



Light and electron microscopical observations on the terminal airways and alveoli of the lung of the SA (Cape) fur seal *Arctocephalus pusillus*

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ABSTRACT

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During activities of the Sea Fisheries Research Institute at Kleinsee, lung samples from six South African fur seals were collected. The terminal airways showed pseudostratified ciliated columnar epithelium with numerous goblet cells and occasional brush cells. Smooth muscle, cartilage and submucosal glands were also present. The epithelium changed over a short distance, in the smaller airways, through pseudostratified columnar non-ciliated to simple cuboidal epithelium with no goblet cells. The columnar non-ciliated cells contained secretory granules, which appeared to be serous. No Clara cells were found. Cartilage and muscle were present throughout, up to the origin of the alveolar ducts, but the glands disappeared together with the goblet cells.

Alveoli were lined by types one and two alveolar epithelial cells, with subepithelial capillaries.

They were divided by an alveolar septum with a well developed alveolar knob. This knob contained elastic fibres and fibroblasts, but not the smooth muscle cells which are present in terrestrial mammals and in Phocidae.

Keywords: *Arctocephalus pusillus*, Cape fur seal, electron microscopy, histology, terminal airways

INTRODUCTION

The South African (SA) fur seal (*Arctocephalus pusillus*) belongs to the family Otariidae or eared seals. They breed along the coast of South Africa and Namibia. Adult SA fur seals spend most of their lives in the sea. Once a year, during the summer breeding season, they come ashore to whelp and mate. In a study of the diving behaviour of two female SA fur seals, over 70 % of the dives were to depths shallower than 50 m and lasted 2–3 min. Less than 10 % were deeper than 150 m. The maximum depth was

about 200 m and these deep dives lasted about 7 min (David 1989).

Several light and electron microscopical descriptions of the terminal airways and alveoli of marine mammals have been published (Bélanger 1940; Simpson & Gardner 1972; Boyd 1973; Dennison & Kooyman 1973; Boshier & Hill 1974; Welsch & Drescher 1982; Drabek & Kooyman 1984; Henk & Haldiman 1990). Only Denison & Kooyman (1973) included the SA fur seal in their study, and they examined only one specimen of this species. They described the different ways in which the terminal airways were reinforced in Phocidae (true seals), Otariidae (eared seals), Odobenidae (walrus) and the sea otter. No description of the airway lining epithelium was given, and no electron microscopy was done by Denison & Kooyman (1973). Other publications that provide information on the pulmonary histology of otariid seals include that of Simpson & Gardner (1972) on the comparative microscopic anatomy of marine mammals, and a paper on the embryological development of the

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terminal airways in pinnipeds and the sea otter (Drabek & Kooyman 1984). None of these refers to the SA fur seal. In the present study we have attempted to illustrate some of the histological characteristics of the distal airways in this species.

MATERIALS AND METHODS

Lung samples were collected from six SA fur seals during activities of the Sea Fisheries Research Institute at Kleinsee on the West Coast of Southern Africa during November 1994. The seals were 10–12 months old. Samples were taken about 30–60 min after death, from the periphery of caudal lung lobes. Slices of 10 mm thickness were fixed in 10% buffered formalin, and embedded in paraffin wax. Sections 5 µm thick were cut with a sliding microtome and stained with haematoxylin and eosin (H & E), periodic acid-Schiff (PAS) (Bancroft & Stevens 1990), Masson's trichrome (Culling 1974), Verhoeff's elastic stain (Culling 1974), and reticulin stain (Lynch, Raphael, Mellor, Spare & Inwood 1969). The sections were examined under a Nikon Labophot light microscope and microphotographs were taken. Five mm thick sections from the lungs of three seals were fixed overnight in 2.5% glutaraldehyde at a temperature of about 4°C, whereafter they were immersed in phosphate-buffered saline (pH = 7.4) and kept refrigerated. These samples were post-fixed in osmium tetroxide, routinely dehydrated, and embedded in epoxy resin. Sections of 1 µm thickness (semithin sections) were cut and stained with toluidine blue (Bancroft & Stevens 1990). Ultrathin sections for electron microscopy were stained with lead citrate and uranyl acetate. These were examined and photographed in a Hitachi H-600 electron microscope. The magnifications of these photographs were known and measurements were made directly from the photographs.

OBSERVATIONS

Light microscopy

Pleura and lobulation

The visceral pleura was 55–82 µm thick and consisted of a simple squamous mesothelium resting on prominent connective tissue, containing blood vessels. Elastic fibres were concentrated below the mesothelium with collagen fibres forming the bulk of the layer.

The lungs were clearly lobulated. Interlobular septa between secondary lobules contained fine strands of collagen with some elastic fibres. These septa were continuous with the pleura and surrounded groups of small bronchioles and their alveolar ducts and sacs. Pairs or small groups of alveolar ducts with their

alveolar sacs were enveloped in thinner connective tissue sheaths (mostly collagen fibres with few elastic fibres) forming primary lobules. The definitions of primary and secondary lobules are according to Jordan (1934).

Airways

Branches of the bronchi with a diameter of about 340–520 µm had a pseudostratified, ciliated, columnar epithelial lining with numerous goblet cells containing PAS positive mucus. The lamina propria was sparse and had elastic fibres concentrated below the respiratory epithelium, with collagen fibres below the elastic fibres.

Deep to the loose connective tissue of the lamina propria was a layer of smooth muscle cells, which appeared to be discontinuous. The arrangement of the muscle fibres seemed to be mainly circular, but some had a different orientation. The smooth muscle layer thus probably consisted of bundles of smooth muscle cells spirally arranged, forming looser and tighter spirals or possibly criss-crossing bundles. On the outside the smooth muscle was closely associated with the cartilage.

The cartilage layer was prominent, and was composed of pieces of hyaline cartilage of various shapes and sizes completely surrounding the airways. A thin collagenous perichondrium was present, and extended between the cartilage plates as a connecting layer. The semithin sections stained with toluidine blue revealed numerous empty cytoplasmic vacuoles in the chondrocytes; presumably these were lipid droplets. Some PAS positivity was also present in the chondrocyte cytoplasm.

Submucosal glands consisting of rounded acini were situated in between the cartilage plates and also deep to the cartilage layer. The glandular epithelial cells were cuboidal or prismatic in shape and their cytoplasm contained PAS positive apical granules. The ducts opening into the bronchial lumen were lined by cuboidal epithelial cells which contained a few PAS positive cytoplasmic granules.

At a diameter of 300 µm the goblet and ciliated cells disappeared from the epithelial lining of the airways, which changed over a short distance from the typical respiratory epithelium mentioned above, through a pseudostratified columnar non-ciliated epithelium to simple cuboidal epithelium with no goblet cells and no submucosal glands (Fig. 1). The cuboidal epithelial cells had little cytoplasm and no cilia. PAS positive intracytoplasmic granules were seen in some. Near the termination of the airway the simple cuboidal epithelium abruptly changed to a simple squamous epithelium. The cytoplasm of these cells was invisible, but dark elongated nuclei parallel to the surface could be seen. Capillaries were situated directly

below the epithelium. The smooth muscle was very prominent at this level, and a well developed cartilage layer was still present.

The smallest cartilaginous airway gave rise to two or more alveolar ducts, ending in alveolar sacs. The cartilaginous airway appeared to form two very short terminal branches just before the cartilage disappeared with the transition to alveolar ducts. In fortuitous sections, this resulted in an isolated piece of cartilage and muscle, covered by squamous epithelium, appearing in between the entrances to the alveolar ducts. This is indicated by the arrow in Fig. 2. Cuboidal epithelium was also present on this structure, on that portion nearest to the larger airway.

Alveoli were 140–150 µm in diameter and opened into a common alveolar duct (Fig. 3). An interalveolar septum with a well developed alveolar knob at the free end, separated the individual alveoli of the alveolar ducts (Fig. 3). The knob was covered by simple squamous epithelium with occasional subepithelial capillaries. Elastic fibres formed the main extracellular component of the alveolar knob. This knob is probably part of a ring-like structure which forms the entrance to the alveolus. The connective tissue framework of the alveolar knobs may well be continuous with that of the terminal airways. Semithin sections stained with toluidine blue revealed the presence of cuboidal cells with foamy cytoplasm in the alveolar walls, as well as numerous capillaries. Other components of the alveoli were indistinct under the light microscope.

Electron microscopy

The descriptions and nomenclature of respiratory airway cells published by Breeze & Wheeldon (1977) were used as a guideline to identify the cells.

Pseudostratified ciliated columnar epithelium with goblet cells (respiratory epithelium)

CILIATED CELL

These cells were columnar, resting on the basal lamina and extending to the lumen (Fig. 4). Their nuclei were vesicular and prominent nucleoli were seen in some. The basal cytoplasm contained many ribosomes, both free and as part of the granular endoplasmic reticulum. Numerous mitochondria were present in the apical cytoplasm. A few small electron-dense granules were also seen in the cytoplasm. Numerous microvilli and typical cilia were observed at the apical surface of the epithelial cells.

GOBLET CELL

The numerous goblet cells appeared to have narrow bases, with swollen apical cytoplasm bulging out at the surface of the epithelium. Junctional complexes were present between the goblet cells and neigh-

bouring cells. A few isolated microvilli were observed at their apical surface. The cytoplasm was electron-dense, with large electron-dense secretory granules about 800 nm to over 2000 nm in diameter (Fig. 4). A nucleus with an irregular outline and a few mitochondria were observed at the base of one of these cells.

BRUSH CELL

According to Breeze & Wheeldon (1977), brush cells are characterized by a group of large microvilli on the luminal surface of the cell. Prominent bundles of filaments extend from the microvilli into the cytoplasm, and numerous apical vesicles and vacuoles are present. The usual organelles (free ribosomes, mitochondria, g.e.r., lysosomes) are sparsely distributed in a moderately electron dense cytoplasm. Owing to the occasional observation of afferent nerve endings associated with brush cells, it has been suggested that they may have a sensory function (Junqueira *et al.* 1992).

Cells which had some of the characteristics of brush cells were occasionally encountered in our specimens. They had an electron-dense cytoplasm and extended from the basal lamina to the lumen. Many microvilli projected from their luminal surfaces. Junctional complexes could be observed in the lateral plasmalemma close to the lumen. The nuclei were elongated and irregular in outline. Microfilament bundles were observed in the cytoplasm adjacent to the nucleus in one of these cells.

BASAL AND INTERMEDIATE CELLS

Basal and intermediate cells were present and were identified by their position (Fig. 4).

Non-ciliated pseudostratified columnar epithelium

EPITHELIAL SEROUS CELL

The serous cells which were present in this epithelium rested on the basal lamina and extended to the lumen. Cell junctions could be seen in their lateral and apical cell membranes. There was a basal nucleus and electron-dense membrane bound secretory granules, most of which were in the apical cytoplasm. Some granules had an electron-dense centre and a peripheral crescent of lighter, granular material. The granular endoplasmic reticulum and mitochondria were concentrated in the basal cytoplasm, but also present in the apical cytoplasm. A few microvilli projected from the luminal surface (Fig. 5).

No goblet cells were present in the unciliated epithelium. Basal cells, a possible brush cell and non-ciliated cells with invaginated nuclei and few secretory granules were also seen. No neuro-endocrine cells (Kulchitsky cells) were seen in this epithelium, nor in any of the other epithelia.

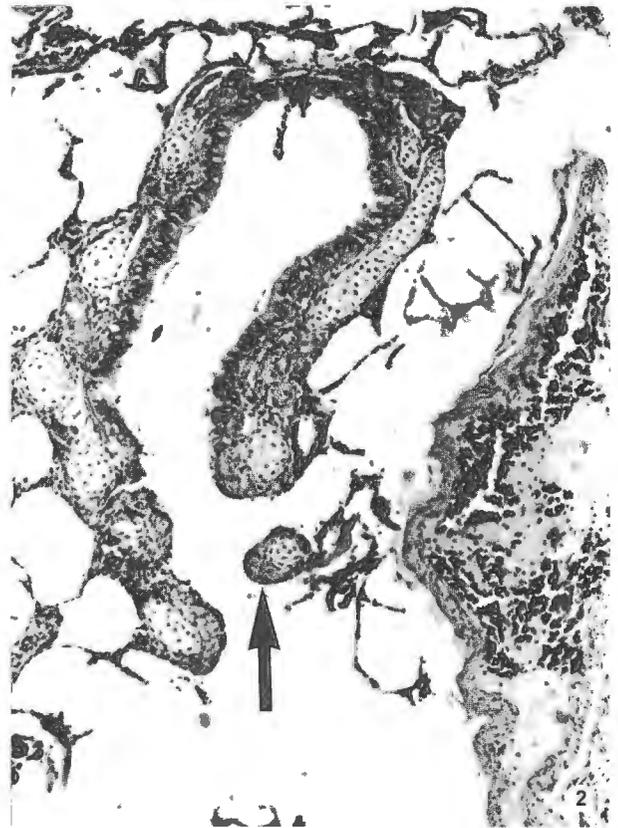


FIG. 1 Pseudostratified ciliated columnar epithelium with goblet cells (C), pseudostratified non-ciliated columnar epithelium (P) and simple cuboidal epithelium (S) can be seen in this airway. Also note the submucosal glands (G) and smooth muscle (M), H and E stain, semithin section, x 200

FIG. 2 The most distal airway, giving rise to alveolar ducts, with the cartilage (arrow) at the entrance to the ducts. H and E stain, paraffin section, x 100

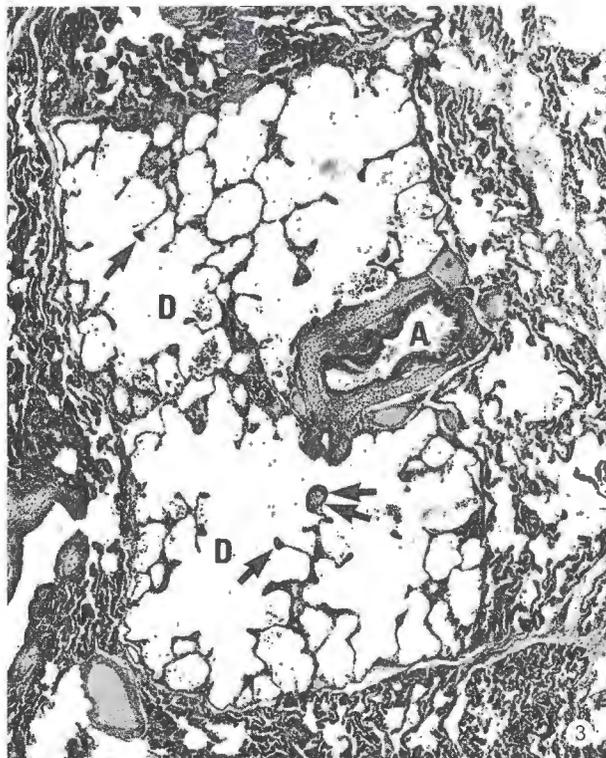
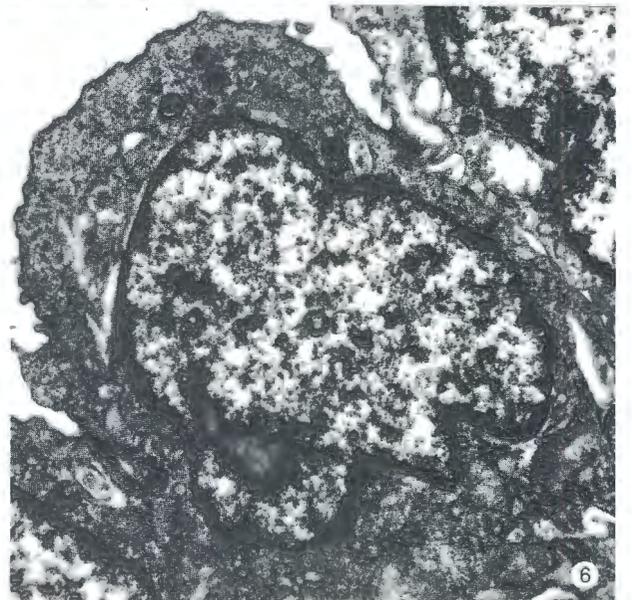
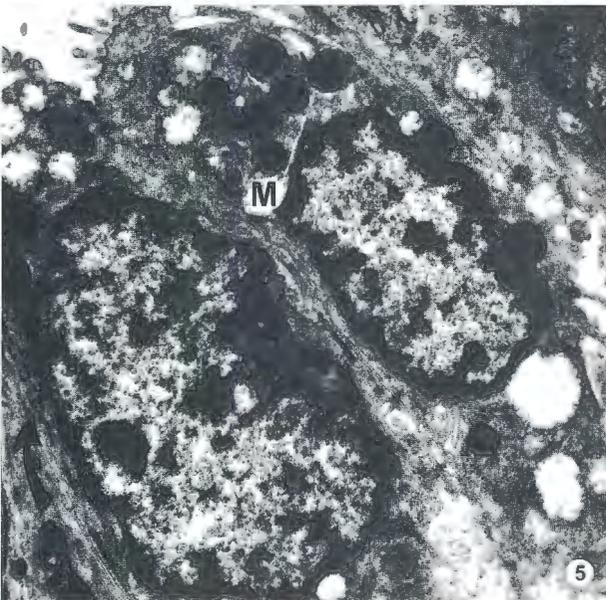
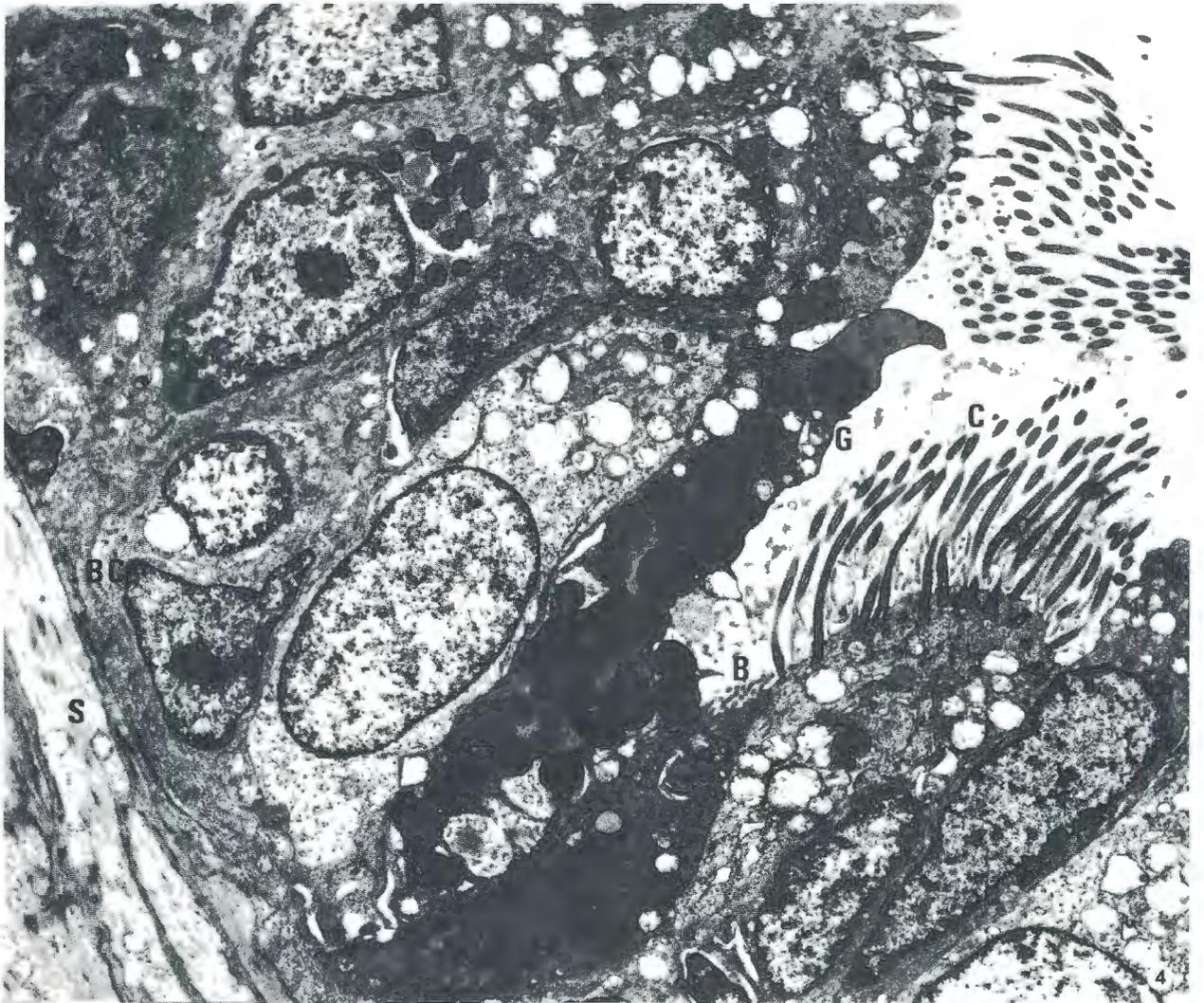


FIG. 3 An intralobular airway (A) with inflated alveolar ducts (D) and prominent alveolar knobs (single arrows). The piece of cartilage at the entrance to two alveolar ducts is also visible (double arrow). H and E stain, paraffin section, x 40

FIG. 4 Pseudostratified ciliated columnar epithelium. Goblet cell with electron dense secretory granules (G), brush cell (B), ciliated columnar cell (C), basal cell (BC), smooth muscle cell (S). Thin section, x 2500

FIG. 5 Two bronchiolar serous cells with apical electron dense granules and a few apical microvilli. Mitochondria (M) and granular endoplasmic reticulum (arrow) are also present in moderate amounts. Thin section, x 6000

FIG. 6 A cuboidal epithelial cell with apical cytoplasm bulging into the lumen of a small bronchiole. Thin section, x 2500



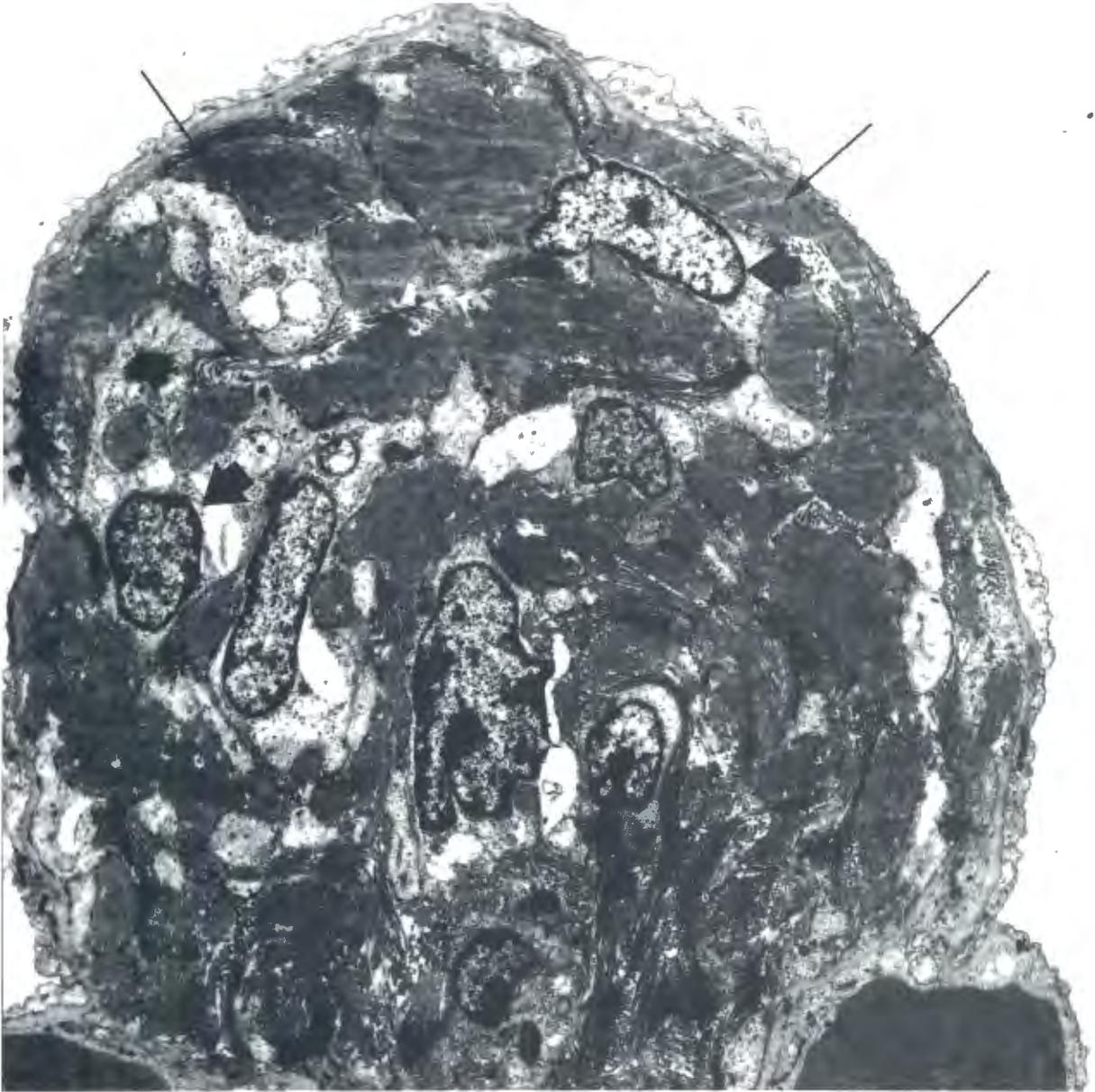


FIG. 7 Alveolar knob with large areas occupied by elastic fibres (thin arrows). Cells which are probably fibroblasts are also present (thick arrows). Thin section, x 2500

Simple cuboidal epithelium

CUBOIDAL EPITHELIAL CELL

The cuboidal epithelial cells were dome shaped, projecting into the lumen of the airway and resting on the basal lamina (Fig. 6). They were joined together by lateral cell junctions. A few dense cytoplasmic granules, about 210–380 nm in diameter, were present in the apical and basal cytoplasm of the cells. Myelin figures were seen in the basal cytoplasm of one of these cells. A few mitochondria and polyribosomes were also present in the basal cytoplasm. The

nucleus was generally round, with one or more clefts or invaginations. The basolateral cell membrane formed complex interdigitations with adjacent cells.

Alveolar knob

The alveolar knobs consisted mainly of elastic fibres, but some collagen was also present. The centrally situated cells were probably fibroblasts (Fig. 7). The alveolar knobs were covered by a simple squamous epithelium, resting on a basal lamina. A few capillaries were visible just below the basal lamina.

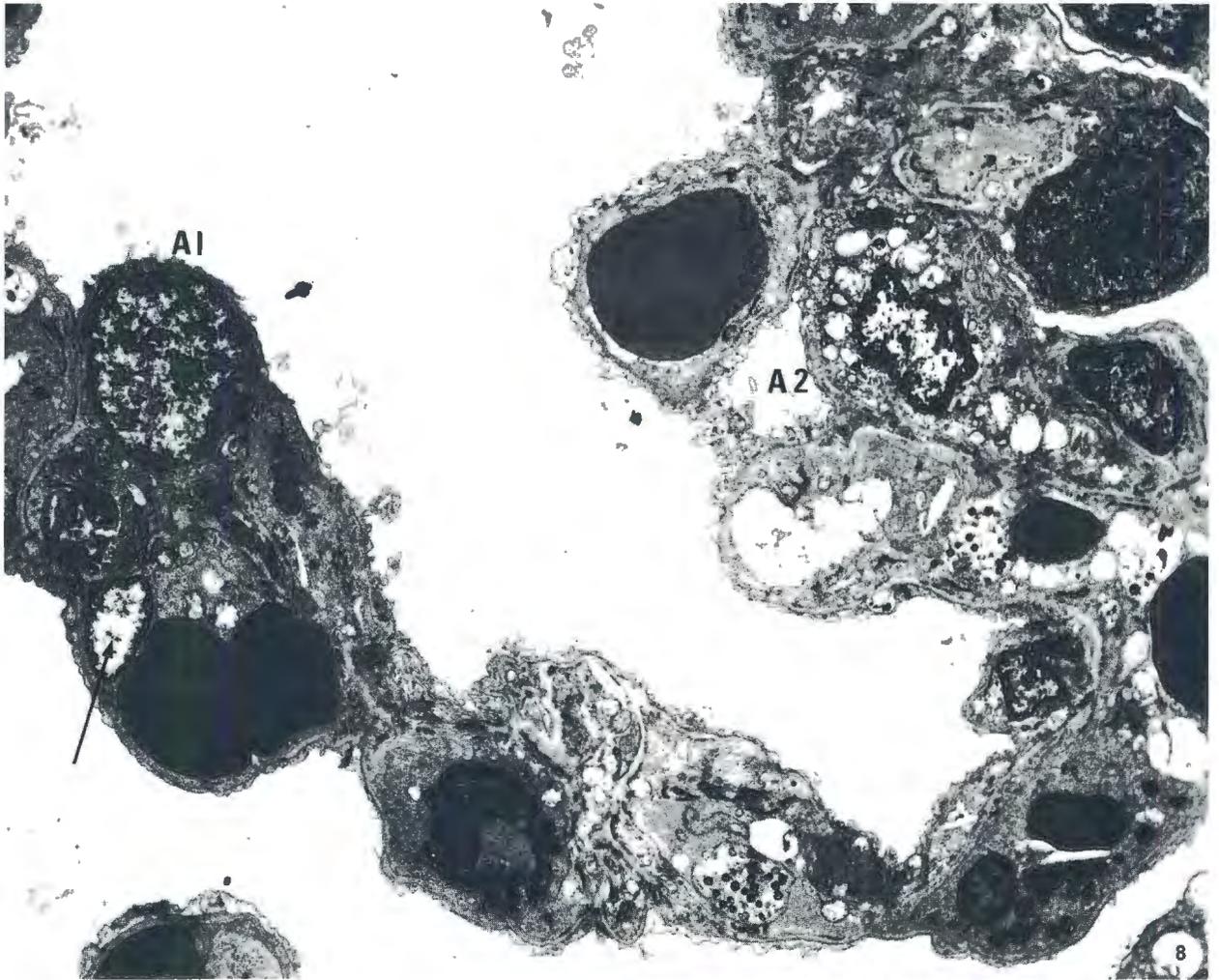


FIG. 8 Alveolar septum. Type I alveolar epithelial cell (A1), type II alveolar epithelial cell (A2), endothelial cell (arrow). Thin section, x 1500

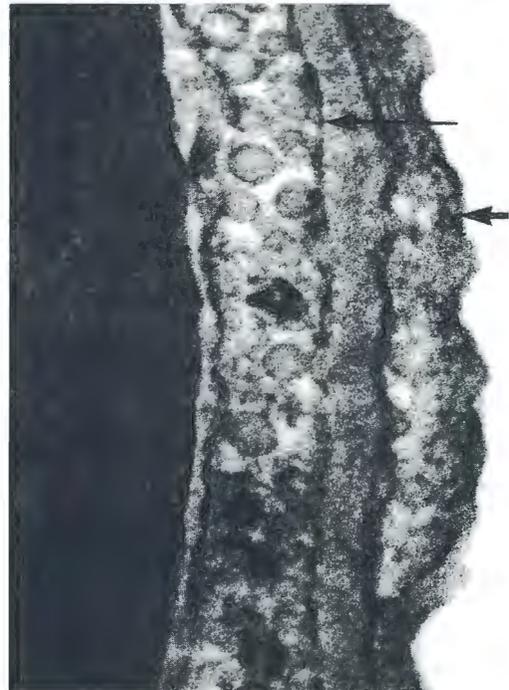


FIG. 9 Blood-air barrier. Cytoplasm of type I alveolar epithelial cell (short arrow) and cytoplasm of endothelial cell (long arrow), with the fused basal lamina in between. Within the capillary is a red blood cell. Note the numerous pinocytotic vesicles in the endothelial cell. Thin section, x 20 000

Alveolar septum

TYPE I ALVEOLAR CELLS

Their attenuated cytoplasm lined large areas of the alveoli (Fig. 8).

TYPE II ALVEOLAR CELLS

The cuboidal cells with foamy cytoplasm seen in semi-thin sections probably corresponded to these cells. They were more-or-less cuboidal, rested on a basal lamina and formed cell junctions with type I alveolar cells (Fig. 8). They had a few apical microvilli. Membrane bound organelles with concentric lamellar inclusions were seen in some of these cells, and clear vacuoles in others. This variation may have been the result of poor fixation of lamellar inclusions, or it may indicate the presence of a different content of more saturated lipids, in the clear vacuoles.

ENDOTHELIAL CELLS

Capillaries formed by endothelial cells with many pinocytotic vesicles were present in the interalveolar septum. They were surrounded by a basal lamina which fused with that of the type I alveolar cells to form a single layer where these two cells were adjacent (Fig. 9).

COLLAGEN AND ELASTIC FIBRES

The extracellular matrix of the alveolar septa contained small numbers of collagen and elastic fibres (Fig. 8). No smooth muscle cells were seen at this site, under the electron microscope.

Blood-air barrier

The thickness of the blood-air barrier ranged between 0,25–1,7 μm which is within the normal range found in mammals (Meban 1980).

DISCUSSION

The light microscopical observations made in this study were similar to those reported by Denison & Kooyman in 1973, although they provided much less histological detail. They referred to 'distal airways' in the otariid lungs, and did not use the term bronchiole.

The histological definition of a bronchiole in marine mammals is controversial because there is a wide variation in mammalian airway histology, with marked differences between terrestrial and marine mammals. Terms such as *small* or *terminal airways* were used by Simpson & Gardner (1972), Denison & Kooyman (1973) and Henk & Haldiman (1990) in their descriptions of the lung histology of marine mammals. To distinguish between primary and secondary lobulation when examining a histological section, is also

very difficult. If one considers a bronchiole as that extension of the bronchus that appears to enter a secondary lobule, the bronchiole of the SA fur seals had a diameter of 340–520 μm with a pseudostratified ciliated columnar epithelium and goblet cells. The terminal bronchiole had a diameter of about 300 μm with a simple cuboidal to squamous epithelium. No respiratory bronchioles could be identified, i.e. no alveoli opened directly from the bronchioles.

The reinforcement of terminal airways by cartilage as seen in this study is found in other Otariidae as well as in Cetacea. (Simpson & Gardner 1972; Denison & Kooyman 1973; Henk & Haldiman 1990), but not in terrestrial mammals (Junqueira, Carneiro & Kelly 1992). Experimental work on isolated lungs of sea lions and dogs has shown that under pressures such as those occurring during dives, the terminal airways of sea lions (Otariidae) remain open, but the alveoli collapse. The alveolar air is therefore displaced to the non-respiratory portions of the airways, preventing absorption of nitrogen and inert gas narcosis. In the dog lung, the terminal airways collapse before the alveoli do, so air is trapped in the alveoli and the lungs collapse less completely (Denison, Warrell & West 1971).

It is also very probable that the more rigid, cartilage-reinforced terminal airways in otariid lungs permit rapid, forceful respiration with high peak flows during surfacing. This would be necessary as these mammals stay at the surface for such a short time in between dives. The Phocidae spend a longer time on the surface between dives. Their smallest terminal airways are reinforced with muscle and not cartilage, presumably because they do not need high peak flows (Denison & Kooyman 1973).

Furthermore, Boshier & Hill (1974) have proposed that plication of the alveolar walls during their collapse, and decreased alveolar capillary blood flow due to shunting, retard absorption of residual alveolar gas during a dive. Thus there may well be multiple mechanisms for prevention of inert gas narcosis in seals. Further research on the diving behaviour of marine mammals and the changes that occur during diving is needed to elucidate the relationships between structure and function.

Considering the cellular composition of airway epithelium, Breeze & Wheeldon (1977) identified non-ciliated secretory cells in the proximal airways (bronchi) of the rat as epithelial serous cells. These cells were columnar and contained numerous electron-dense granules and extensive granular endoplasmic reticulum. Some of them had a dome-shaped apical portion which protruded above the adjacent ciliated cells into the bronchial lumen. This description is similar to that of the cells seen in the short section of non-ciliated pseudostratified columnar epithelium in our seal airways, and we therefore called them serous cells.

The other possibility is that they are Clara cells, which are numerous in the distal airways of many mammals, e.g. rat, sheep and rhesus monkey. Clara cells are present throughout the airways of certain other mammals, viz. mice, hamsters and rabbits. There is a large interspecies variation in the ultrastructure of these cells. In many species, e.g. rodents, pigs, sheep, horses, abundant smooth endoplasmic reticulum is a characteristic feature of these cells. In primates, however, there is very little smooth endoplasmic reticulum, but larger amounts of granular endoplasmic reticulum are present. Numerous mitochondria and secretory granules are a common feature of Clara cells in most of the species studied. This has been reviewed by Plopper, Hyde & Buckpitt (1991).

In marine mammals, Welsch & Drescher (1982) mentioned cells 'which may be termed Clara cells' in the bronchioles of Antarctic seals (*Lobodon carcinophagus* and *Leptonychotes weddelli*). They noted the presence of dense bodies and vesicles in the protruding apical region of these cells, but no further ultrastructural detail was given. Boyd (1973), as well as Boshier & Hill (1974) who studied the ultrastructure of the distal airways of *Leptonychotes weddelli*, did not mention either serous or Clara cells. These seals are Phocidae. No electron microscopical studies on otariid airways have been published, so the Clara cell in these species remains undefined.

If the columnar granulated cells we have described are serous cells, then we found no Clara cells in the pseudostratified epithelium in our samples. Another obvious place to look for Clara cells is in the simple epithelium distal to the pseudostratified type. Here we found a uniform population of cuboidal epithelial cells with relatively little cytoplasm, few mitochondria and few dense granules. These cells are not likely candidates for classification as Clara cells, and the granules are probably lysosomes.

In the absence of Clara cells, it is possible that other cell types are responsible for their functions. For example, secretion of antiprotease by Clara cells has been demonstrated in man (Plopper *et al.* 1991). An antiprotease has also been demonstrated in the serous cells of human submucosal glands (Kramps, Franken, Meijer & Dijkman 1981). This type of gland is also present in the proximal airways of the SA fur seal. The Clara cell acts as a stem cell for the airway epithelium (Ayers & Jeffery 1988). Basal cells also have this function, which is therefore available in all the pseudostratified epithelia. In the cuboidal epithelium lining the airways just proximal to the alveolar ducts in our material, there were no basal cells and we presume that the cuboidal cells themselves are able to divide. The phospholipids produced by Clara cells probably have important antibacterial and lubricating functions (Girod, Zahn, Plotkowski, Beck & Puchelle 1992). Serous and mucous granules in the

secretory cells of human submucosal glands also contain phospholipids (Girod *et al.* 1992), and presumably the serous cells of the surface epithelium will have similar granules. In the cuboidal epithelium of the distal airways of the SA fur seal, where Clara cells were conspicuously absent, it is possible that alveolar surfactant could supply the necessary phospholipid, as this area is so close to the alveoli. Thus it appears that a number of Clara cell functions can also be performed by other cell types, making the absence of Clara cells in the SA fur seal a reasonable proposition.

Another area of confusion in the nomenclature of airways of marine mammals is the alveolar duct/sac. Denison & Kooyman (1973) described alveolar sacs but implied the absence of alveolar ducts in otariid lungs. The presence of rows of alveolar knobs in a longitudinal section in our material suggests that this structure is actually an alveolar duct. This duct would end in an alveolar sac with knobs at the entrance only, as in terrestrial mammals (Junqueira, Carneiro & Kelly 1992). Because we did not study serial sections, we cannot be dogmatic on this point, but in some fortuitous sections we did see structures which conformed with this description; viz. alveolar duct opening into an alveolar sac.

In the alveolar knobs of the SA fur seal we found numerous elastic fibres, whereas in terrestrial mammals (Junqueira *et al.* 1992) and in the Weddell seal (Boshier & Hill 1974) there is smooth muscle in these knobs. The elastic fibres would allow expansion without overstretching, and help to maintain the structure of the delicate alveolar ducts during the collapse and reinflation caused by diving.

These preliminary observations on the ultrastructure of the distal airways of the SA fur seal open up an area of research which has been extensively studied in terrestrial mammals, but not in marine mammals. This is presumably for ethical reasons and because the marine mammals are less accessible. If, and when, further material becomes available, a more detailed study than the present one needs to be done, including some investigation into the relative numbers of each type of cell in the epithelium. Histochemical methods are also required to establish the contents of secretory granules, thereby relating structure to function.

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