their translocation onto the respiratory surface was assessed by serial lavage.
- The location of the FARMs was investigated by separately lavaging the lung and the air sacs.
- The morphological and morphometric attributes of the FARMs were studied by:
    - fluorescence microscopy
    - transmission and scanning electron microscopy
    - stereological analysis

- The surface area of the "phagocytic epithelium" of the atrial muscles, atria and infundibulae was determined stereologically.

The abbreviations used in the text are listed and explained in appendix 1 for quick reference.

2.0 LITERATURE REVIEW

2.1 THE STRUCTURE OF THE AVIAN LUNG

The avian respiratory system is structurally more complicated than the mammalian one and functionally differs from it in many fundamental ways (e.g. King, 1966; Duncker, 1971; Scheid, 1979). The most important differences are that the avian respiratory system has large air sacs that connect to the secondary bronchi of the lung via multiple openings, the ostia. The compact, inexpansile lung is ventilated continuously and unidirectionally by synchronized action of the air sacs, in contrast to the in-and-out (tidal) flow of air in the mammalian lung. The unique design of the avian respiratory system is not a prerequisite for flight since bats, which have a normal mammalian system (e.g. Maina, 1985), are capable of sustained flight.

The avian lungs, small, flattened and in most birds quadrilateral in shape, are located on the dorsal aspect of the coelomic cavity where they are firmly attached to the ribs (e.g. King, 1966; Duncker, 1971). The cranial end of the lung reaches the movable rib that is attached to the last cervical vertebrae while the caudal end extends to the cranial border of the ilium. In some other species (e.g. geese) it extends almost to the level of the hip joint (e.g. King and McLelland, 1984). In transverse section, the lung is wedge shaped giving rise to 3 surfaces:

The dorsolateral surface: it is in contact with the ribs and is thus known as the costal surface.

The dorsomedial surface: it is in contact with the vertebrae and is known as the vertebral surface.

The ventromedial surface: it is in contact with the horizontal septum, is thus named septal surface.

Blood supply to the avian lung occurs via the pulmonary arteries which give rise to numerous interparabronchial arteries that run alongside and obliquely around the parabronchi (e.g. Abdalla and King, 1975, Abdalla, 1989). Each of these arteries forms a series of intraparabronchial arterioles that penetrate the exchange tissue and terminate in the blood capillary network that interdigitates with the air capillaries (Maina, 1982; 1988). Nerve supply to the avian lung is from the vagus and thoracic sympathetic system (e.g. King and McLelland, 1984).

2.1.1 PRIMARY BRONCHUS

The extrapulmonary bronchus is formed by the bifurcation of the trachea at the syrinx. Each perforates the septal surface of the lung and then continues as the intrapulmonary primary bronchus to the caudal extremity of the lung. Its widest part occurs at the point where it enters the lung, tapering progressively to its termination. Histologically, the respiratory epithelium of the primary bronchus consist of a pseudo-stratified columnar epithelium with goblet cells, intraepithelial mucous alveoli and projecting ridges carrying cilia (e.g. McLelland, 1989).

2.1.2 SECONDARY BRONCHI

These arise from the primary bronchus. There are four groups of secondary bronchi named according to the region of the lung they supply with air (e.g. King, 1966; Duncker, 1971; King and McLelland, 1984): medioventral, mediodorsal, lateroventral and laterodorsal.

- The mediodorsal and medioventral secondary bronchi together with their interconnecting tertiary bronchi (parabronchi) form the thick medial part and the whole of the cranial part of the lung.
- The lateroventral and laterodorsal secondary bronchi and their parabronchi form the thin lateral region of the lung.

In three-dimensions, the broncho-parabronchial transition appears as an oval window opening into the parabronchial vestibulum. Towards the transitional zone, the mucous (goblet) cells gradually disappear and the ciliated pseudostratified columnar epithelium of the bronchus continues as a non-ciliated epithelium toward the crest of the fold (McLelland, 1989). The site of transition is distinct, with the narrow zone of non-ciliated columnar epithelium passing into simple cuboid epithelium formed by granular cells. The connective tissue is formed by loose connective tissue with numerous plasma cells, mast cells and tissue macrophages. The presence of macrophages at this site shows the significance of protecting the parabronchial gas exchange tissue from pathogens and harmful particulate matter (Klika, Scheuermann, De Groodt, *et al.*, 1999).

2.1.3 THE PARABRONCHI (TERTIARY BRONCHI)

After running for a few millimeters, the four groups of secondary bronchi give off a large number of tubes (300-500 in chicken) of small but uniform caliber (0.5-2mm in diameter), the parabronchi (e.g. Maina, Abdalla and King, 1982). They are not blind ending but rather join end to end to form long curved hoop-like structures. Each parabronchus is the central axis of a respiratory unit of the lung (e.g. Salt and Zeuthen, 1960; King, 1966; Duncker, 1971). The cranial and dorsomedial parts of the lung are made up of layers of parabronchi running between the mediodorsal and medioventral secondary bronchi. Together, these secondary bronchi and their parabronchi form an integrated functional unit: the medioventral-mediodorsal system of bronchi. This system and its connection to the first 2-3 lateroventral secondary bronchi constitute the paleopulmo (i.e. the old lung). The anastomosing network in the ventrolateral part of the lung, together with the connection of this network to the caudal air sac, forms the neopulmo (i.e. the new lung) (e.g. Duncker, 1971). The smooth muscles that delineate the parabronchial lumen are penetrated by numerous more or less pentagonal or hexagonal openings (between 100-200 μm in diameter), the atria (e.g. King and Molony, 1971).

2.1.3.1 Atria

The epithelium of the atrial compartment is composed of granular and squamous cells. Granular cells correspond closely to type II pneumocytes in mammalian pulmonary alveolus (Corral, 1995). They produce and discharge osmiophilic lamellar bodies that are the precursor of the surfactant that imparts alveolar stability. The pulmonary surfactant is a complex of lipid and protein, which lines the respiratory surface of the lung. By

spreading in a monolayer, it lowers the surface tension at the air-liquid interface, preventing alveolar collapse. In the fixed (inexpansile) lungs of birds (e.g. Jones, Effman and Schmidt-Nielsen, 1985), the role of the surfactant is rather that of preventing transudation of blood plasma into the air spaces. Furthermore, it has been suggested that the surfactant may be involved in the defense of the lung (Lorz and Lopez, 1997). The squamous atrial cells produce and discharge a trilaminar substance, a specific lamellated material encountered only in avian parabronchial units (e.g. Scheuermann, Klika, De Groodt *et al.*, 1997). To some extent, it contributes to the defense of the avian lung. Stearns, Barnas, Walski *et al.* (1987) found iron oxide particles trapped within the trilaminar substance of all the ducks that they exposed to aerosol. The lamellated bodies like the trilaminar substance, have been postulated to play a surface tension reduction role in turkeys and chickens (Scheuermann *et al.*, 1997). Klika *et al.* (1996) reported that the trilaminar substance is discharged in a form of a 15-nm thick acellular lining layer that is uniquely adapted to the extremely thin respiratory epithelium: it is formed in the cytoplasm of squamous respiratory cells from profiles of granular and smooth endoplasmic reticulum. Epithelial cells lining the atria and infundibulae were shown to be phagocytic by Stearns *et al.* (1987). Earlier histochemical studies have demonstrated a high concentration of hydrolytic enzymes associated with phagocytosis in atrial epithelial cells found on the surface of the atria and infundibulae (Stearns *et al.*, 1987). The atria open into 3-6 air ducts, the infundibulae (Klika *et al.*, 1999).

2.1.3.2 Infundibulae

These are narrower and much less well defined air spaces than the atria. As many as 8 infundibulae arise from an atrium (Maina *et al.*, 1982). At the transition between the atria and the infundibulae, a squamous epithelium changes to an intermediate one. Infundibulae are lined by squamous respiratory cells that continue to the air capillaries (e.g. King, 1966).

2.1.3.3 Air capillaries

From an infundibulum originates a complex network of fine anastomosing tubules known as air capillaries (3-10 μm in diameter) (Duncker, 1971; Maina, 1982; 1988) that are intimately interlocked with a network of blood capillaries to form the gas exchange tissue. The three dimensional interdigitation between the air and the blood capillaries, to a large extent, imparts the integrity and stability to the avian lung while providing an extensive respiratory surface area (Maina, King and Settle, 1989; Maina, 2000).

2.1.4 AIR SACS

The air sacs are poorly vascularised and do not participate in gas exchange (e.g. Scheid, 1979). Their main function is to act as bellows in moving air through the complex system of passageways and ventilating the parabronchial exchange tissue (e.g. Schmidt-Nielsen, 1975). In adult birds, on average there are 9 air sacs: 4 are paired (2 cervical, 2 cranial thoracic, 2 caudal thoracic and 2 abdominal) and a single clavicular one, (e.g. King, 1966; Duncker, 1971). Functionally, the air sacs are divided into cranial and caudal groups.

-The cranial sacs that arise from the medioventral secondary bronchi include cervical, clavicular and cranial thoracic ones. During normal inspiration, the caudal air sacs draw air across the lung through the primary bronchus while the expanding cranial air sacs "suck it out" of the lung. The entry of fresh air into the parabronchi is therefore primarily due to the action of the cranial air sacs. Only at higher flow rates such as those that might occur during exercise do the cranial and caudal sacs act synergistically to move air into the dorsobronchi (Brackenbury, 1979).

-The caudal sacs include caudal thoracic and abdominal sacs which arise from the lateroventral secondary bronchi or the primary bronchus (e.g. King, 1966; Duncker, 1971): they drive fresh air into the mediodorsal secondary bronchi and parabronchi during expiration (Brackenbury, 1979).

2.1.5 AIRFLOW PATTERN IN THE RESPIRATORY TRACT

The single most unique characteristic of the avian lung is that it permits through-flow of air. In this regards the bird lung is fundamentally different from the mammalian lung which is tidally ventilated (e.g. Scheid, 1979). In the avian lung-air sac system, it is not possible to correctly predict the direction of air flow through the parabronchi from morphological observations only, as the lungs are open at both ends through ostia. During inspiration, when air reaches the mediodorsal secondary bronchi, part of it goes into these bronchi and the rest through the neopulmonic parabronchi to the caudal air sacs. The air flowing into the mediodorsal secondary bronchi enters the paleopulmonic parabronchi and then into the cranial group of air sacs. No air enters the medioventral secondary bronchi from the intrapulmonary primary bronchus, nor does it leave these bronchi to

reenter the intrapulmonary primary bronchus during inspiration. The shunting of air is due totally to aerodynamic valving at the junction between the medioventral secondary bronchi and the intrapulmonary primary bronchus (e.g. Banzett, Nations, Wang *et al.*, 1991; Wang, Banzett, Nations *et al.*, 1992): no anatomical valves (sphincters) occur at the origin of the medioventral secondary bronchi as was once envisaged. A constriction caused by an epithelial swelling was described by Maina and Africa (2000) and taken to constitute a segmentum accelerans (Wang *et al.*, 1992). During expiration, air moves from the caudal air sacs through the neopulmo and into the mediodorsal secondary bronchi. Expiratory aerodynamic valving regulates the volume of air that flows through the intrapulmonary primary bronchus during expiration (Fedde, 1980): it varies during a bird's activity. Ultimately, i.e. at the second expiratory cycle, air moves from the cranial air sacs through the medioventral secondary bronchi out into the primary bronchus and trachea to the atmosphere, completing the complex respiratory circuit.

2.1.6 MECHANISM RESPONSIBLE FOR UNIDIRECTIONAL AIRFLOW

Anatomical valves which could easily explain the unidirectional airflow in the avian lung have never been found (e.g. King and Payne, 1960). Brackenbury (1979) demonstrated that there is a small pressure difference between caudal and cranial air sacs groups. Scheid (1982) found unidirectional flow to persist in paralyzed, pump-ventilated, and even lungs of dead animals. He demonstrated that these pressure differences couldn't be solely responsible for the rectification of airflow. An interesting observation was made by Molony, Graf and Scheid (1976) who found the airflow resistance across the orifices of the medioventral secondary bronchi into the primary bronchus to be higher at low carbon

dioxide tensions in the bronchial gas than at high levels of carbon dioxide. They suggested that carbon dioxide tension could, through initiating constriction of air passages, constitute part of the functional valving mechanism. The unidirectional airflow in avian parabronchial lungs enhances respiratory efficiency by sustaining a high-pressure gradient in the exchange tissue, i.e. between the air and the blood capillaries.

2.2 MACROPHAGES

They are extremely diffuse cells, found in different forms in practically every tissue in the body where they participate in a wide-ranging number of biological processes that range from development to bone remodeling and wound healing. However, it is as sentinels of the immune system that macrophages express their full functional repertoire (e.g. Morisette, Gold and Alan, 1999).

2.2.1 Production of macrophages

In parallel with the production of other hemoietic cells, macrophages are produced throughout life. The production of tissue macrophages is the end result of a series of events in which multipotential stem cells (colony forming units of the spleen, CFU-S) generate progenitor cells committed to granulocyte and monocyte production (GM-colony forming cell, GM-CFC). Individual progenitor cells generate clones of progeny cells that progressively lose the capacity for further division and produce maturing monocytes that are released into circulation from the bone marrow and the spleen.

Circulating monocytes seed in the tissues either to die or to form relatively long-lived tissue macrophages (e.g. Metcaff, 1982).

2.2.2 Pulmonary macrophages

Macrophages play a central role in the defense of the respiratory tract against deposited particles and pathogenic microorganisms (e.g. Geiser, Serra, Cruz *et al.*, 1995). Pulmonary macrophages consist of several subpopulations that can be defined by their anatomical location, as well as by other criteria such as being free or fixed macrophages.

2.2.2.1 Alveolar macrophages (AMs)

They constitute the majority of pulmonary macrophages in mammals. Under normal condition, they represent 3-5 % of all the cells in the lung of non-smoking humans as well as rats (Sebringand and Lehret, 1992). They reside predominantly on the alveolar epithelial surface where they provide phagocytic defense against deposited particulate agents and perform a variety of other diverse activities. Mediators released from the alveolar macrophages after phagocytosis of particulate matter induce a systemic inflammatory response that includes stimulation of the bone marrow (Mukae, Hogg, English *et al.*, 2000). A novel role was recently attributed to AMs by Edwards, Sutherland and Murray (2000) who stated that the nitric oxide secreted by AMs may function as part of a physiological antiapoptotic mechanism that prevents alveolar type II cells from undergoing stretch-induced death in the lung. Upon activation, AMs produce significantly higher amount of superoxide anion, nitric oxide and tumor necrosis factor alpha compared to pleural and peritoneal macrophages (Dorger, Munzing, Allmeling *et*

al., 2001). As poultry lung do not have alveoli, the avian counterparts to the mammalian AMs have been called respiratory macrophages or respiratory phagocytes (e.g. Fulton, Reed and Denicola, 1990). Absence of free avian respiratory macrophages (FARMs) has been reported by, e.g. Klika *et al.* (1996). However, other researchers were able to demonstrate the cells by lavage only (e.g. Ficken, John and Lay, 1986) and by lavage and morphology (e.g. Maina and Cowley, 1998). Despite scarcity of FARMs on the surface of the respiratory tract, experimental challenge of the lung with pathogenic microorganisms leads to translocation of macrophages onto the respiratory tract (e.g. Toth, Robert, Caceci *et al.*, 1988).

2.2.2.2 Pulmonary intravascular macrophages (PIMs)

Present in the mammalian lung (e.g. Brain, Molina, Decamp *et al.*, 1999), PIMs were found for the first time in the lung of the domestic fowl (*Gallus gallus* variant *domesticus*) by Maina and Cowley (1998). They reported that avian PIMs resemble the analogous cells in mammals (e.g. Dehring and Wisman, 1989). PIMs are large (20-80 μm in diameter) mature macrophages that are bound to the pulmonary capillary endothelium. They have the characteristic morphological features of phagosomes or phagolysosomes, tubular micropinocytic vermiform structures and a fuzzy glycocalyx (Brain *et al.*, 1999). They phagocytise bacteria and particulate matter (Dehring and Wisman, 1989). In mammals, they collectively account for about 2% of all cells in the parenchymal region or about 40% of all pulmonary macrophages (Sebringand and Lehret, 1992). PIMs are as metabolically active as other populations of macrophages and they have a full range of

secretory and immune capabilities, apparently different from alveolar macrophages and Kupffer cells (Longworth, 1997).

2.2.2.3 Interstitial macrophages (IMs)

They constitute the majority of avian pulmonary macrophages and are predominantly located in the loose connective tissue immediately beneath the epithelium lining the floor of atria at the entrance to the infundibulum and gas exchange tissue proper (Klika *et al.*, 1996). In mammals IMs are found in the interstitium of the interalveolar septa (Crowell, Heapy, Valdez *et al.*, 1992). In rats, IMs are smaller and morphologically more heterogeneous than AMs that have a higher phagocytic capacity, faster attachment, and ingestion processes (Fathi, Johansson, Lundborg et *al.*, 2001).

2.2.3 CHARACTERISTICS OF MACROPHAGES

2.2.3.1 Morphological Characteristics

Wahneit, Hill and Brody (1984) reported that in rats, most resident macrophages (73%) are flat, and exhibit ruffled surfaces. Macrophage surface features correlate with the phagocytic capacity of the cells: macrophages with ruffled membrane phagocytise a significantly greater number of particles than do those with smooth unruffled one. Actively phagocytising macrophages have a cytoplasm differentiated into a continuous ectoplasmic zone with numerous fingerlike processes and pseudopodia. The most outstanding feature is the rich lysosomal apparatus of varying structure depending on the functional state (Klika, Rychterova, Tesik *et al.*, 1975). FARMs observed on the surface of the atria and infundibulae of the rock dove by Maina and Cowley (1998) had large s-

shaped heterochromatic nuclei, long, thin filopodia, rough endoplasmic reticulum, interspersed vesicular bodies, and small diffuse mitochondria.

2.2.3.2 Functional Characteristics

2.2.3.2.1 Secretion

Mononuclear phagocytes constitutively secrete lysozymes and can be induced to secrete several neutral proteases (Silverstein, 1982). The kind and amount of product secreted depend upon the state of differentiation or activation of the macrophages. In early stages of challenge by invading microorganisms or tissue injury, macrophages defend local and systemic homeostasis by initiating a complex series of cellular, biochemical, and behavioral events. These pathophysiological adjustments are mediated by an extensive variety of communication molecules, including cytokines, cytokines inhibitors, endocrine hormones, cicosanoids, neurotransmitters, and reactive oxygen intermediates (Silverstein, 1982). Compared with the mammalian ones, the cytokines produced by avian macrophages have not been well characterized, but a variety of chemokines, pro-inflammatory, and colony-stimulating factors have been described (e.g. Klasing, 1998).

2.2.3.2.2 Homeostatic Function

The roles of mononuclear phagocytes in bone remodeling and resorption, in lipid accumulation in the atherosclerotic plaque and in wound healing demonstrate that cells of this lineage play essential roles in many other homeostatic processes unrelated to immune defense. One of the major daily function of the mononuclear phagocytes is the removal of

senescent red blood cells (Silverstein, 1982). Another important function is that of regulating the turnover of surfactant in the lung (Nichols, 1982).

2.2.3.2.3.Phagocytosis

Phagocytosis is the most important defense mechanism of macrophages in all animal phyla. Notwithstanding the increasingly sophisticated humoral and cellular immune defense components that have evolved in vertebrates which culminated in the complex immune systems of birds and mammals, phagocytosis still remains the principal effector mechanism for the ultimate disposal of invading, foreign effete, or otherwise unwanted cells or particles (Carel, 1986). In a study on pulmonary macrophages in birds, Klika *et al.* (1996) reported that the transport of small airborne particles occurs via the squamous atrial epithelium to the underlying macrophages. Twenty minutes after the end of aerosol inhalation, 32–53% of the particles retained in the conducting airways are already phagocytized by macrophages (Gehr, Schurch, Geiser *et al.*, 1990). Avian respiratory macrophages were observed to phagocytise *Aspergillus fumigatus* spores and disseminate them into the blood stream (Toth and Siegel, 1986). Particle displacement by surfactant might be the first step in the defense mechanism promoting particle-macrophage contact. This will probably facilitate particle phagocytosis as well as preparation and presentation of deposited antigens to other cells of the defense system (Gehr *et al.*, 1990). It is the phagocytic and lytic potential of pulmonary macrophages that constitute most of the known bactericidal properties of the lungs (Brain, Gehr and Robert, 1984). The broad range of effector functions carried out by avian monocytes and macrophages (e.g. chemotaxis, phagocytosis, bacterial and tumor cell killing, antigen presentation,

monokine, and reactive oxygen and nitrogen intermediates production signify the importance of these cells in resistance to diseases (Qureshi, Marsh, Dietert *et al.*, 1994).

3.0 MATERIALS AND METHODS

Twenty-two mature healthy chickens (*Gallus gallus* variant *domesticus*), twenty-four domestic ducks (*Cairina moschata*) and twenty rats (*Rattus rattus*) were obtained through Central Animal Services of the University of the Witwatersrand. Considering the fact that the environment in which an animals lives is important regarding the numerical density of pulmonary macrophages, the animals were all acquired from the same supplier who retained the same hygienic husbandry level. The animals were killed by an intravenous overdose of Euthanase® (200 $mg.cm^{1}$ pentobarbitone sodium - Centaur Labs) into the brachial vein (chickens and ducks) or peritoneal cavity (rats). The animals were immediately weighed.

3.1 LAVAGE OF THE RESPIRATORY SYSTEM

The lavage was done with the animal in a supine position at room temperature. The trachea was cannulated and the lungs instilled with a known volume of prewarmed (40 °C for birds and 37 °C for rats) phosphate buffer saline (PBS), pH 7.4 (Appendix 2) at a pressure head of 30 cm H_2O (3 kPa) until the instilled fluid stopped flowing into the lung-air sac system. During instillation, in birds, the caelomic cavity was gently massaged to expel trapped air. This was done to ensure maximum penetration of the fluid into the various compartments of the lungs and air sacs with their extensive diverticulae. The instilled fluid was left in the system for 2 minutes after which it was slowly aspirated with a 50 ml syringe and the volume of the recovered fluid determined. The short time over which the lavage was performed allowed collection of free cells on the surface of

the lungs and air sacs eliminating possibility of flux of cells from the subepithelial space and the vascular system.

3.2 CELL COUNTS

Ten ml of the collected fluid was spun for 10 minutes at 350g using a Hermle Z 200A centrifuge. The supernatant was removed and the pellet resuspended in 1 ml of PBS. The aliquot was then stained with 1% trypan blue (Appendix 3) in a 1:2 dilution. The cell suspension was subsequently loaded onto the hemocytometer. A coverslip was placed on the grid area of the hemocytometer. Using a plastic pipette, a drop of the cell suspension was placed at the edge from where it moved under the coverslip by capillary action, filling the 5 chambers of the hemocytometer. The cells in the four 1 mm corner squares were counted (Fig. 3.1), the total number of collected macrophages calculated (Appendix 4) and the total count was normalized with body mass to allow comparison between chickens, ducks and rats, animals that differed in body size.

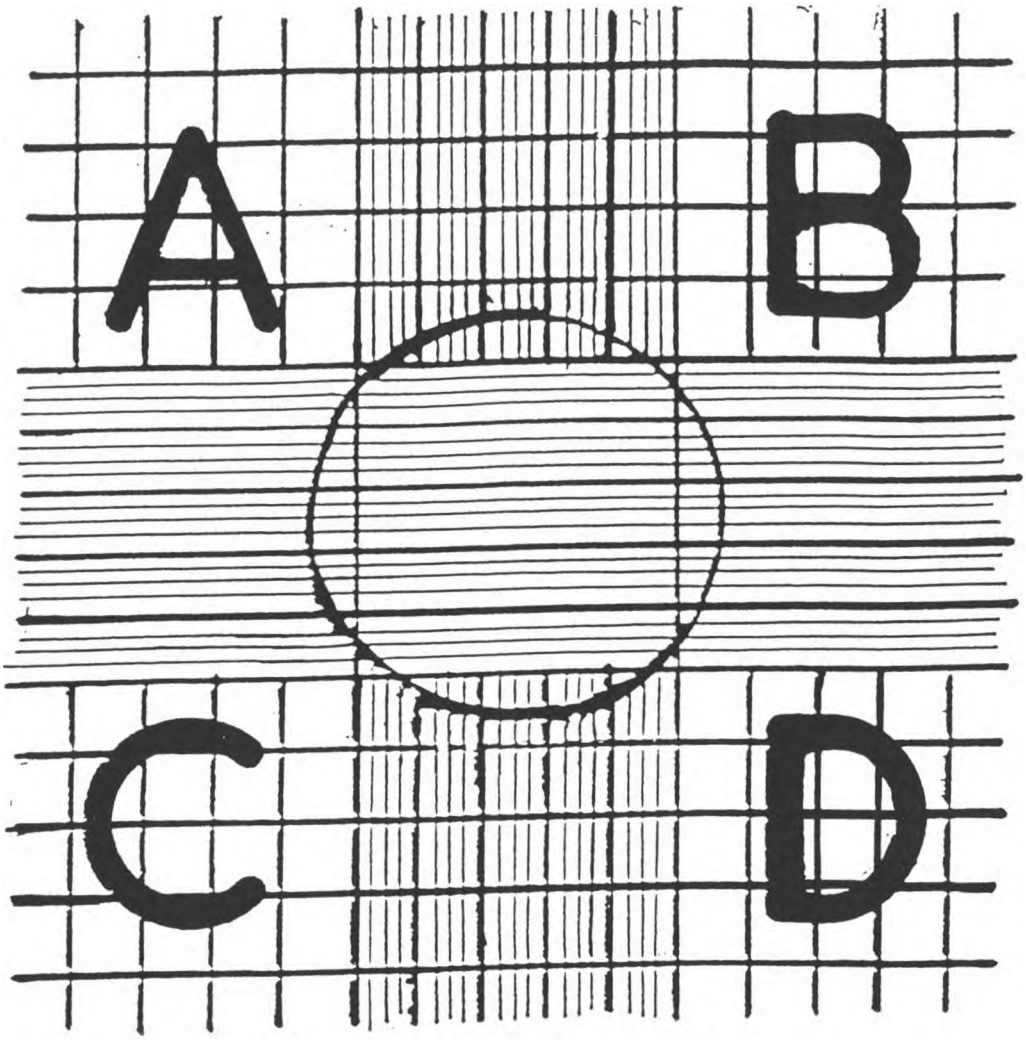


Fig.3.1: Illustration of the hemocytometer showing the four corner squares (A, B, C, D) in which the cells were counted.

3.3 EVALUATION OF THE LONGEVITY OF THE MACROPHAGES

This experiment was performed to allow determination of the cells (from the chicken, duck and rat) that have the greatest longevity (*in vitro*) when exposed to fairly similar environmental conditions. However, to minimize the effects of temperature on cell longevity, in birds, the cells (in PBS) were kept at 40 °C and at 37 °C in rats (normal

body temperatures of the animals) in thermostatically controlled incubator. Every 30 minutes over a period of three hours, 10ml of the fluid was taken from the incubator and centrifuged at 350g for 10 minutes. The pellet was resuspended in PBS and cells in the cell suspension stained with a 1:3 dilution of 0.04% neutral red and 0.5% trypan blue (Appendix 5) as follows: to 0.5 ml of the cell suspension, 0.5 ml of 0.04 % neutral red was added. The mixture was incubated at 40 °C (birds) or 37 °C (rats) for 10 minutes. After incubation, 0.5 ml of 0.5% trypan blue was added, mixed well, and the new solution allowed to stand for 3 minutes. Live cells stain red while dead cells stain blue (Gretchen, 1979). Using a hemocytometer, the live cells were counted.

3.4 FLUX AND DYNAMICS OF PULMONARY MACROPHAGES

With the animals at room temperature (25 °C), pulmonary lavage was repeated 5 times at intervals of two and a half minutes. The collected fluid was kept in an incubator (at 40 °C in the case of birds and 37 °C in the case of rats). At the end of the lavages, the collected fluid were centrifuged at 350g for 10 minutes, the supernatant was aspirated, the pellet resuspended in PBS and the cells stained with 1% trypan blue in a 1:2 dilution. Using a hemocytometer, cells from each collected sample were counted.

3.5 LOCATION OF FREE PULMONARY MACROPHAGES IN CHICKENS AND DUCKS

With the birds in a supine position, incisions were made through the pectoral muscles and the sternum to expose the lungs and the air sacs. The trachea and lungs were removed intact. This task was carried out with extreme precaution so as to preserve as many air

sacs as possible. To find out whether FARMs occur in the air sacs, prewarmed (40 °C) PBS was injected in the remaining intact air sacs (mainly cranial thoracic and abdominal air sacs). After two minutes the fluid was aspirated, centrifuged at 350g and the cells stained with 1% trypan blue. Free macrophages were checked in the recovered fluid under a microscope. To investigate whether FARMs are found in the lungs, the trachea was canulated and the lungs instilled with prewarmed (40 °C) PBS. An attempt was made to block the ostia using forceps to enhance flow of PBS into the bronchial system of the lung. The instilled fluid was collected in a beaker, centrifuged at 350g, and the cells stained with 1% trypan blue. Free macrophages were checked in the recovered fluid under a microscope.

3.6 EVALUATION OF LYSOSOMAL BODIES IN THE MACROPHAGES

This was done by fluorescence microscopy and transmission electron microscopy (TEM) of free macrophages in the three groups of animals.

3.6.1 Fluorescence microscopy

Fluorescence staining of lysosomes was done by using a lysotracker probe (Lysotracker Red DND- 99®, L - 7528: Molecular Probes). This is a fluorescent acidotropic probe for labelling and tracking acidic cell organelles. It has high selectivity for acidic organelles and effective labelling of live cells at nanomolar concentrations. This probe can also be used to investigate the biosynthesis and pathogenesis of lysosomes (Haugland, 1996). The lavage fluid was centrifuged for 5 minutes at 60g (to obtain a cell pellet) and the supernatant was aspirated. The cells were then resuspended gently in prewarmed (40 °C

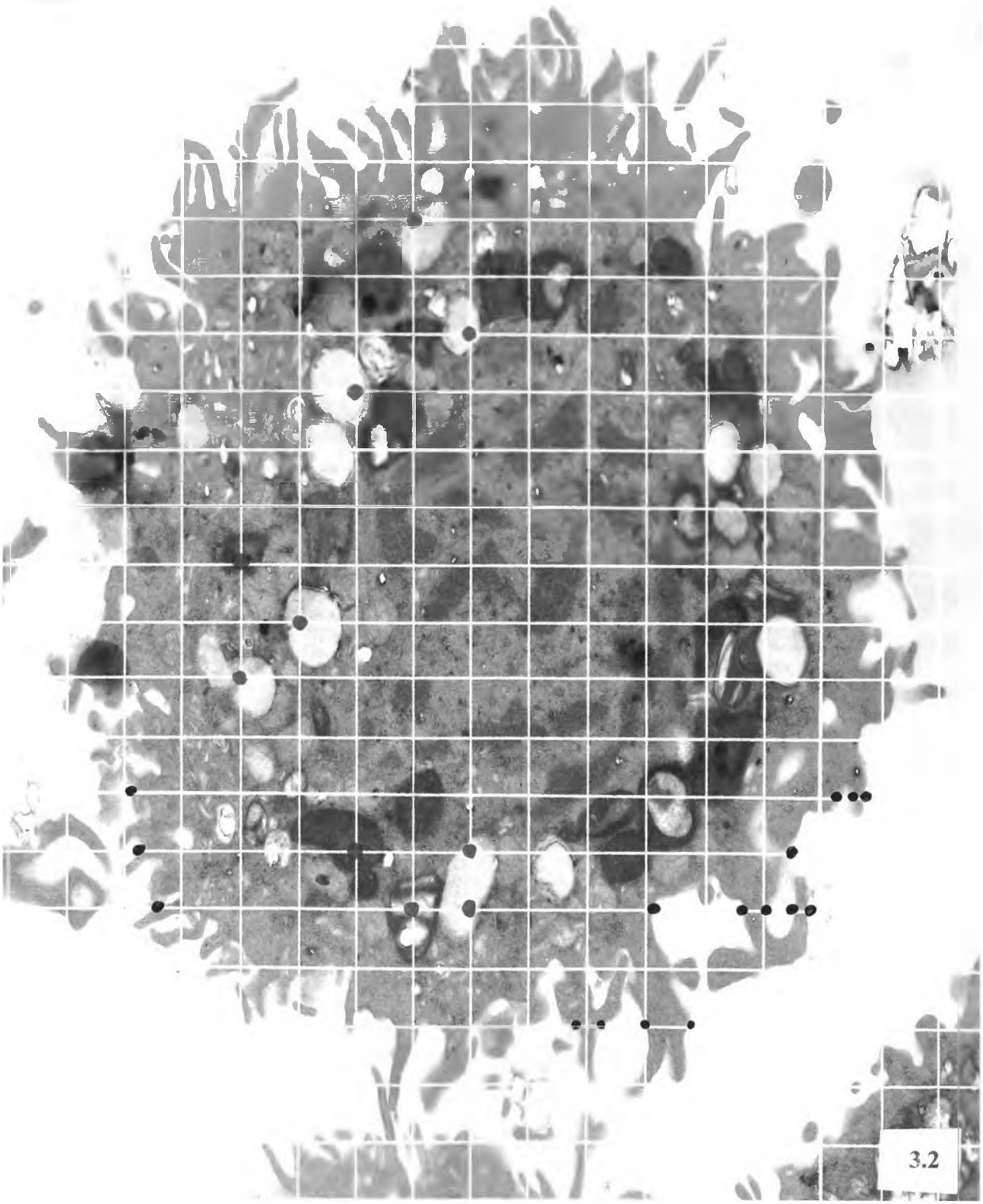
for chickens and ducks, 37 °C for the rats) probe (Lysotracker Red DND-99®) containing medium (Appendix 6) and incubated for 1 hour at the appropriate normal body temperature (40 °C for chickens and ducks, 37 °C for the rats). Following incubation, they were re-pelleted by centrifugation at 60g for 5 minutes and the excess dye washed off with PBS. The pellet was resuspended in fresh and prewarmed medium, fixed with 3 % buffered formalin and mounted on slides with wells (Appendix 7). The cells were then viewed using a confocal laser-scanning microscope (Zeiss LSM 410 Invert). Red fluorochrome Lysotracker Red DND-99® was excited at λ = 568 nm and was detected at $\lambda > 585$ nm.

3.6.2 Processing of free macrophages for transmission electron microscopy (TEM)

The lungs were infused with 2.5 % glutaraldehyde. After 2 minutes, the fluid was aspirated and centrifuged at 350g for 10 minutes. The pellet was fixed with fresh 2.5% glutaraldehyde in PBS (pH 7.4) for 3 hours and processed through standard laboratory techniques (Appendix 8). Thereafter, the specimens were embedded in epoxy resin (Epon). The blocks were sectioned on a Reichert-Jung Ultracut E ultramicrotome. To select the area of interest on the block, semithin sections were first cut at 1μm and stained with toluidine blue. Subsequently, ultrathin sections were cut at 100 nm from the selected area of the block, mounted on 200 wire mesh copper grids, and stained with uranyl acetate and lead citrate (Appendix 9). The stained sections were then viewed with a Joel JEM-100 S transmission electron microscope and pictures taken. The negatives were printed on a "8x10" photographic paper with a superimposed quadratic lattice grid. The volume density of vesicular bodies of the macrophages was estimated by point counting

(Appendix 10) and the surface density of the filopodia by intersection counting (Fig. 3.2) (Appendix 11).

Fig. 3.2: Electron micrograph of a rat alveolar macrophage with a superimposed quadratic lattice grid. Some of the intersections counted to estimate surface densities of filopodia are shown with black dots. Some of the points used to determine the volume density of the vesicular bodies are shown with red dots. Mag. x 16538



3.2

3.7 FIXATION OF THE LUNGS FOR MICROSCOPY

The lungs were fixed *in situ* for 3 hours by instillation of 2.5% glutaraldehyde buffered in phosphate (Appendix 12). Subsequently, they were removed intact and kept in fresh 2.5% glutaraldehyde. Pieces were sampled and processed for light microscopy and transmission electron microscopy.

3.8 PROCESSING OF THE LUNG TISSUE FOR TRANSMISSION ELECTRON MICROSCOPY (TEM)

Small pieces (1 mm^3) of the lung were taken from the medioventral and mediodorsal secondary bronchi and fixed overnight in 2.5 % glutaraldehyde. The fixed tissues were then processed through standard laboratory techniques for TEM (Appendix 8). Thereafter, the specimens were embedded in epoxy resin. The blocks were sectioned on a Reichert- Jung Ultracut ultramicrotome. To select the area of interest on the block, semi thin sections were first cut at 1μm and stained with toluidine blue. Thereafter, thinner sections were cut at 100 nm from the selected area of the block, mounted on 200 wire mesh copper grids and stained with uranyl acetate and lead citrate. The stained sections were then viewed with a JEOL JEM-100 S transmission electron microscope.

3.9 PROCESSING OF LUNG TISSUES FOR SCANNING ELECTRON MICROSCOPY (SEM)

Small pieces (1 cm^3) of the lung were taken along the area of the medioventral and mediodorsal secondary bronchi. The tissues were fixed in 2.5% glutaraldehyde, dehydrated in alcohol, critical point dried in liquid carbon dioxide and mounted on aluminium metal stubs. The mounted specimens were sputter-coated with gold-palladium

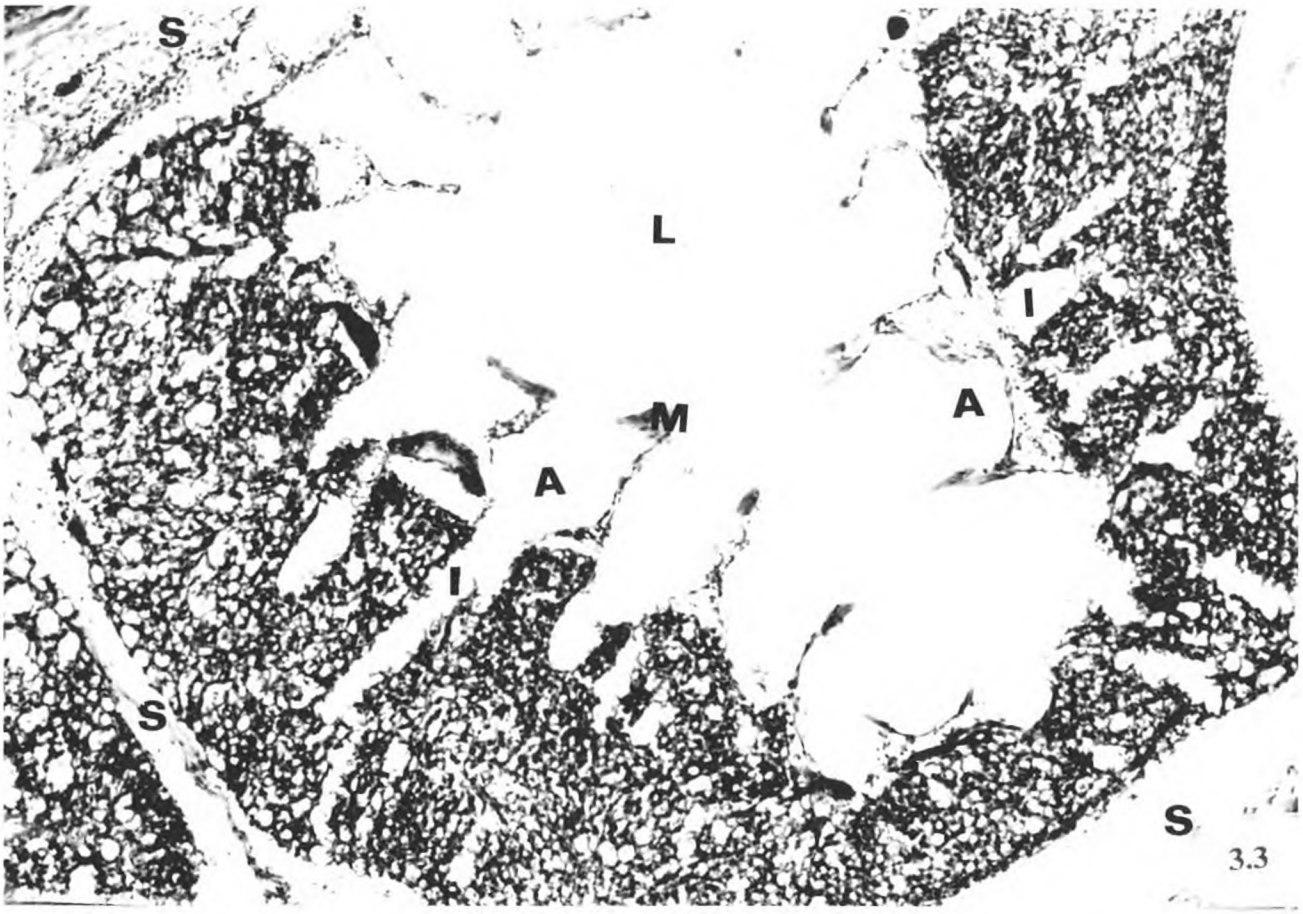
complex before viewing on a JEOL 840 scanning electron microscope at an accelerating voltage of 15 kV.

3.10 PROCESSING OF LUNG TISSUES FOR LIGHT MICROSCOPY

The lung was sampled by sectioning along the costal sulci. The sampled parts were then fixed in 2.5 % glutaraldehyde and processed through routine histological technique for light microscopy in a Shandon Citadel 1000 automatic processor (Appendix 13). The specimens were then embedded in paraffin wax. Sections were made at 10 μm with a microtome and stained with haematoxylin and eosin (Appendix 13). Using an image analyzer (Videoplan Image Processing System) the surface density of the phagocytic surface of the lung (atrial muscles, atria floor and infundibulae, Fig. 3.3) was determined by the intersection counting (Fig. 3.4) method (Appendix 11). The surface area was calculated from the volumes of the atria and infundibulae which were calculated from the volume densities of the lungs of the chicken and the duck (Maina *et al.*, 1989) and the volumes of the lungs which were directly determined.

Fig.3.3: Photomicrograph of a histological section of the lung of the chicken showing the parabronchial lumen (L), atrial muscle (M), atrium (A), infundibulum (I) and the interparabronchial septum (S). Mag. x 208

Fig. 3.4: Photomicrograph of a histological section of the lung of the chicken (*Gallus gallus* variant *domesticus*) with a superimposed quadratic lattice grid for intersection counting to estimate the surface density of the phagocytic epithelium of the atrial muscles, atria and infundibulae (shown in Fig. 3.3). The grid was generated from the image analyzer. Mag. x 269



3.3


3.4

3.11 DETERMINATION OF THE LUNG VOLUME IN THE CHICKEN AND THE DUCK

This was done by using the weight displacement method of Scherle (1970). A large beaker filled to about two-thirds with PBS was placed on a balance, the first reading was tarred (Fig. 3.5a) and the lung put in the beaker . The specific density of the organ being lighter than the PBS, it was kept totally submerged by means of a wire spiral. The weight of the fluid displaced (Vo) by the lung (Fig.3.5b) was then recorded after making sure that the organ did not touch the walls of the beaker.

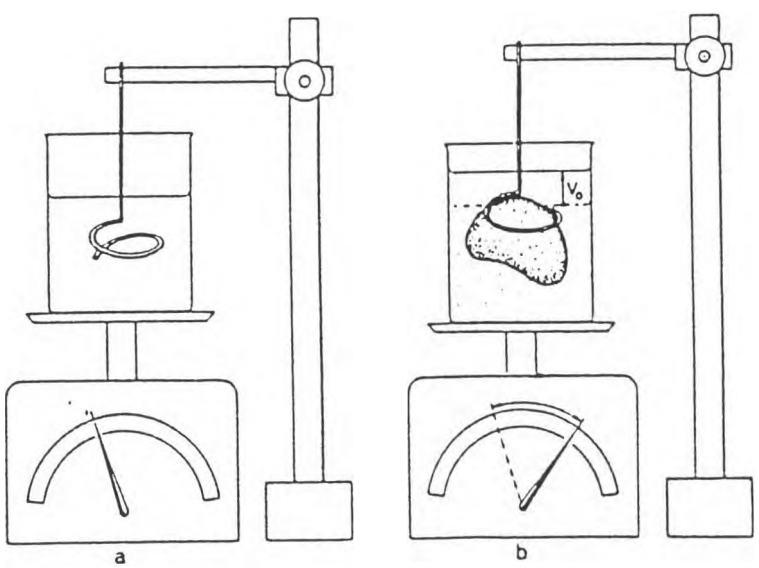


Fig.3.5: Lung volume determination by the method of Scherle (1970).

3.12 STATISTICAL ANALYSIS OF THE DATA

The student t-test was used to compare the estimated values of the mean values for chickens, ducks and rats in the various experiments. The level of significance was set at 5% (0.05).

4.0 RESULTS

4.1 LAVAGE OF THE LUNGS

The recovery rate of the fluid instilled in the chicken was 90.8 %, in the duck 88.5 % and in the rat 83.1 %. (Tables 4.a, 4.b and 4.c).

Table 4.a Volume of the PBS instilled in the lung-air sac systems of chickens, volume recovered and recovery rate

Animal	Body mass (g)	Instilled fluid (ml)	Recovered fluid (ml)	recovery (%)
Chicken 1	1541.7	349	320	91.6
Chicken 2	3282.2	458	452	98.6
Chicken 3	2988	416	376	90.3
Chicken 4	2605.2	555	465	83.7
Chicken 5	2010	501	450	89.8
Mean	2485.4	455.8	412.6	90.8
SD	635	70.5	55.8	4.7

Table 4.b Volume of PBS instilled in the lung-air sac system of ducks, volume recovered and recovery rate

Animal	Body mass (g)	Instilled fluid (ml)	Recovered fluid (ml)	Recovery (%)
Duck 1	2026	600	530	88
Duck 2	2676	880	665	75
Duck 3	2499	714	650	91
Duck 4	2723	823	765	92
Duck 5	2479	774	732	94
Duck 6	3368	939	855	91
Mean	2628.5	788.3	699.5	88.5
SD	438.2	121.2	111.2	6.8

Table 4.c Volume of PBS instilled in the lungs of rats, volume recovered and recovery rate

Animal	Body mass (g)	Instilled fluid (ml)	Recovered fluid (ml)	Recovery (%)
Rat 1	704.1	20	18.5	92.5
Rat 2	306.47	20	13	65
Rat 3	338.22	14	12.5	89.2
Rat 4	311.14	16	12	75
Rat 5	387.7	17	16	94
Mean	409.5	17.4	14.4	83.14
SD	150	2.3	2.4	11.2

4.2 CELL COUNT

The number of free macrophages per gram body mass in rat (2933 ± 579) was significantly higher ($P< 0.05$) than in the duck (1430 ± 211) and also significantly higher ($P< 0.05$) than in the chicken (1413 ± 345) (Tables 4.d, 4.e, 4.f). However, there was no significant difference ($P> 0.05$) in the number of FARMs between the chicken (1413 ± 345) and the duck (1430 ± 211) (Tables 4.d and 4.e) (Fig. 4.1).

Table 4.d Number of cells in the lavaged fluid from the lung-air sac systems of chickens

Animal	Body mass-BM (g)	Cells/ml	Total cell number	Cells/g BM
Chicken 1	1541.7	8400	2688000	1744
Chicken 2	3282.2	11600	5243200	1597
Chicken 3	2988	8000	3008000	1007
Chicken 4	2605.2	9200	2604000	1642
Chicken 5	2010	4800	2160000	1074
Mean	2485.4	8400	3140640	1413
SD	635	2190	1085621	345

Table 4.e Number of cells in the lavaged fluid from the lung-air sac systems of ducks

Animal	Body mass (g)	Cells/ml	Total cell number	Cells/g BM
Duck 1	2026	5600	2968000	1465
Duck 2	2676	4800	3192000	1193
Duck 3	2499	5200	3380000	1363
Duck 4	2723	6000	4590000	1686
Duck 5	2479	5600	409900	1654
Duck 6	3368	4800	4104000	1219
Mean	2628.5	5333	3107316.7	1430
SD	438.2	484.4	1453872.1	211

Table 4.f Number of cells in the lavaged fluid from the lungs of rats

Animal	Body mass (g)	Cells/ml	Total cell number	Cells/g BM
Rat 1	704.1	78000	1443000	2049
Rat 2	306.47	75000	975000	3181
Rat 3	338.22	72000	900000	2660
Rat 4	311.14	90000	1080000	3471
Rat 5	387.7	80000	1280000	3301
Mean	409.5	79000	1135600	2933
SD	150	6131	199868.5	579

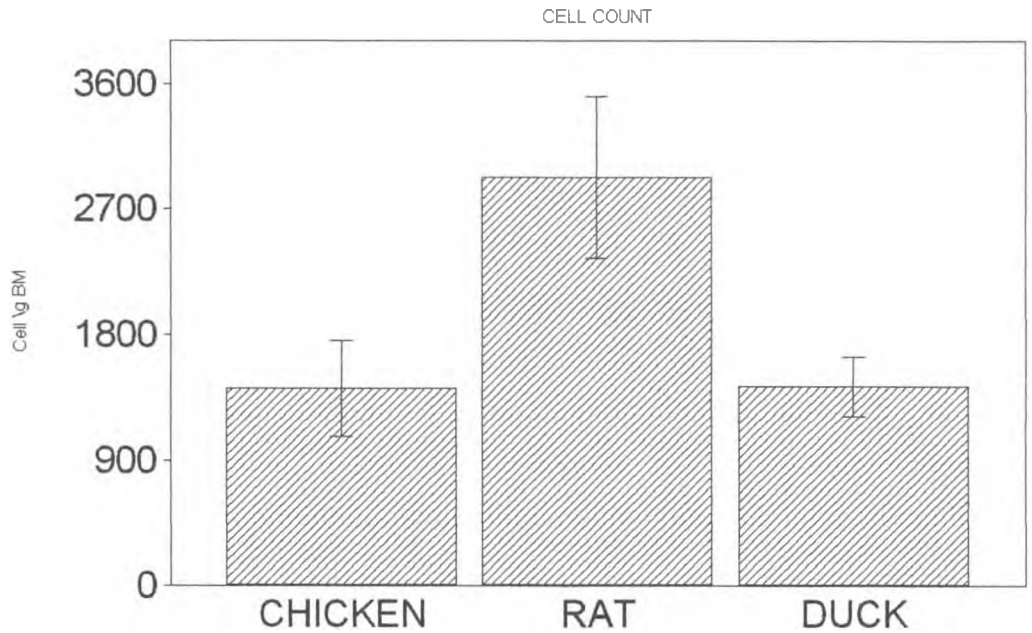


Fig.4.1. Histogram showing comparison of the number of surface pulmonary macrophages per gram body mass in chicken, rat and duck. Bars show standard deviations.

4.3 CELL LONGEVITY

The most robust FRMs (free respiratory macrophages) to changes in the experimental environmental conditions such as oxygen tension and availability of nutrients were those from the chicken, followed by those of the rat with the duck's being least resistant to changes (Fig. 4.2). The death rate of chickens and rats FRMs was relatively slower (slope 0.20 for chicken, 0.52 for rat) compared with those of the duck (slope 0.65).

Table 4.g Number of cells per gram body mass counted after every thirty minutes over 3 hours in the chicken

Time (min) Cells/g BM	0	30	60	90	120	150	Body mass (g)
Chicken 1	1847	1239	1074	922	826	578	3282.2
Chicken 2	1510	906	679	604	528	503	2988.1
Chicken 3	601	481	420	240	210	180	2384
Chicken 4	1386	970	900	762	692	415	2078.4
Chicken 5	930	857	572	500	429	358	1928.6
Mean	1255	891	729	606	537	406	2532.2
SD	440	244	232	232	213	136	522

Table 4.h Number of cells per gram body mass counted after every thirty minutes over 3 hours in the duck

Time (min) Cells/g BM	0	30	60	90	120	150	Body mass (g)
Duck 1	5735	3924	3622	3018	1962	1811	3836
Duck 2	6325	5091	4474	2777	2468	2005	3538.9
Duck 3	2073	1629	1037	888	740	444	2026
Duck 4	1789	1192	1043	745	596	447	2676
Duck 5	2029	1717	1561	1092	936	642	2499
Mean	3590	2711	2347	1704	1340	1070	2915
SD	2003	1523	1427	984	740	772	672

Table 4.i Number of cells per gram body mass counted after every thirty minutes over 3 hours in the rat

Time(min) Cells/g BM	0	30	60	90	120	150	Body mass (g)
Rat 1	2049	1497	1182	788	630	394	704.1
Rat 2	3691	3054	2672	1527	1400	763	306.4
Rat 3	2661	1884	1219	1108	776	506	338.2
Rat 4	2930	2051	2100	1709	976	733	552.8
Rat 5	1935	1393	1209	1128	564	403	483.7
Mean	2653	1976	1676	1252	869	560	476.9
SD	638	591	607	327	300	159	154

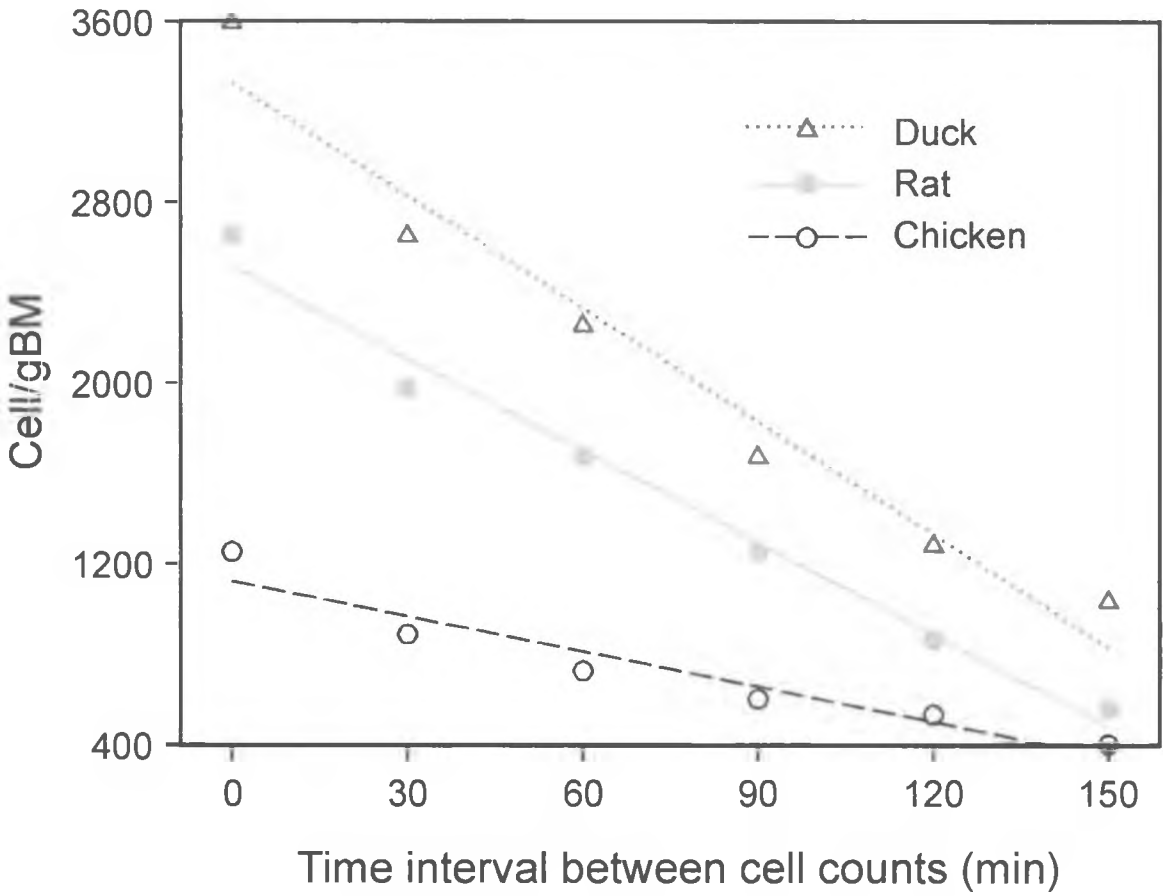


Fig.4.2. Graph showing progressive death of macrophages per gram body mass at different time intervals in the duck, the rat and the chicken.

4.4 CELL DYNAMICS

While in the rat the number of FRMs decreased steadily with progressive lavages, in the chicken, a significant flux of the cells ($P< 0.05$) onto the respiratory surface was observed between the first (879 ± 81) and the second lavages (1222 ±70). The same observation was made in the duck ($P< 0.05$) between the first (1277 ± 340) and the second lavages (1842 ± 322) (Tables 4.j, 4.k, 4.l and Fig. 4.3).

Table 4.j Number of cells per gram body mass collected after every 2.5 minutes over a period of 10 minutes in chickens

Time (min)/ Cells/g BM	0	2.5	5	7.5	10	Body mass (g)
Chicken 1	931	1196	732	1038	446	22082
Chicken 2	845	1112	572	538	572	2067
Chicken 3	831	1218	930	471	369	2141.8
Chicken 4	781	1261	757	900	584	2219
Chicken 5	1009	1324	636	529	315	1473.8
Mean	879	1222	725	695	457	2021.9
SD	81	70	122	229	107	279.3

Table 4.k Number of cells per gram body mass collected after every 2.5 minutes over a period of 10 minutes in ducks

Time (min)/ Cell/g BM	0	2.5	5	7.5	10	Body mass (g)
Duck1	1686	2248	1359	740.3	624	2723
Duck 2	800	1378	800	667	384	1829
Duck 3	1653	2161	1461	1210	877	2479
Duck 4	1027	1680	870	750	415	2469
Duck 5	1219	1743	1268	1033	584	3368
Mean	1277	1842	1152	880	577	2573.6
SD	340	322	267	231.3	176	554

Table 4.1 Number of cells per gram body mass collected after every 2.5 minutes over a period of 10 minutes in rats

Time (min)/ Cell/g BM	0	2.5	5	7.5	10	Body mass (g)
Rat 1	1985	1876	1782	1657	1501	415.6
Rat 2	3301	2527	1096	595	570	387.7
Rat3	999	698	629	512	419	345.5
Rat 4	956	723	654	589	539	342.4
Rat 5	1335	1077	839	646	388	634.8
Mean	1715	1380	1000	800	683	425.2
SD	874	714	452	431	415	108.2

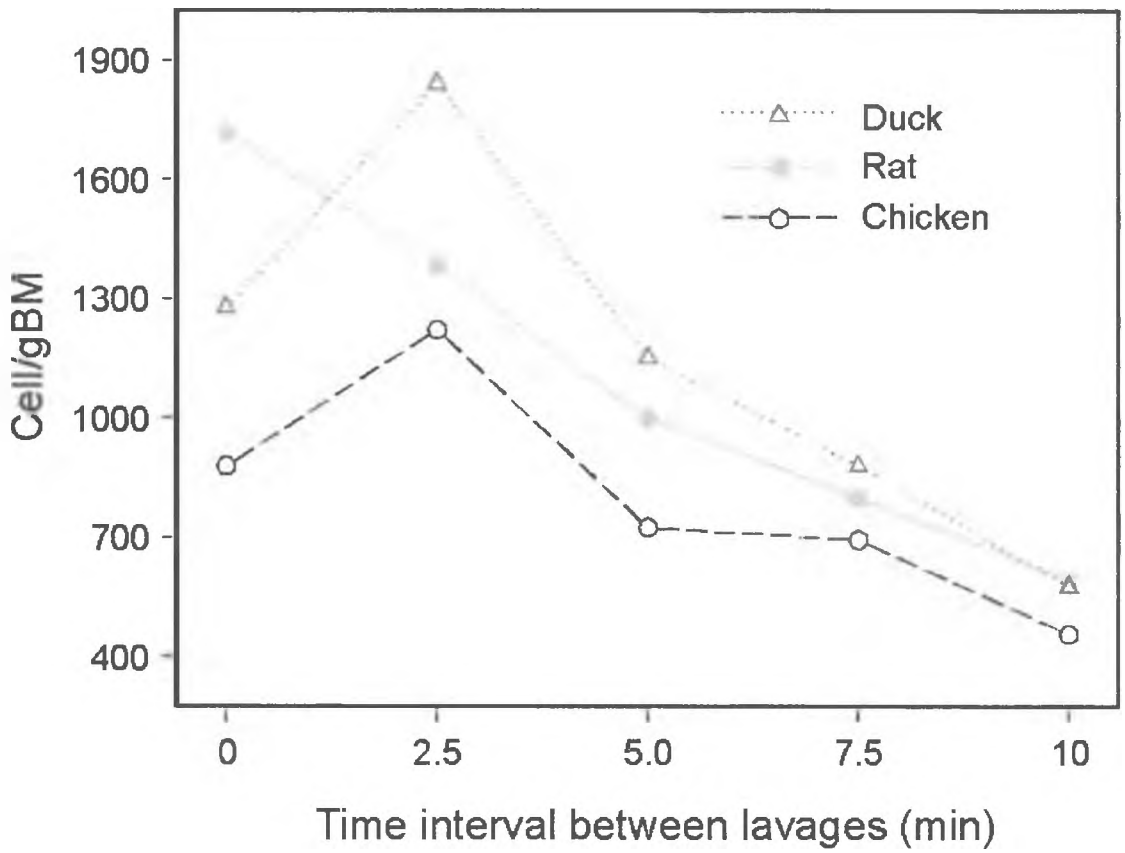


Fig.4.3. Graph showing the number of cells per gram body mass collected after every 2.5 minutes over a period of ten minutes in the duck, rat and chicken.

4.5 SURFACE DENSITY OF FILOPODIA OF FREE RESPIRATORY MACROPHAGES (FRMs)

The surface density of the filopodia of the macrophages of the rat (7104 $cm^2.cm^{-3} \pm 2952$) was significantly higher ($P< 0.05$) compared to those of the chicken (5074 $cm^2.cm^{-3} \pm 2055$). The value for the rat macrophages was also significantly higher ($P< 0.05$) than that for the duck (5045 $cm^2.cm^{-3} \pm 1815$).The value for the chicken (5074 $cm^2.cm^{-3} \pm 2055$) and the duck (5045 $cm^2.cm^{-3} \pm 1815$) were not significantly different ($P> 0.05$). (Table 4.m; Appendix 14).

Table 4.m Surface density (S_V) of the filopodia of FRMs in chickens, ducks and rats

	Chicken ($cm^2.cm^{-3}$)	Duck ($cm^2.cm^{-3}$)	Rat ($cm^2.cm^{-3}$)
Mean	5074	5045.2	7104.3
SD	2055.5	1815.2	2952.5

4.6 VOLUME DENSITY OF VESICULAR BODIES OF FRMs

There was no significant difference ($P< 0.05$) between the volume density of vesicular bodies in rat (25.5 % ± 9.2), chicken (22.2 % ± 8.9) and duck (23.8 % ± 9.4) (Table 4.n)

Table 4.n Volume density (V_V) of vesicular bodies of FRMs in chickens, ducks and rats.

	Chicken (%)	Duck (%)	Rat (%)
Mean	22.2	23.8	25.5
SD	8.9	9.4	9.26

4.7 SURFACE DENSITY AND SURFACE AREA OF THE PHAGOCYTIC EPITHELIUM OF THE CHICKEN AND DUCK LUNG

There was no significant difference ($P< 0.05$) between the surface density of the epithelial surface of the atrial muscles, atrial floor and infundibulae of the chicken lung (8.5 $cm^2.cm^{-3} \pm 0.7$) and that of the duck (8.3 $cm^2.cm^{-3} \pm 2$). However, the surface area (Sa) of the three structural components in the duck (46298.5 $cm^2 \pm 12291$) was significantly greater ($P< 0.05$) than that in the chicken (21556.1 $cm^2 \pm 3156$). The volume per gram of body mass of the duck lungs (0.028 cm^3/g) was 2.3 times greater than that of the chicken (0.012 cm^3/g), (Table 4.o and 4.p).

Table 4.o Lung volume, surface density (Sv) and surface area (Sa) of the atrial muscles (AM), atrial floor (A) and infundibulae (I) of the chicken lung.

Animal	Body mass (g)	Volume of both lungs (cm^3)	Sv of AM, A, and I ($cm^2.cm^{-3}$)	Sa of AM, A and I (cm^2)
Chicken 1	2713	26.6	9.01	18432.7
Chicken 2	2801	37.4	8.65	24881.15
Chicken 3	2920	35.2	7.87	21305.91
Chicken 4	2605	33.4	9.6	24660.42
Chicken 5	2010	30.8	7.81	18500.5
Mean	2609.8	32.6	8.5	21556.1
SD	354.6	4.16	0.7	3156

Table 4.p Lung volume, surface density (Sv) and surface area (Sa) of the atrial muscles (AM), atrial floor (A) and infundibulae (I) of the duck lung.

Animal	Body mass (g)	Volume of both lungs (cm^3)	S_V of AM, A, and I ($cm^2.cm^{-3}$)	Sa of AM, A and I (cm^2)
Duck 1	2253.5	66.06	7.6	43533.27
Duck 2	2221.8	66	9.01	51562.96
Duck 3	1264	58.06	5.17	26027.75
Duck 4	2469	61.4	10.32	54943.6
Duck 5	2832	68	9.4	55425
Mean	2208.06	63.9	8.3	46298.5
SD	581.1	4.06	2	12291

4.8 LOCATION OF FREE MACROPHAGES IN CHICKENS AND DUCKS

Macrophages were found in lavage fluids of both the lung and the air sacs of the chickens and the ducks while exfoliated epithelial cells were only present in lavage fluid from the lung. This shows that FARMs in bird occur in both parts of the respiratory system.

4.9 FLUORESCENCE MICROSCOPY

Lysosomes were found scattered around the cytoplasm in both FARMs (Fig. 4.4 a, b and Fig. 4.5 a, b) and AMs (Fig. 4.6 a, b). Exfoliated epithelial cells from both chicken (Fig. 4.7 a, b) and ducks (Fig. 4.8 a, b) had a concentration of lysosomes at the apical end as well as within the cytoplasm. This shows a potentially high lytic capacity of the respiratory epithelium.

4.10 TRANSMISSION ELECTRON MICROSCOPY OF FRMs

FRMs from chicken showed large, blunt filopodia, an eccentrically located nucleus and numerous vesicular bodies (Fig. 4.9). The same features were found in the duck FRMs

(Fig. 4.10). In rats, AMs showed numerous thin filopodia, abundant mitochondria as well as vesicular bodies (Fig. 4.11). The presence of numerous lysosomal bodies on the apical aspect of the epithelial cells of the chicken and duck was confirmed by TEM (Fig. 4.12. and Fig. 4.13). In rats, exfoliated epithelial cells were not observed.

4.11 SCANING ELECTRON MICROSCOPY OF THE PARABRONCHI

Scanning electron micrographs of birds' parabronchi showed presence of red blood cells, some of which were in various stages of being engulfed by the epithelial lining of the atria and infundibulae (Fig. 4.14).

4.12 TRANSMISSION ELECTRON MICROSCOPY OF THE EPITHELIAL SURFACE OF THE PARABRONCHI

Electron microscopy confirmed the phagocytic nature of the epithelium lining of the atria and infundibulae of the avian lung. Particulate matter was seen being engulfed from the surface of the chicken's lung (Fig. 4.15 a, b). Interstitial (subepithelial) pulmonary macrophages were observed in both ducks (Fig. 4.16) and chickens (Fig. 4.17).

Fig. 4.4a and **Fig. 4.4b:** Fluorescence staining of FARMs in the chicken showing the distribution of the lysosomes (intensely stained areas) within the cytoplasm. Scale bar, 5 μm.

Fig. 4.5a and Fig. **4.5b**: Fluorescence staining of FARMs in the duck showing the distribution of the lysosomes (intensely stained areas) within the cytoplasm. Scale bar, 5 μm.

Fig. 4.6a and Fig 4.6b: Fluorescence staining of a single (Fig. 4.6a) and cluster (Fig. 4.6b) of alveolar macrophages in the rat. Note the distribution of the lysosomes (intensely stained areas) within the cytoplasm. Scale bar, 5 μm.

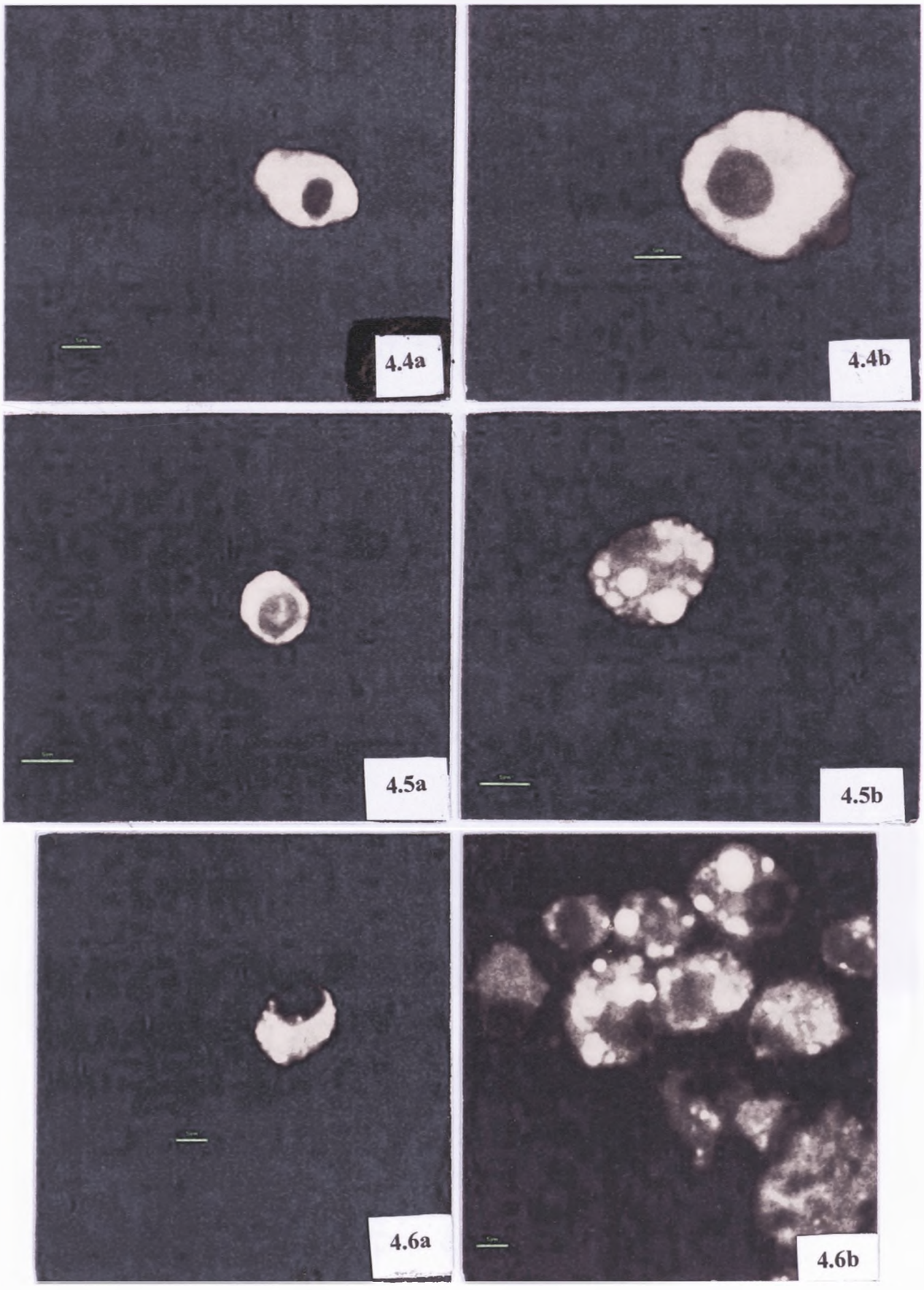

4.4a
4.4b
4.5a
4.5b
4.6a
4.6b

Fig. 4.7a and **Fig. 4.7b**: Fluorescence staining of a single (Fig. 4.7a) and clusters (Fig. 4.7 b) of exfoliated epithelial pulmonary cells in the chicken showing lysosomes (intensely stained area) concentrated at the apical end. Scale bar, 5 μm.

Fig. 4.8a and **Fig. 4.8b**: Fluorescence staining of a single (Fig.4.8a) and two (Fig. 4.8b) exfoliated pulmonary epithelial cells in the duck showing lysosomes (intensely stained area) concentrated at the apical end. Scale bar, 5 μm.

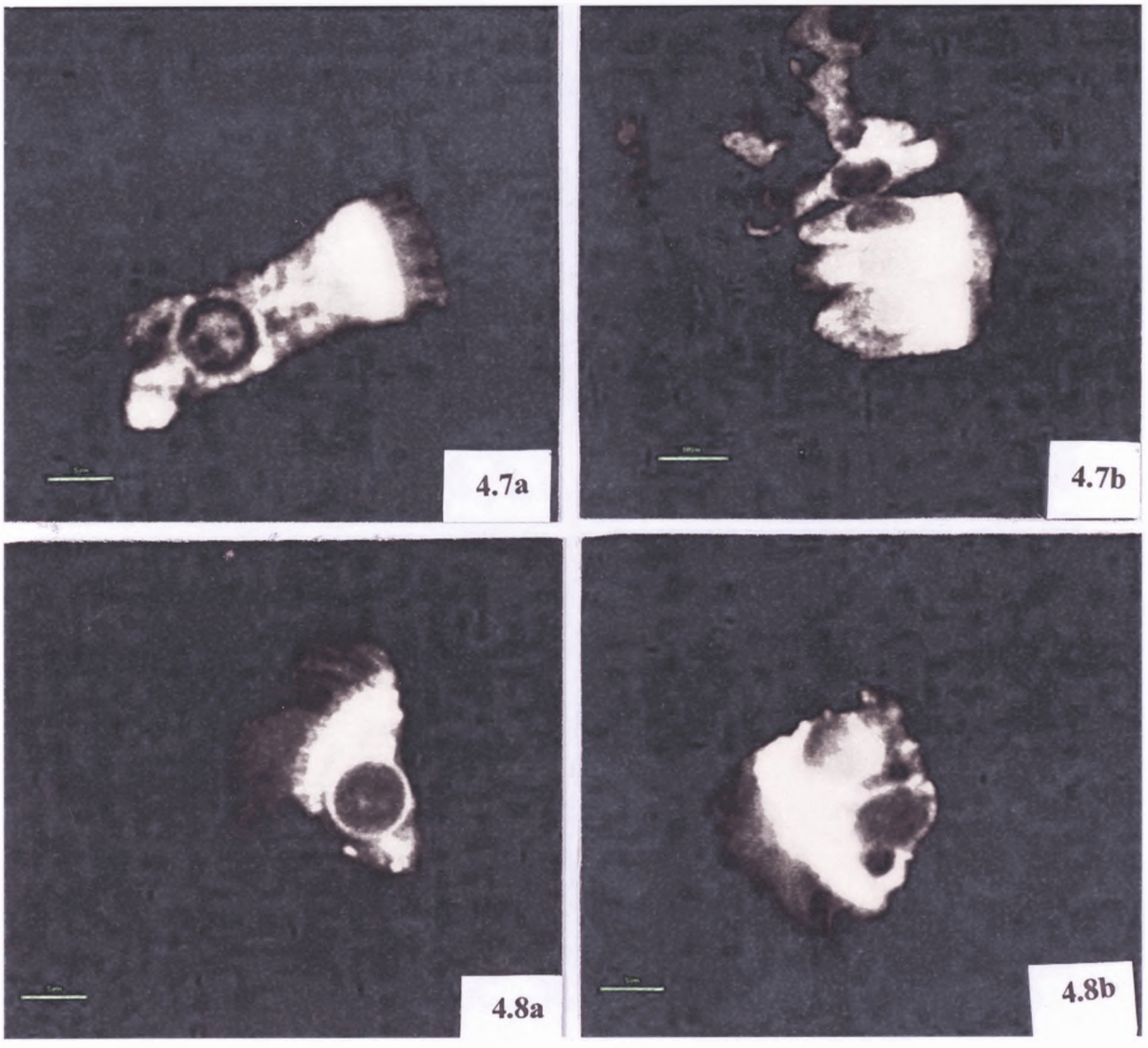
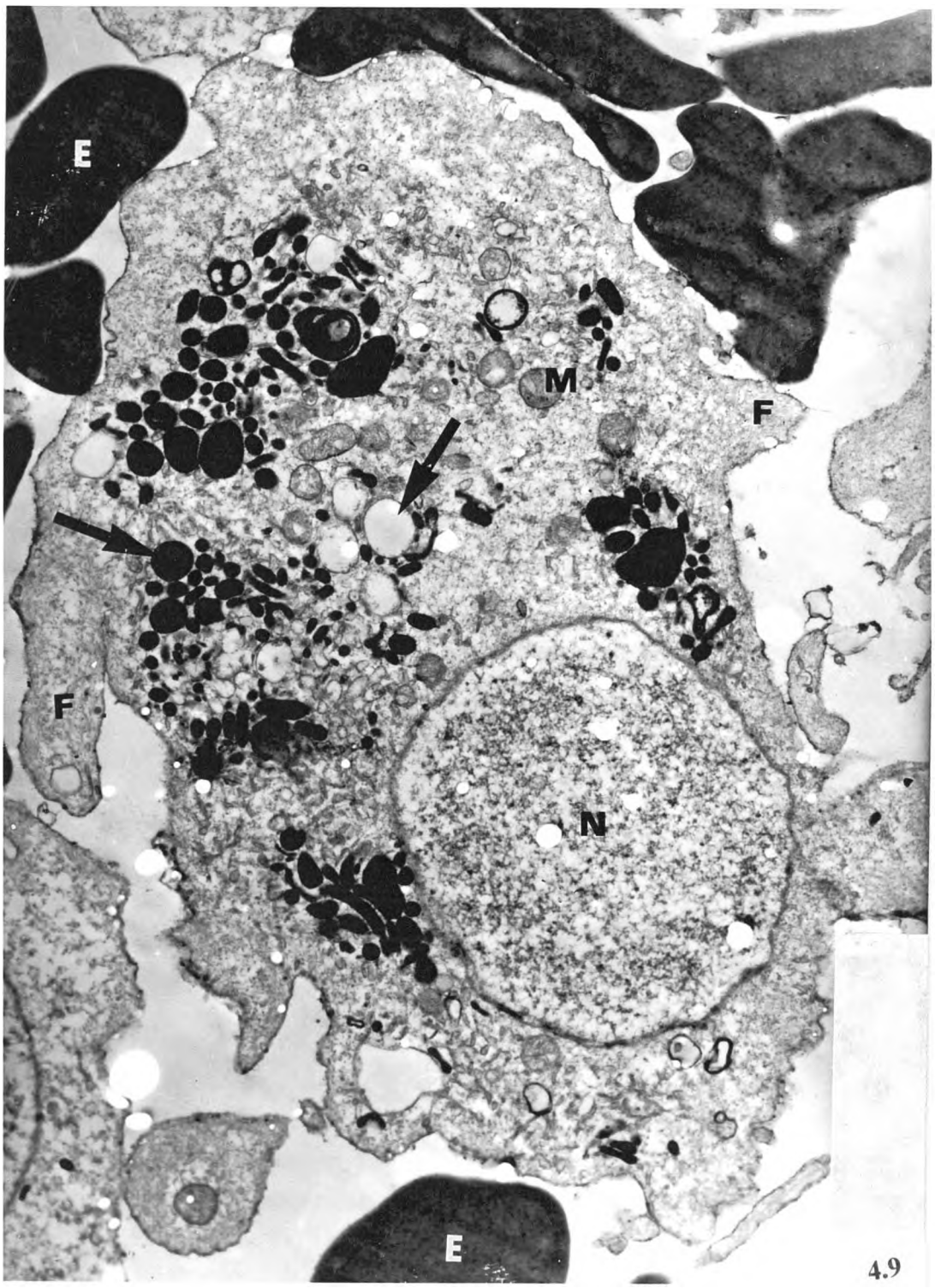
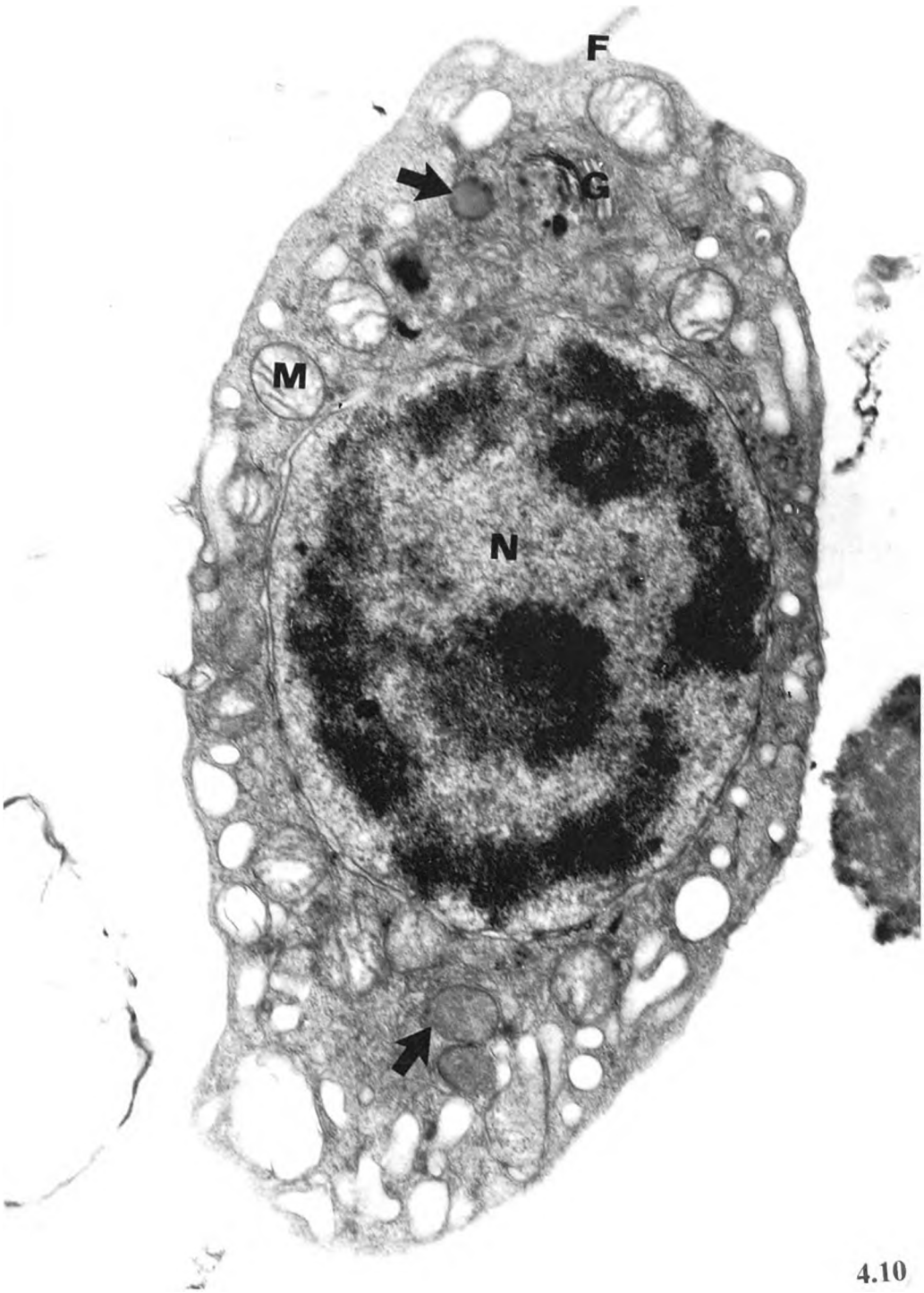

4.7a
4.7b
4.8a
4.8b

Fig. 4.9: Electron micrograph of a FARM in a chicken showing filopodia (F), nucleus (N), erythrocytes (E) and vesicular bodies (arrows). Mag. x 11500



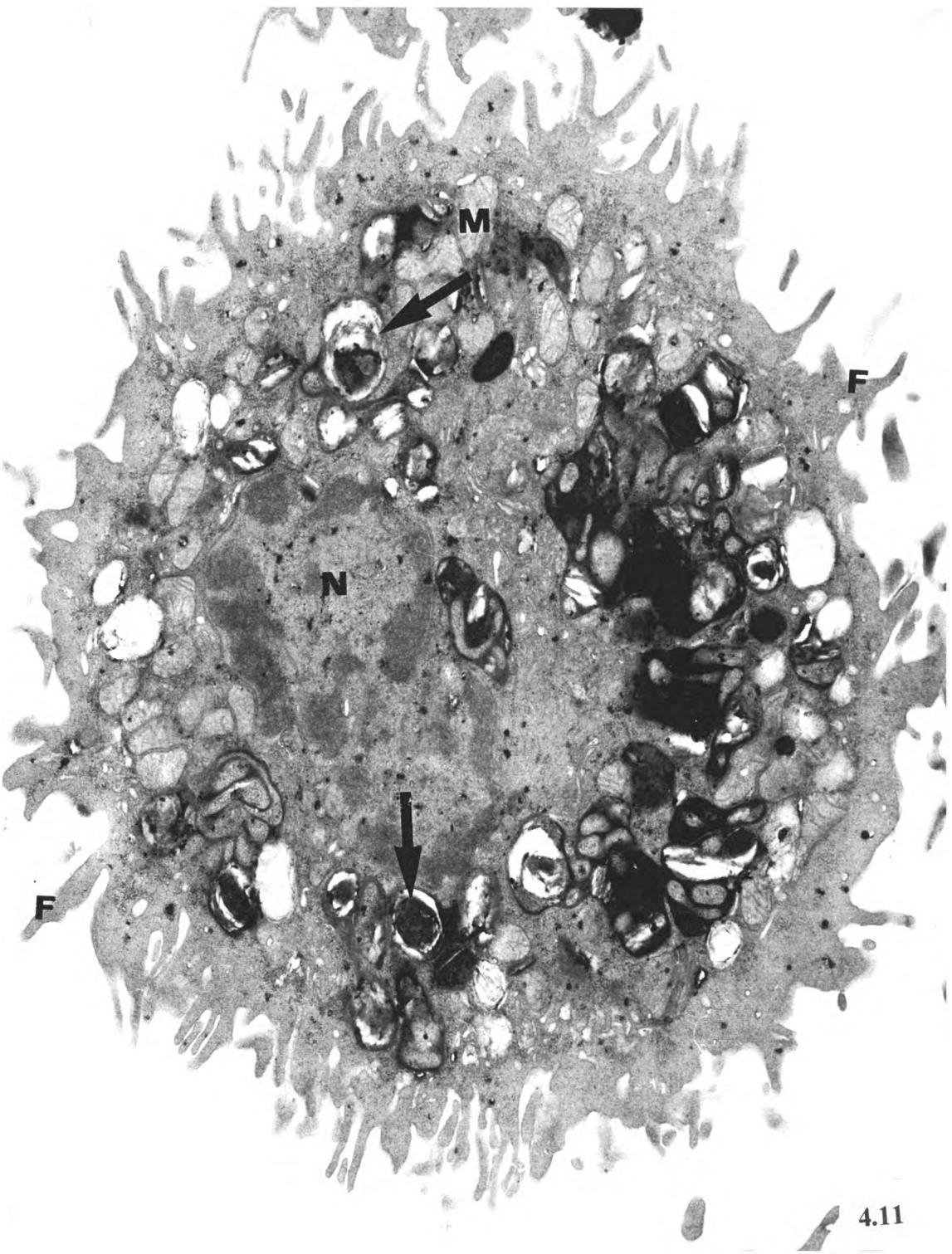
4.9

Fig.4.10: Electron micrograph of a FARM in a duck showing a filopodium (F), a mitochondrion (M), Golgi complex (G), vesicular bodies (arrows) and nucleus (N). Mag. x 21600



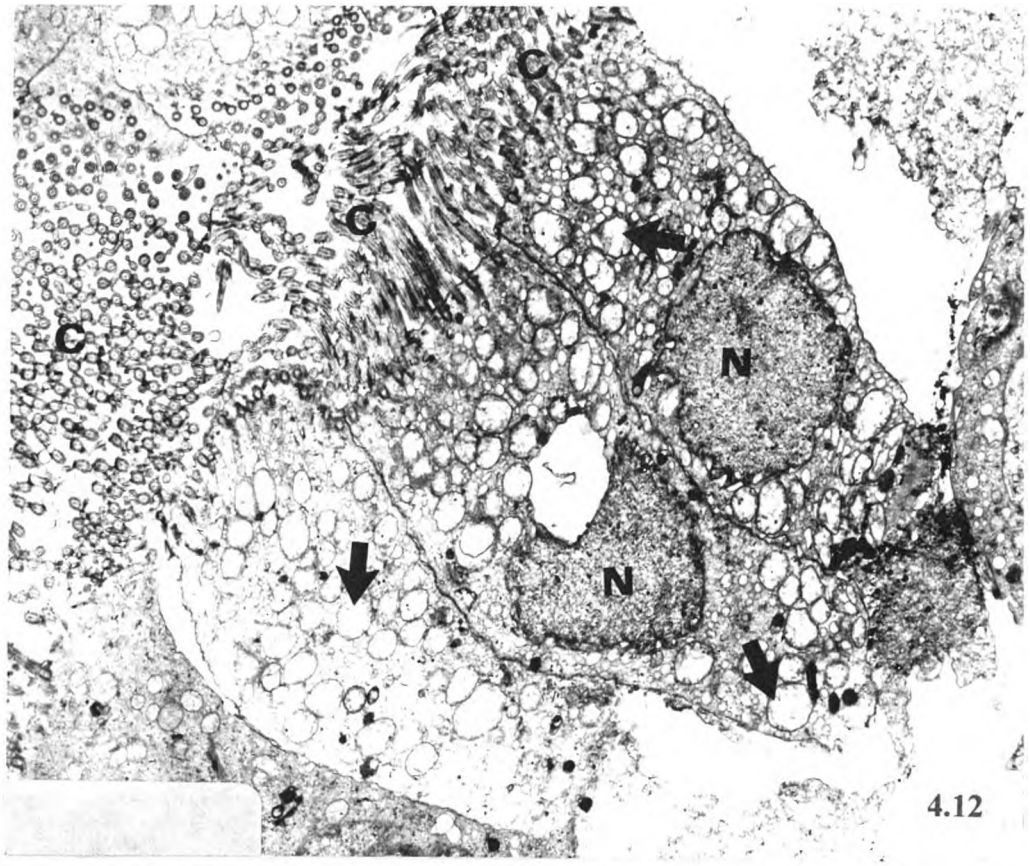
4.10

Fig. 4.11: Electron micrograph of a rat AM showing filopodia (F), a mitochondrion (M), vesicular bodies (arrows) and nucleus (N). Mag. x 17179

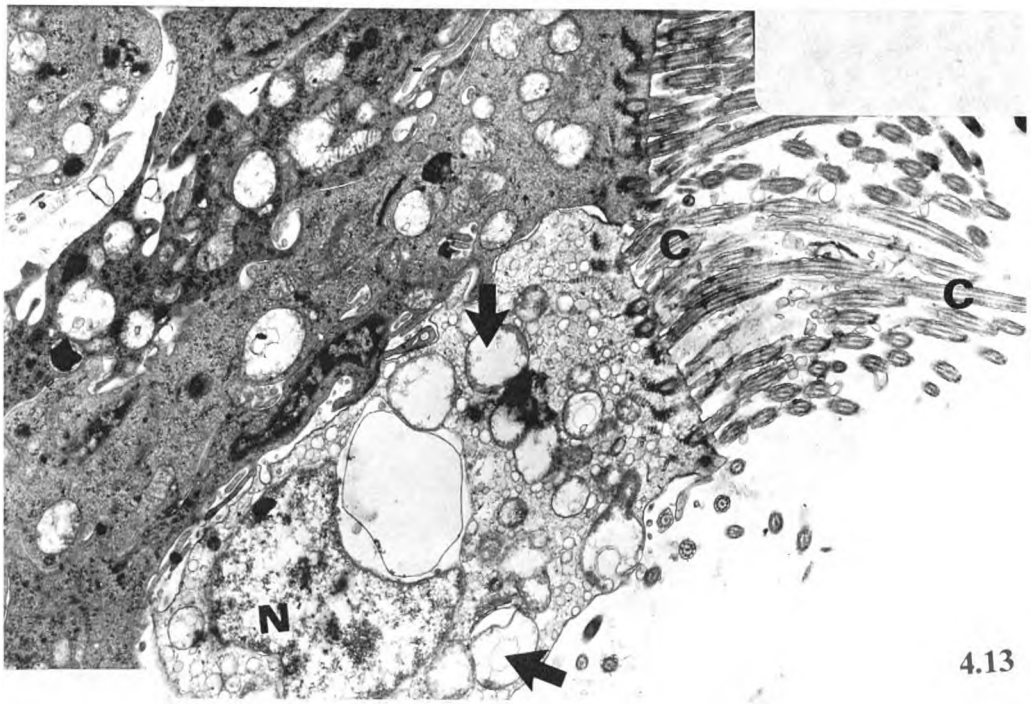


4.11

Fig. 4.12 and **Fig. 4.13**: Electron micrographs of exfoliated pulmonary epithelial cells from the chicken (Fig. 4.12) and the duck (Fig. 4.13) showing vesicular bodies (arrows), cilia (C) and the nuclei (N). Fig. 4.12, Mag. x 5906; Fig. 4.13, Mag. x 8189



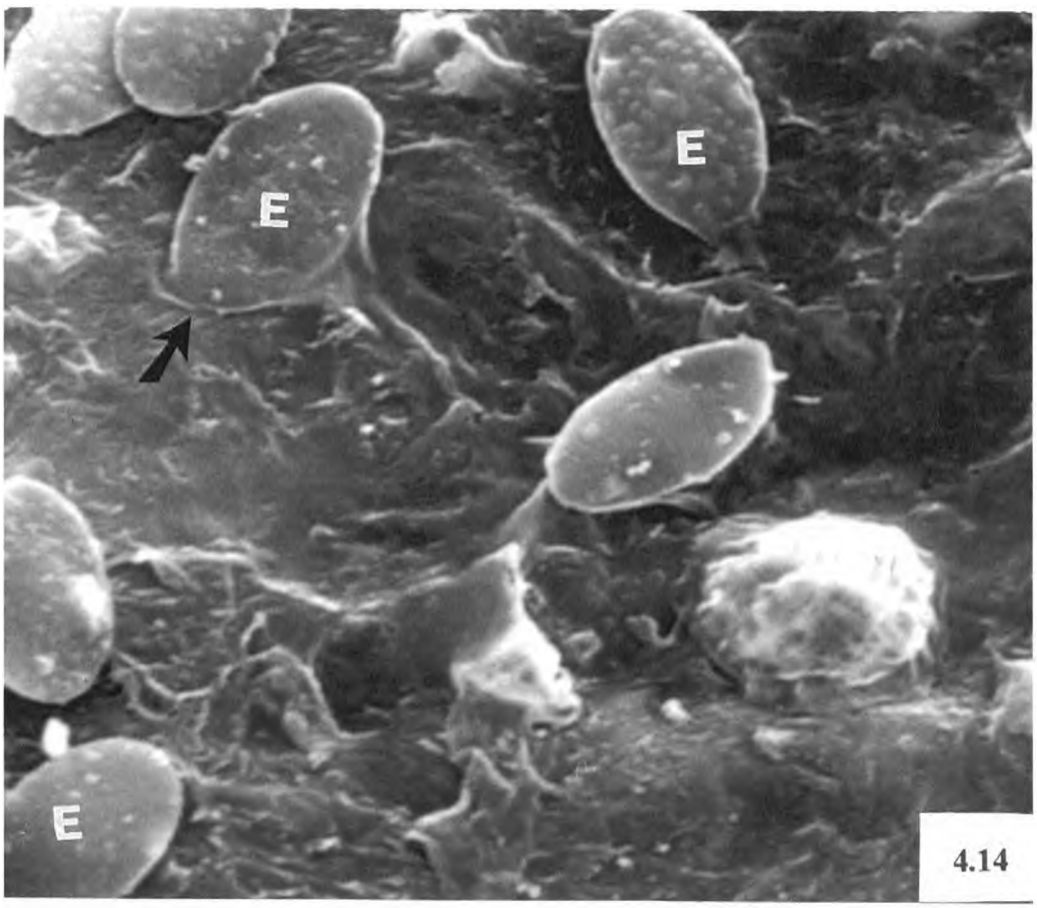
4.12



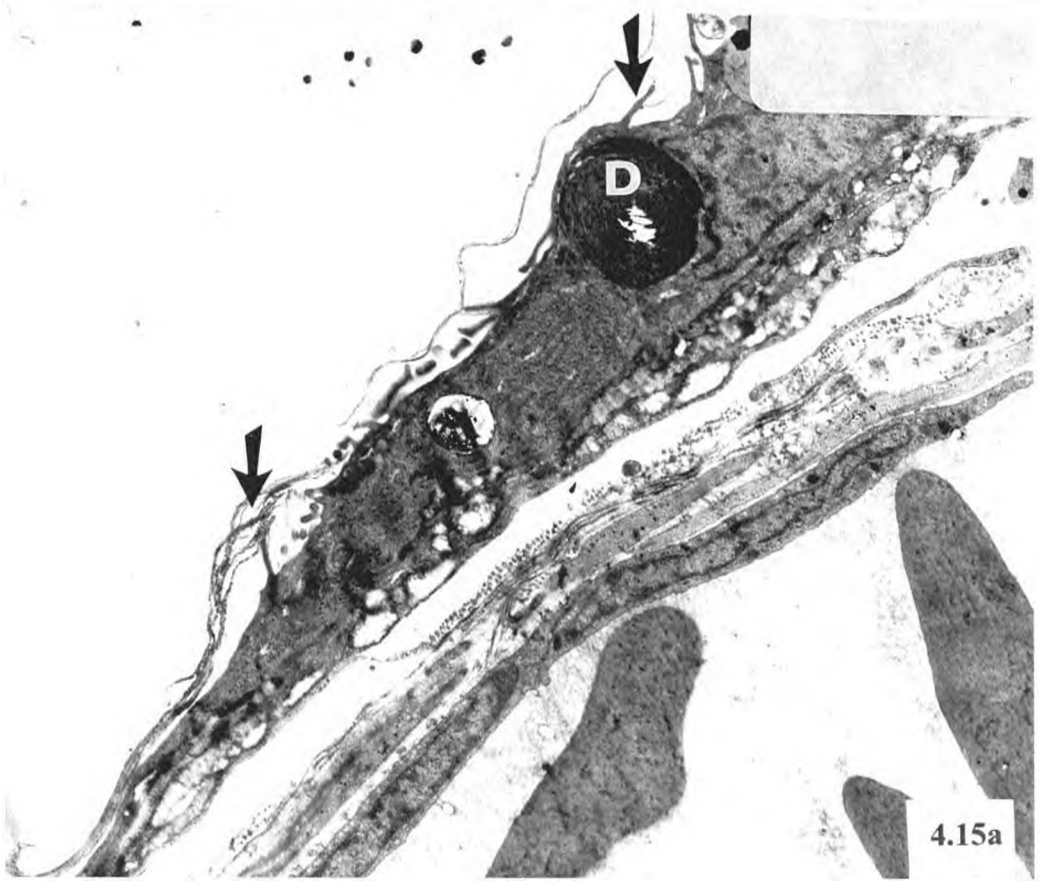
4.13

Fig. 4.14: Electron micrograph (scanning) of the respiratory surface of a chicken's lung showing erythrocytes (E) lying on the epithelial surface. One of the erythrocytes is seen being engulfed (arrow) by the epithelial cells. Mag. x 4500

Fig. 4.15a: Electron micrograph (TEM) of a section cut along the parabronchial epithelium of the lung of the chicken showing long microvilli (arrows) with a lamellated particulate body (D) being engulfed by the epithelium. Mag. x 11100



4.14



4.15a

Fig. 4.15 b: High power micrograph of a putative foreign particulate (D) being engulfed by the phagocytic epithelial surface of the chicken's lung. Mag. x 37000

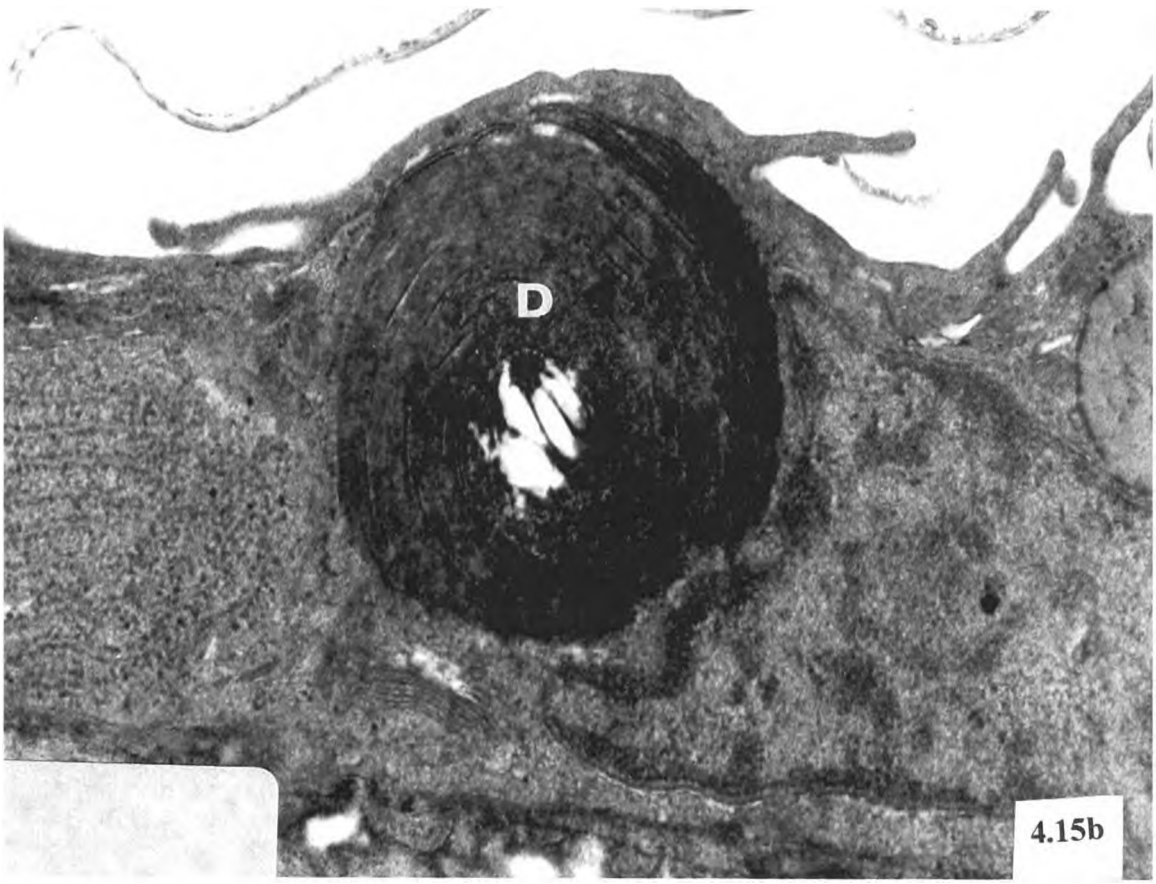
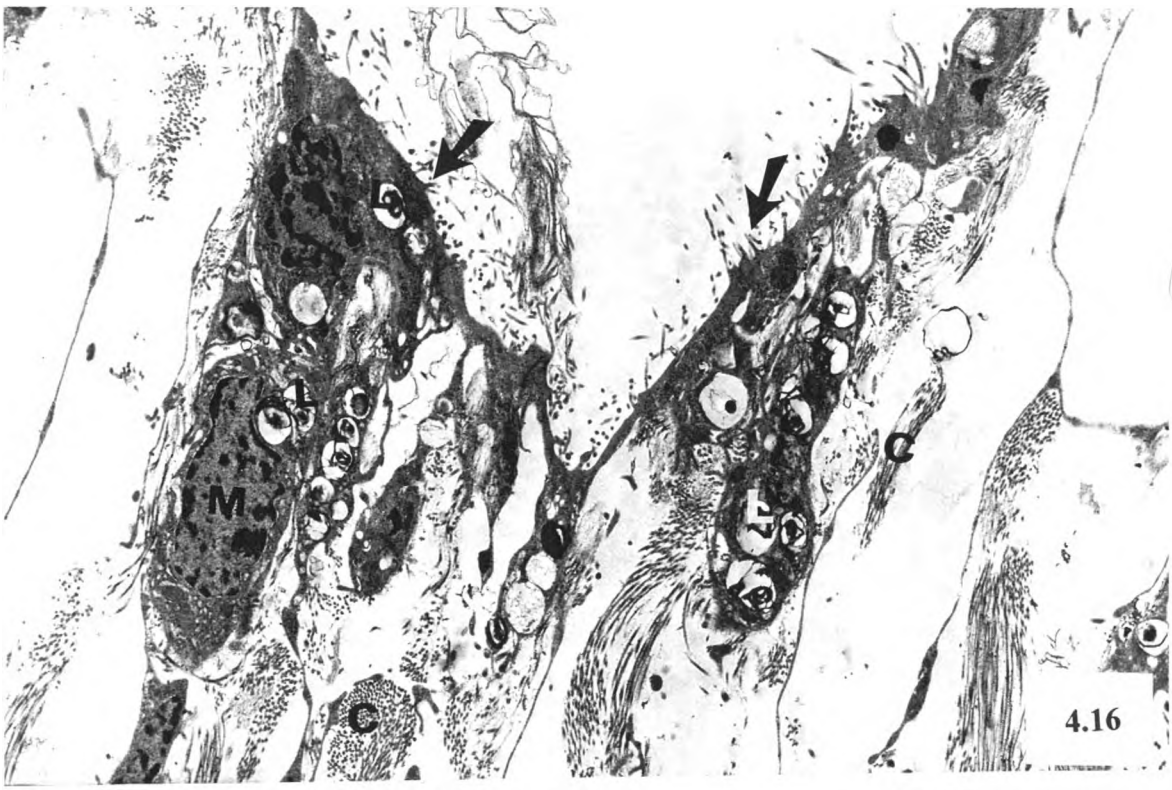
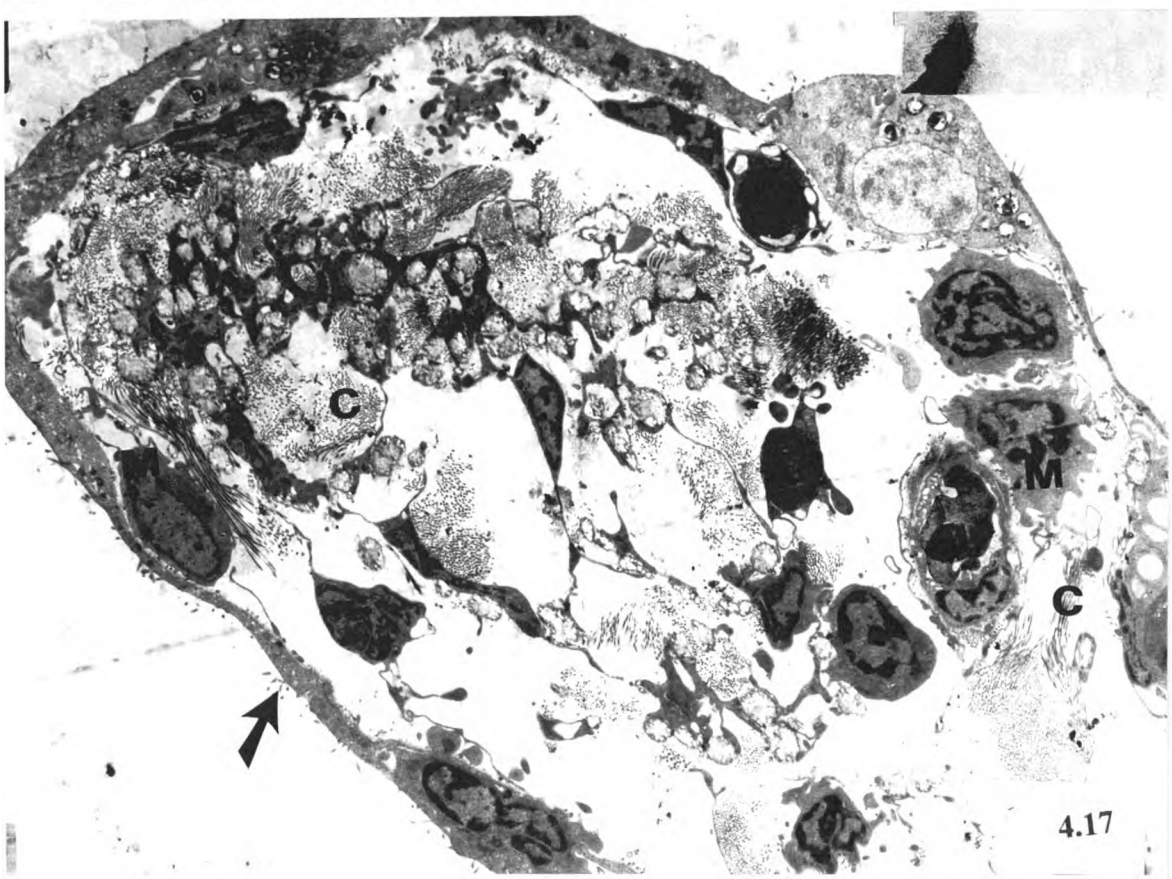

D
4.15b

Fig. 4.16: Section of the lung of the duck taken at the level of a parabronchus showing interstitial macrophage (M), collagen fibers (C), microvilli of the respiratory epithelium (arrows) and lamella-like bodies (L). Mag. x 11700

Fig. 4.17: Section of the lung of the chicken taken at the level of a parabronchus showing an interstitial macrophage (M), collagen fibers (C) and microvilli of the epithelial cells (arrow). Mag. x 4033



4.16



4.17

5.0 DISCUSSION

Scarcity (e.g. Ficken *et al.*, 1986, Maina and Cowley, 1998) and even lack (e.g. Qureshi *et al.*, 1994; Klika *et al.*, 1996; Lorz and Lopez, 1997) of free macrophages have been reported on the surface of the avian respiratory system. Ficken *et al.* (1986) and Maina and Cowley (1998) reported 1.15 x 10^6 and 1.6 x 10^5 FARMs in the turkey and the rock dove respectively. In mammals, alveolar macrophages were estimated by e.g. Holt (1979) to range from 0.55 to 1.55 x 10^6 in mice, 2.86 to 4.46 x 10^6 in rats, and 1.08 to 1.77 x 10^7 in guinea pigs. The values obtained from mammals are remarkably higher than those from birds, especially when the values are normalized with body mass. Chicken selected for large body size were reported by Toth and Siegel (1986) to have more respiratory macrophages than those from a line selected for small body mass: the minimum yield was 10,000 and the maximum was 2 million. The present study confirms previous observations that birds have fewer free pulmonary macrophages compared to mammals. The number of alveolar macrophages in the rat per gram of body mass (2933) was two times greater than that recorded in the chicken (1413) and the duck (1430) (this study). Since the respiratory surface area of active birds exceeds that of mammals of equivalent body mass by a factor of 15% and the blood gas barrier is 56 – 67% thinner (Maina *et al.*, 1989), all other factors being the same, the avian lung should conceivably be more susceptible to pulmonary infections and afflictions than the mammalian one. Accordingly, more FARMs would be expected to occur on the avian lung to provide adequate defense. Abdalla, Maina, King *et al.* (1982) reported a mass-specific surface area of the blood-gas (tissue) barrier of 8.7 $cm^2.g^{-1}$ and a harmonic mean thickness of the barrier of 0.318 μm in the domestic fowl, *Gallus gallus* variant *domesticus*: equivalent values on the domestic muskovy duck (*Cairina moschata*) were 30

$cm^2.g^{-1}$ and 0.119 μm respectively (Vidyadaran, 1987). In the rat, the surface area of the blood-gas barrier and the thickness of the barrier are 28 $cm^2.g^{-1}$ and 0.37 μm respectively (Gehr, Mwangi, Amman *et al.*, 1981). Assuming that the defensive capacity in the avian lung is similar to that in mammals, it would be expected that auxiliary defense lines occur to compliment the scarce FARMs or that the FARMs may be relatively more efficient in destroying pathogens than those of mammals. Moreover, there may be other intrinsic structural/functional features of the avian lung that minimize prospects of infection.

The epithelial cells lining the atria and infundibulae were shown to be phagocytic by, e.g. Stearns *et al.* (1987). They reported high concentration of hydrolytic enzymes associated with phagocytosis in atrial epithelial cells and in the general regions of atria and infundibulae. Mensah and Brain (1982) reported that 46% of particles deposited on the respiratory tract of the chicken is cleared within 1 hour. This is more rapid than comparable data for the same aerosol in mammalian or reptilian species. For example, Gehr *et al.* (1990) reported the clearance in hamster and horse to occur within 24 hours. In the boa constrictor, less than 8% of deposited particles are cleared over a period of 5 hours (Grant, Brain and Vinegar, 1981). Through fluorescence staining, the present study demonstrated abundant presence of lysosomes on the apical ends of the epithelial cells lining the respiratory passages of the chicken and the duck. Furthermore, phagocytosis of extravasated red blood cells by the epithelial lining of the chicken lung was observed. Sorokin and Brain (1975) reported little evidence of activation of the lysosomal system in squamous alveolar (type I) cells regarding uptake of iron oxide: there was no indication that these cells destroyed the ingested particles. Here, it was estimated that the surface area of the phagocytic epithelium

(i.e. the surface area of the atrial muscles, atrial floor and infundibulae) of the chicken and the duck constituted respectively 71% and 70% of that of the blood-gas (tissue) barrier. These values were estimated from values of the surface area of the blood-gas barrier per gram body mass reported by Duncker (1972), Abdalla *et al.* (1982) and Vidyadaran (1987) on the two avian species that were investigated in this study (Appendix 15.a and 15.b). With a phagocytic epithelium constituting such a high proportion of the pulmonary respiratory epithelium, conceivably, a large resident population of FARMs may not be necessary on the surface of the avian lung.

The complex sieve-like arrangement of the air passages in the avian lung starting with the primary bronchus, a series of secondary bronchi, many parabronchi (tertiary bronchi), atria and infundibulae may constitute an efficient sieve of pathogens and particulate matter. The foreign materials may be entrapped onto the respiratory epithelium of the major air conduits of which the cells have abundance of lysosomes before they reach the parabronchial level or onto the phagocytic epithelium of the atria and infundibulae close to the blood-gas barrier of the air capillaries. Large particles (3.7 to 7μm) are trapped in the nasal cavities and proximal trachea, midsize particles (1.1μm) are trapped in the lung and cranial air sacs, while smaller particles (0.091μm) pass through the lung and are trapped in the abdominal air sacs (Hayter and Besch, 1974). Moreover, in contrast to the bronchial tree of mammals, the air capillaries of the avian lung are not blind ending (Maina, 1982) as compared to alveoli. While the mammalian lung is tidally ventilated, the airflow in the avian lung is continuous and unidirectional (e.g. Scheid, 1979). It is plausible that this process may flush out harmful factors, minimizing their chances of landing and causing damage to the pulmonary tissue.

Furthermore, the trilaminar substance, a lamellated material seen only in avian parabronchial units (e.g. Scheuermann *et al.*, 1997) is reported to participate in particle clearance in the avian lung (Stearns *et al.*, 1987).

Klika *et al.* (1996) reported that avian respiratory macrophages located in the connective tissue do not seem to migrate to the surface of conducting airways. However, some researchers like Ficken *et al.* (1986) and Toth and Siegel (1986) demonstrated FARMs influx to the surface of the lung. In this study, movement of FARMs onto the respiratory surface was observed after serial lavage. Toth *et al.* (1988) showed that the number of FARMs in chickens activated with *Pasteurella multocida* vaccine increase considerably compared with FARMs from non-stimulated control birds. Not only did the number of FARMs increases in the *P. multocida* inoculated birds, indicating cell influx, but the phagocytic capacity of individual cells also increased in terms of number of engulfed microspheres. Alveolar macrophages were reported by White, Lanser, Nelson *et al.* (1985) to react similarly. Toth and Siegel (1986) reported that two of the birds used in their study presented no clinical signs of respiratory disease and yet had an exceptionally high number of FARMs. They concluded that the influx of FARMs must play a very important role in defense against pathogens as it occurs before clinical signs of a disease develop. According to Toth (2000), the normal steady-state avian respiratory system is refractory to elicitation by inert stimulants but responds efficiently to replicating bacteria, leading to a very rapid influx of large numbers of activated FARMs with increased phagocytic capacities. The potential for efficient translocation of subepithelial macrophages to the epithelial surface could be another explanation of the lack of large numbers of resident macrophages on the respiratory surface.

Though it is arguable that in the present study the observed increase of the lavaged macrophages could be attributed to cells that were not washed off during the preceeding lavage, the increase was statistically significant and a gradual decrease in cell numbers occurred in the subsequent lavages. This strongly suggests that the large number of macrophages collected in the second lavage emanated from elsewhere and not from the respiratory surface itself. Unquestionably, the cells came from the subepithelial compartment, where interstitial macrophages were observed in large numbers in tissues prepared for microscopy.

The turn over time of pulmonary macrophages is known to be about 6 days (Blusse *et al.*, 1983). Considering that mammalian and avian respiratory systems are structurally and functionally different and the two taxa evolved from reptilian stock at different times, possible variations regarding their free macrophages are to be envisaged. In that regard, the longevity of the cells from the two taxa when exposed to similar environmental conditions was evaluated: the FARMs of chickens were the most robust, followed by alveolar macrophages of rats and lastly FARMs of ducks. Interestingly, the lysosomal content of macrophages from mammals was not different from that of birds. This suggests that the phagocytic capacity of the FARMs compares with that of the alveolar macrophages. Toth *et al.* (1988) demonstrated that the proportion of phagocytic avian respiratory phagocytes increased more than five-fold within 24 hr in the *P. mucocida*-stimulated birds. They observed a higher proportion of engulfed microspheres in larger number per cell from stimulated chicken respiratory macrophages as opposed to non-stimulated ones: alveolar macrophages have been reported by e.g. White *et al.* (1985) to react similarly. White *et al.*

(1985) noted that in mammals, elicited macrophages show increased chemotaxis, spreading, phagocytic and microbicidal activities compared with nonelicited macrophages.

The greater surface density of alveolar macrophages compared to FARMs possibly indicates their greater motility and phagocytic ability. Considering that in birds the epithelium immediate to the respiratory surface (the epithelium lining the atria nad infundibulae) is phagocytic, highly mobile FARMs may not be necessary. Within the air sacs where defense is only provided by the FARMs, air sacs being non-vascularised and lacking an elaborate ciliated epithelium, clearance of foreign particles occurs within 24 to 48 hours (e.g. Ficken *et al.* 1986). This indicates that on their own, FARMs are very efficient in clearing deposited particles from the respiratory surface. The low numerical density of FARMs could also be explained by the fact that macrophages can cause severe damage to normal lung tissue through generation of reactive oxygen radicals (RORs) and initiation of autoimmune reactions (e.g. Brain, 1986): the degree of injury, is dependent on the concentration of oxygen at the site of activity. It is hence plausible that since birds have a higher oxygen consumption even at rest that is 60 % higher than that of mammals (27 %) (e.g. Prosser, 1950), scarcity of FARMs may curtail damage to the lung through production of RORs.

6.0 CONCLUSION

Morphologically, FARMs and AMs are not significantly different except for the filopodia that appear to be more numerous in those of rats. This study confirms that compared with mammals, the FRMs are scarcer in birds. Though mammals may have more FRMs than birds, it doesn't *ipso facto* mean that scarcity of FARMs constitutes a handicap in cellular respiratory defense. The greater robustness of FARMs in the chicken and apparently more efficient translocation of FARMs onto the respiratory surface of the chicken and duck may explain why presence of large population of FARMs on the respiratory surface unnecessary. In birds, the phagocytic capacity of FARMs during invasion by inhaled particles is complimented by those of the atrial and infundibular epithelia, the microbial activity of the trilaminar substance, and the influx of subepithelial macrophages to the respiratory surface: this constitutes an efficient, complex defense arsenal. Furthermore, in birds, the Harderian gland, located behind the eye and within the orbit is a major source of antibodies (e.g. Ewert, Barger and Edison, 1979). The locally produced antibodies that bathe the cells lining the nasal cavity, trachea and bronchi constitute a major defense mechanism of the avian respiratory system (Bang and Bang, 1969). Presence of such peculiar features in the avian lung may explain the low numerical density of FARMs. Paucity of FARMs is not inexorably responsible for the high puported mortality that may afflict the poultry industry. The explanation for the high death rate of cage birds could be attributed to other factors that include management and husbandry practices as well as intense genetic manipulation for fast growth and productivity, features that may weaken resistance to pathogenic microorganisms. Tully and Shane (1996) observed that some bacterial, viral and parasitic diseases emerge from production settings, as newly

introduced birds may contaminate soil and facilities with pathogens such as *Mycobacterium* and *Salmonella*. Proper hygiene, good management, and balanced nutrition will reduce likelihood of flock exposure to diseases, limiting mortality.

APPENDIX 1

List of abbreviations used in the text

FRMs: Free respiratory macrophages

MPS: Mononuclear phagocyte system

AMs: Alveolar macrophages

FARMs: Free avian respiratory macrophages

CFU-S: Colny forming units of the spleen

GM-CFC: Granulocytes and monocytes-colony forming cell

PIMs: Pulmonary intravascular macrophages

IMs: Interstitial macrophages

PBS: Phosphate buffered saline

TEM: Transmission electron microscopy

SEM: Scanning electron microscopy

RORs: Reactive oxygen radicals

APPENDIX 2

Preparation of 1000 ml phosphate buffer saline (PBS) pH 7.4

Solution A:

6.24 g of 0.2 M of NaH_2PO_4 (MW 156) in 200 ml distilled water.

Solution B:

25.47 g of 0.2 M of $NaHPO_4$ (MW 142) in 900 ml distilled water.

PBS (pH 7.4): 190 ml of solution A + 810 ml of solution B

APPENDIX 3

Preparation of 1% trypan blue.

1 g trypan blue mixed with 100 ml of distilled water

APPENDIX 4

Macrophage counts

The macrophages were counted using a heamocytometer. Each square of the heamocytometer, with cover slip in place, represents a total volume of $0.1mm^3$. Macrophages were counted in 4 chambers of the heamocytometer. These 4 chambers constituted a volume of 0.4 mm^3. The count was repeated five times within the 4 chambers and the average of the five counts was used to determine the total number of cells counted.

Example

- first count: 3 macrophages
- second count: 3 macrophages
- third count: 2 macrophages
- fourth count: 3 macrophages
- fifth count: 2 macrophages

Average count: (3+ 3+2 + 3 +2) / 5 = 2.6 cells in 0.4 mm^3

The number of cells in 1ml of the collected fluid needs to be known in order to determine the total number of cells collected.

Number of cells in 1 mm^3

2.6 / 0.4 = 6.5 cells

Number of cells in 1cm^3

6.5 x 1000 = 6500 cells/ cm^3

The cells were counted in a concentration of 10 ml and the lavage fluid diluted with 1% trypan blue in a 1:1 dilution giving a dilution factor of 2. Taking these factors into consideration, the number of cells per ml of the original fluid will therefore be:

6500 (cells /ml) x 2 (dilution factor) / 10 = 1300 cells/ml

Total number of macrophages in the lavage fluid of 300 ml

1300 (macrophages /ml) x 300 ml (lavage fluid volume) = 390,000 macrophages

APPENDIX 5

Staining solutions for longevity evaluation

- Preparation of 0.04 % neutral red

0.04g neutral red in 100 ml distilled water

- Preparation of 0.5 % trypan blue

0.5g trypan blue in 100 ml distilled water

APPENDIX 6

Lysosomes staining solution

❖ *Preparation of the probe-containing medium (PBS – Lysotracker Red DND 99)*

The Lysotracker from Molecular Probes comes in a vial containing 50µl of a 1 mM stock solution.

Working concentration was 20 nM.

1mM = 1000 nM

With 1000 nM, 20 nM were needed

Dilution factor will therefore be:

1000 / 20 = 50

1: 50 dilution, i.e. 1µl (lysotracker) + 49 µl (PBS)

For 100 µl probe- containing medium the dilution was done as follows:

100 / 20 = 5 therefore,

5 µl (lysotracker) + 245 µl (PBS) = concentration of the probe- containing medium

APPENDIX 7

Preparation of slides for evaluation of lysosomal content of the free macrophages

Wells were made on the slides in order to put drops of the stained cell suspension that were to be viewed using the confocal microscope. The wells were made as follows:

Two separate drops of glycerin were put on a slide, then a separating agent Kontaflon 85 was spread on the slide avoiding the area with the drops of glycerin. This gave rise to a dry lubricating film on the slide. Twenty minutes after spreading the separating agent, the

drops of glycerin were then washed off with warm water. Vacuum shaping moulds were formed.

APPENDIX 8

Tissue processing for transmission electron microscopy

❖ *FIXATION*

Small pieces (1 mm^3) of the lung were:

Fixed in 2.5% glutaraldehyde for 3 hours

Washed in PBS (3 changes of 10 minutes each)

❖ *DEHYDRATION*

The fixed tissue was dehydrated trough graded series of alcohol as follows:

70 % alcohol for 10 minutes

95 % alcohol 10 minutes

100 % alcohol 15 minutes (x2)

❖ *CLEARING*

The tissues were cleared in 2 changes of propylene oxide. Each change lasted for 15 minutes.

❖ *INFILTRATION AND EMBEDDING*

The tissue was passed through mixtures of propylene oxide: resin as follows:

Propylene oxide: resin 3: 1 (1 hour)

Propylene oxide: resin 1: 1 (1 hour)

Propylene oxide: resin 1: 3 (1 hour)

Resin 1 hour

The tissues were embedded in separate numbered blocks containing fresh resin and kept in the oven at 60 °C for 48 hours.

APPENDIX 9

Staining of ultrathin sections on grids for TEM

MATERIALS

- Petri dishes were kept moist with wet pieces of filter paper on base, and strips of dental wax above.
- Six small beakers filled with double- distilled water
- Uranyl acetate
- Lead citrate
- Clean forceps washed in acetone and air dried

METHOD

Staining with uranyl acetate

- Large drops of uranyl acetate were placed on dental wax strips in a petri dish.
- Each grid section was floated down on a separate drop for 10 minutes.
- Each grid was washed separately three times, once in each of three beakers of double distilled water, 15 seconds in each beaker.
- The section was dried on filter paper.

Staining with lead citrate

- Drops of lead citrate solution were placed on wax in a different petri dish and the grid sections were stained and washed as for uranyl acetate.

APPENDIX 10

Point counting

Using a quadratic lattice test system, the number of points falling on the vesicular bodies (P_{vb}) and those falling onto the entire cell (P_c) was counted. The volume density of the vesicular bodies (Vv_{vb}) was calculated as:

$Vv\ (vb) = P_{vb} / P_c$

APPENDIX 11

Intersection counting

Intersection (I): contact between the test line and the object surface or profile boundary.

Surface density (Sv) of filopodia of macrophages

$$Sv = 2I / Lt / Magnification$$

$Lt = Pt \times d$

Lt: length of the test line, Pt: test point set and d: length of the test system

Surface area of the phagocytic epithelium of the lung (atrial muscle, atrial floor and infundibulae):

Sa = Sv x reference volume (Rv)

Surface density (Sv) of atrial muscles, atria and infundibulae:

Sv = 2I / Lt / Magnification

Lt = Pt x d

Reference volume (Rv) of the lung

Rv = VL x Vv (PS)

With VL : lung volume and Vv (PS): volume density of the exchange tissue that contains the phagocytic surface

Vv (PS) = Vv (ET) x Vv (PSB) with:

Vv (ET): volume density of the gas exchange tissue and

Vv (PSB): volume density of the parabronchi and secondary bronchi.

In chicken:

Vv (PSB) = 30.56%, Vv (ET) = 46.35%

Therefore, Vv (PS) = Vv (ET) X Vv (PSB)

= 46.35 + 30.56 = 76.91%

In duck:

Vv (PSB) = 37.47%, Vv (ET) = 49.24%

Therefore, Vv (PS) = Vv (ET) X Vv (PSB)

= 49.24 + 37.47 = 86.71%

APPENDIX 12

Preparation of 100 ml of 2.5 % glutaraldehyde in PBS

90 ml PBS in 10 ml of 25 % glutaraldehyde.

APPENDIX 13

Tissue processing for light microscopy

PROGRAM OF THE AUTOMATIC PROCESSOR

- 70 % alcohol 2 hours
- 95 % alcohol 2 hours
- 95% alcohol 2 hours
- Absolute alcohol 2 hours
- Absolute alcohol 2 hours
- Absolute alcohol 1.5 hours
- Chloroform 2 hours
- Chloroform 2 hours
- Wax 1 2 hours
- Wax 2 2 hours

STAINING WITH HEAMATOXYLIN AND EOSIN

- xylene : 5 minutes
- Absolute alcohol : 1 minute
- 95% alcohol : 1 minute
- 95% alcohol : 1 minute
- Heamatoxylin : 5 minutes
- 1% acid alcohol : 2 to 3 dips
- Running water : 5 minutes

- Eosin : 1 minute
- Running water : briefly
- 70% alcohol : 1 minute
- 95% alcohol : 1 minute
- absolute alcohol : 1 minute
- Xylene : 1 minute

APPENDIX 14a

Volume densities of vesicular bodies and surface densities of filopodia of macrophages in the duck

Micrograph Number	Volume density of vesicular bodies (%)	Surface density of the filopodia of the macrophages ($cm^2\ cm^{-3}$)
1	23	5666
2	19	6833
3	27	3333
4	34	3444.4
5	18	4300
6	13	4941
7	22	2400
8	21	5600
9	18	3466
10	38	6300
11	26	5138
12	30	6400
13	15	5700
14	18	5866
15	21	7272
16	16	5000
17	32	7545
18	42	5200
19	13	4444
20	13	4666
21	32	6666
22	18	9341
23	22	2900
24	16	3733
25	41	7578
26	38	5500
27	16	10500

28	39	6833
29	38	3800
30	14	2900
31	13	4400
32	18	4666
33	Mean: 23.8 ± 9.4 SD	4000
34		4235
35		6200
36		3900
37		2350
38		2066
39		2900
40		6000
41		4375
42		3300
43		4000
44		6333
		Mean: 5045 ±1815 SD

APPENDIX 14b

Volume densities of vesicular bodies and surface densities of filopodia of macrophages in the chicken

Micrograph Number	Volume density of vesicular bodies (%)	Surface density of macrophages ($cm^2.cm^{-3}$)
1	41	2600
2	23	3600
3	29	8906
4	15	10666
5	17	4642
6	20	2833
7	17	3125
8	42	4333
9	29	4200
10	12	6640
11	19	2947
12	19	3900
13	13	4833
14	15	6666
15	24	7142
16	21	4148
17	22	4266
18	36	7428
19	20	7044
20	24	10769
21	24	4300

22	14	3384
23	17	6333
24	16	4923
25	18	4526
26	16	8400
27	12	5808
28	12	2800
29	25	3300
30	14	5300
31	41	3696
32	39	2785
33	27	3933
34	Mean: 22.2 ± 8.9 SD	3444
35		7142
36		4307
37		5666
38		4941
39		3200
40		4875
41		4285
42		Mean: 5074 + 2055.5 SD

APPENDIX 14c

Volume densities of vesicular bodies and surface densities of filopodia of macrophages in the rat

Micrograph Number	Volume density of vesicular bodies (%)	Surface density of macrophages ($cm^2.cm^{-3}$)
1	23	4736
2	29	6000
3	50	5047
4	21	10153
5	27	7555
6	30	7500
7	15	5809
8	46	5600
9	26	7500
10	41	6777
11	31	5529
12	16	6555
13	40	2857
14	32	3037
15	34	7142
16	31	3304
17	31	4421
18	24	4222
19	32	3384

20	25	4347
21	34	3000
22	14	5500
23	10	15666
24	23	5478
25	14	7130
26	28	9000
27	29	14909
28	30	8272
29	27	14352
30	24	5578
31	16	8222
32	30	11058
33	4.6	7400
34	28	9000
35	27	7333
36	14	7578
37	14	9666
38	17	6000
39	18	3793
40	14	7111
41	15	8875
42	26	8823
43	22	6080
44	19	10125
45	29	10625
46	36	4000
47	26	6400
48	33	8333
	Mean: 25.533 ± 9.26 SD	Mean:7104.3 + 2952.5 SD

APPENDIX 15.a

Surface area of the blood-gas barrier of the chicken (Sa)

This was derived from the average values of the surface area of the blood gas barrier per gram of body weight of the *Gallus gallus* var. *domesticus* as follows:

8.70 cm^2/ g (Abdalla *et al.* 1982)

13.6 cm^2/ g (Duncker, 1972)

12.5 cm^2/ g (Vidyadaran, 1987)

Average: 8.70 + 13.6 + 12.5 = 11.6 cm^2/ g

Sa of the blood gas barrier of the chicken = mean weight of the chickens x average of the surface area of the blood gas barrier per gram of body weight of the *Gallus gallus*

Sa = 2609.8 x 11.6 = 30273.68 cm^2

Percentage proportion of the Sa of the phagocytic surface area to that of the blood-gas barrier.

% = Surface area of the phagocytic epithelium (atrial muscle, atrial floor and infundibulum) / Sa of the blood gas barrier

% = 21556.1 / 30273.68 = 71%

APPENDIX 15.b

Surface area of the blood-gas barrier of the duck (Sa)

This was derived from the surface area of the blood-gas barrier per gram of body weight of the *Cairina Moschata* (30 cm^2/ g) reported by Vidyadaran (1987).

Sa blood-gas barrier of the ducks = mean weight of the ducks x average of the surface area of the blood gas barrier per gram of body weight of the *Cairina moschata*

Sa blood gas barrier = 2208.06 x 30 = 66241.8 cm^2

Percentage proportion of the Sa of the phagocytic surface area to that of the blood-gas barrier.

% = Surface area of the phagocytic epithelium (atrial muscle, atrial floor and infundibulum) / Sa of the blood gas barrier

% = 46298.5 / 66241.8 = 69.89%

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