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ULTRASTRUCTURAL EVALUATION OF BRONCHIAL LYMPHOID AGGREGATES AND LYMPHOID NODULES IN CALVES AT DIFFERENT AGES*

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SUMMARY

This study was conducted with the aim of defining the ultrastructure of bronchial lymphoid aggregates and lymphoid nodules of the bronchus-associated lymphoid tissue (BALT) in 3-, 6- and 8-month-old calves. A total of 9 calves divided into 3 age groups of 3 calves in each group was used in this study. Samples of the lungs were taken systematically and processed for scanning electron microscopic (SEM) and transmission electron microscopic (TEM) examinations. The results revealed differences between the epithelium of lymphoid aggregates and lymphoid nodules. SEM showed the absence of cilia over the lymphoid nodules but not over aggregates. Flattened, non-ciliated epithelial cells which may be precursors of the M cells were found in lymphoepithelium (LPE) of 3-month-old calves over nodules. The non-ciliated epithelial cells over nodules in 6- and 8-month-old calves were columnar in bronchi, but in bronchioles they were cuboidal. However, in the 3-month-old calves, some were columnar and some flattened in bronchi and in bronchiole, they were cuboidal. Transmission electron microscopic examination showed the presence of two types of M cells found in the LPE. Type 2 M cells were found in 3- and 6-month-old calves while type 1 M cells predominated in the bronchi in 6- and 8-monthold calves. This work represents the first systematic investigation of the similarities and differences in the ultrastructure of lymphoid aggregates and lymphoid nodules in calves and changes therein as a function of age.

Keywords: Ultrastructure, bronchial lymphoid aggregate, lymphoepithelium, lymphoid nodule, calves

INTRODUCTION

Bronchus-associated lymphoid tissue (BALT) consists primarily of lymphoid cells aggregates and nodules covered by epithelium that is structurally different from typical bronchial epithelium. This special epithelium, or lymphoepithelium (LPE), covers the BALT surface and modifies it in ways that facilitate contact between foreign materials and the surface of epithelial cells surfaces (Fournier *et al.*, 1977). LPE contains 'membranous' or M cells (Owen and Jones, 1974) which take up and transport macromolecules and pathogenic antigens into the BALT (Sicinski *et al.*, 1990). The passage of antigens through the M cell is an essential step for the induction of mucosal immune responses that prevent or modulate many infectious diseases (Amerongen *et al.*, 1991; Morin *et al.*, 1994). The lymphoid cells compartment of BALT typically contains lymphocytes, macrophages and dendritic cells, plasma cells as well as specialised venules with 'high' endothelium (Anderson *et al.*, 1986). LPE is often heavily infiltrated with intraepithelial lymphocytes (IEL) from underlying follicles (Bienenstock and Johnston, 1976). Knowledge of the normal surface topography of the epithelium of lymphoid aggregates and LPE is essential for understanding the physiological functions and pathological alterations of the intrapulmonary airways. The purpose of this study is to define, in relation to their age, the ultrastructure of LPE of lymphoid aggregates and lymphoid nodules of the BALT in calves, while also exploring and defining the morphology of lymphoid cell compartments.

MATERIALS AND METHODS

Animals

A total of nine calves divided into three age groups of 3-, 6- and 8-month-old and consists of 3 calves in each group was used in this study. The calves were divided into three groups (n=3) according to age. The calves were supplied by the Research Park, Universiti Putra Malaysia.

Tissues sampling, processing and examinations

Upon slaughtering the animals, the lungs were removed and washed with phosphate buffer saline solution (pH 7.3). Tissue samples from the right anterior cranial lobe (ac), right posterior cranial lobe (pc) and right caudal lobe (ca) were collected. The samples from each region were trimmed to approximately 1 cm^3 for scanning

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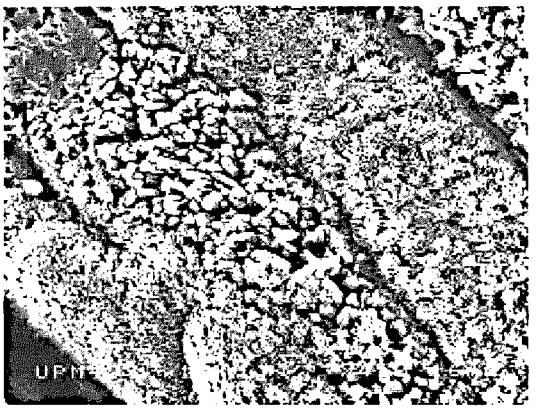

Figure 1: Scanning electron micrograph of the epithelium overlying the lymphoid nodules of bronchi in 6-month-old calf. Note that the ciliated epithelial cells area (CEA) and non ciliated cells (cuboidal shaped) area (NCEA) are found on this epithelium(x400).

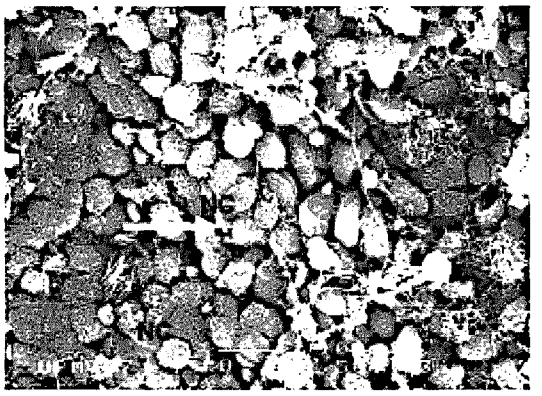

Figure 2: Scanning electron micrograph of the central area of lymphoepithelium (LPE) overlying the lymphoid nodules in bronchi. Note that the non-ciliated cells (NC) are found in this area and have small membrane projections (arrows)(x500).

electron For SEM, the tissues were dehydrated in graded series of acetone solutions (35%, 50%, 75%, 95% and 100%) and dried using a critical point dryer (CPD 030 Bal-TEC, Switzerland), mounted on an aluminum stub and coated with gold (SEM Coating Unit ES100, Polaron Equipment, England). The samples were viewed under a JSM 6400 SEM and photographed. For TEM, the dehydrated samples were embedded with resin-acetone mixture. The fixing and rinsing stages were carried out at 4°C. For TEM, the samples were rinsed and dehydrated in the same manner as the SEM samples, infiltrated with resin-acetone mixture at 4°C, embedded in a capsule with resin mixture and polymerised in oven at 60°C for 24 - 48 hours. Semi-thin sections (1μm thick) were cut by ultramicrotone (Ultracut E Recher-jung, Austria). Ultra-thin sections of 80-90 nm were also cut from selected areas and mounted onto 400-mesh-coper grids. Semi-thin and ultra-thin sections were stained with toludine blue and double-stain with uranyl acetate and lead citrate respectively and examined under TEM (Hitachi H7100, Japan).

RESULTS

SEM of the epithelium of lymphoid nodules

The lymphoepithelium areas of lymphoid nodules in 3- month-old calves were fewer than those in the 6- and 8- month-old calves. The lymphoid nodules of calves in all ages were dome-shaped. The epithelium around the dome area in the 6- and 8- month-old calves was covered by ciliated epithelium, while the dome epithelium or lymphoepithelium was lined by non-ciliated epithelial cells (Figure 1). Scanning electron microscopy of the bronchi in all ages revealed a ciliated surface with small islands of interspersed microvillous cells or M cells. These non-ciliated cells varied in numbers of short, irregular microvilli or microfolds, which were sometimes flattened to form irregular ridge like microplicae. In the central area of the dome, the region of flattened, relatively smooth epithelium was present and the epithelial cells, while devoid of cilia, consistently possessed small membrane projections (Figure 2). These non-ciliated cells

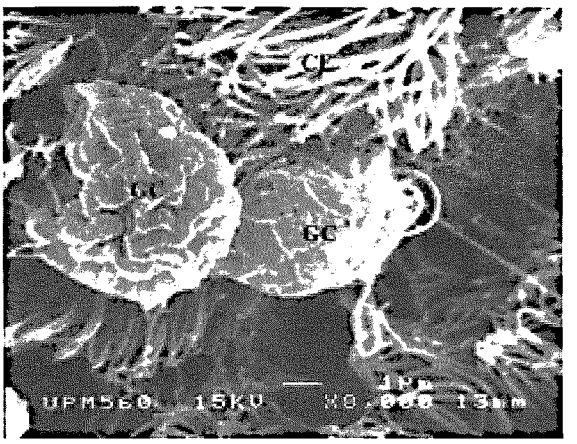

Figure 3: Scanning electron micrograph shows the ciliated epithelial cells area of the bronchi. Note that this area is comprised of ciliated epithelial cells (CE) and goblet cells (GC) (x 8, 000).

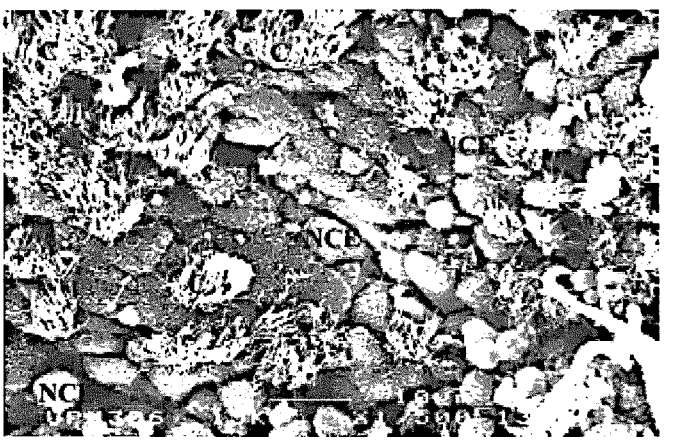

Figure 4: Scanning electron micrograph of the epithelium overlying lymphoid nodules of bronchioles in 6-month-old calf. Note that the epithelium is lined by non-ciliated cuboidal epithelial cell (NCE2) and ciliated cuboidal epithelial cells (CC) (x 1,500).

showed marked heterogeneities in the length and number of microvilli. Outside the LPE, the epithelium was composed of ciliated cells, microvillous cells and goblet cells in varying proportions (Figure 3). The LPE of bronchi of the 3-month-old calves consists of the non-ciliated columnar and flattened epithelial cells. The contours of luminal surfaces of the non-ciliated cells in calves of this age were varied with very few ciliated cells present in this epithelium.

The LPE of lymphoid nodules in the 6- and 8-month-old calves consisted of both non-ciliated cuboidal and columnar epithelial cells with the latter being most numerous. The columnar cells were often scattered in small groups and increased in number slightly in the 8-month-old calves. The LPE of bronchioles in all calves consisted of a single layer of cuboidal epithelial cells which were both ciliated and non-ciliated (Figure 4).

The LPE in bronchiole of calves in all ages included a well-defined population of non-ciliated cells. These cells, which covered the dome area of the LPE, were easily distinguished from the adjacent respiratory epithelium and had numerous short microvilli that showed only slight variation in number, density and length. Non-ciliated cells were seen more scattered among ciliated ones and increased slightly in number and changed progressively with increasing age from cuboidal to columnar. Some of the non-ciliated cells in the 3- and 6-month-old calves have cytoplasmic protrusions along their cell boundaries while others were characterised by an almost smooth luminal surface. However, in contrary, some of the non-ciliated cells in the 8-month-old calves were marked by numerous pinpoint pits in the surface (Figure 5).

SEM of the epithelium of lymphoid aggregates

The epithelium of aggregated lymphoid tissue in the bronchi in all age groups was covered by pesudostratified columnar ciliated epithelium with goblet cells (Figure 6). The deeper layer consisted of mostly basal and intermediate cells. Some of the epithelial cells could be seen to be attached to the basement membrane.

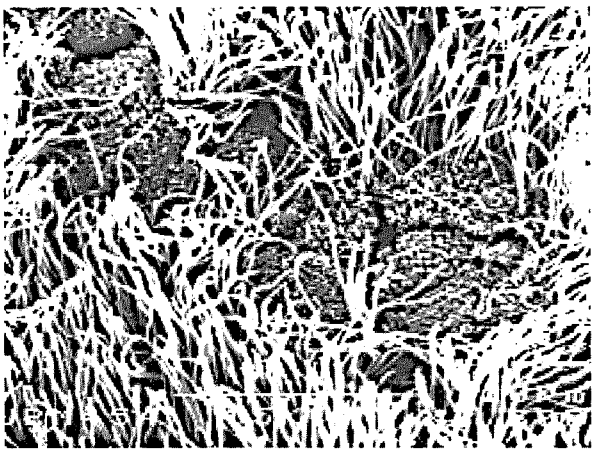

Figure 5: Scanning electron micrograph shows non-ciliated cells of lymphoepithelium in bronchiole in the 8-month-old calf. Non-ciliated epithelial cells can be distinguished from the adjacent ciliated epithelial cells and their luminal surfaces have a numerous pinpoint pits (arrows) (x 3,500).

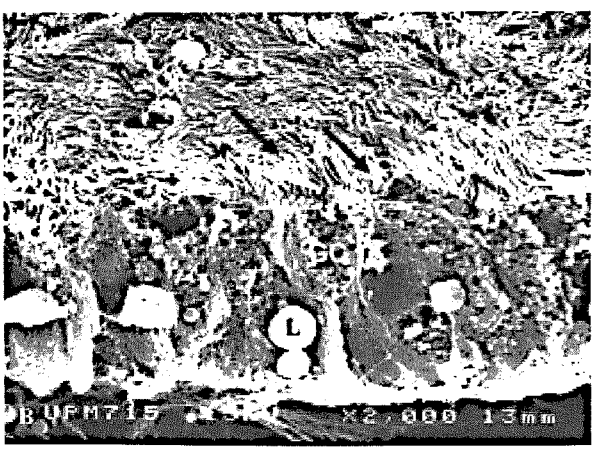

Figure 6: Scanning electron micrographs show the epithelium of dense aggregated lymphoid tissues. B) The epithelium is lined by pseudostratified columnar ciliated epithelium with IELs (L) and goblet cells (GC).

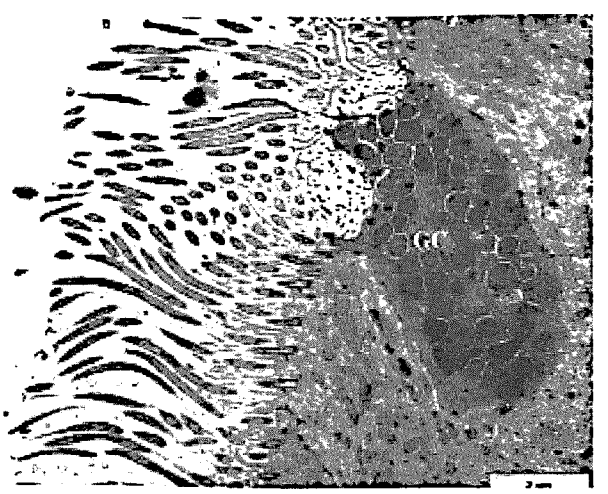

Figure 7: Transmission electron micrograph shows the ciliated cell and goblet cell in epithelium of the dense aggregated lymphoid tissue of BALT in all age groups. Note that the ciliated epithelial cells frequently contain necrotic organelles in their cytoplasm and the goblet cell has electron-dense granules. Bar= 2μm

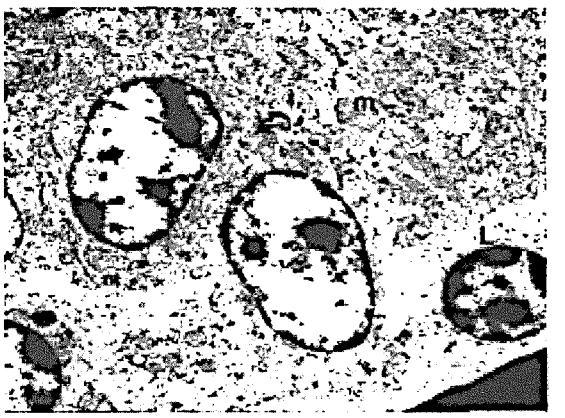

Figure 8: Transmission electron micrograph shows the M cell (Type 1) in lymphoepithelium of lymphoid nodule in BALT. Note that the large number of mitochondria (m) increases towards the luminal surface of the M cell and the M cell associates with lymphocytes (L). Bar= 2μm.

Figure 9: Transmission electron micrograph shows the M cell (Type 2) in lymphoepithelium of lymphoid nodule in BALT. Note that the nucleus of the cell is cuboidal shape and its cytoplasm contains with vesicles (V), electron dense granules and abundant mitochondria. This cell is connected with adjacent cell by desmosome. The luminal surface has short microvilli (arrows). Bar= 2μm

TEM of the epithelium of lymphoid aggregates

The epithelium of lymphoid aggregates in calves of all ages were covered by ciliated columnar epithelial cells, non-ciliated cells and goblet cells (Figure 7). The cytoplasm of goblet cell contained more electron-dense than that of the ciliated columnar cells. The latter contained autophagosome and degenerating organelles in their cytoplasm. The cilia and microvilli of the tall columnar cells were long, uniform and oriented in parallel.

TEM of the epithelium of lymphoid nodules

The LPE of the lymphoid nodules was lined by non-ciliated or microvillous (M) cells and intraepithelial lymphocytes. Two types of microvillous cells were found in the LPE. Type 2 M cells were found in the 3- and 6-month-old calves. These cells were generally scattered, cuboidal with a nucleus that had one or more eccentric nucleoli and a clump of chromatin near the inner nuclear membrane. Type 1 M cells were present mostly in the bronchi of 6- and 8- month-old calves. They were columnar with tall and cylindrical nuclei that have clumps of chromatin (Figure 8). The cells also consisted of highly electron-dense particles and appeared darker than the respiratory epithelial cells. Their cytoplasm contained large numbers of oval to elongated mitochondria, lacked smooth endoplasmic reticulum and few Golgi bodies. The mitochondria were found predominantly in the supernuclear part. The apical surfaces of cell had few long microvilli and were associated with lymphocytes.

The apical surface of both types of M cells contained variable numbers of microvilli. Elongated mitochondria which were predominantly supranuclear were abundant together with numerous small vesicles towards the luminal surface (Figure 9). Smooth and rough endoplasmic

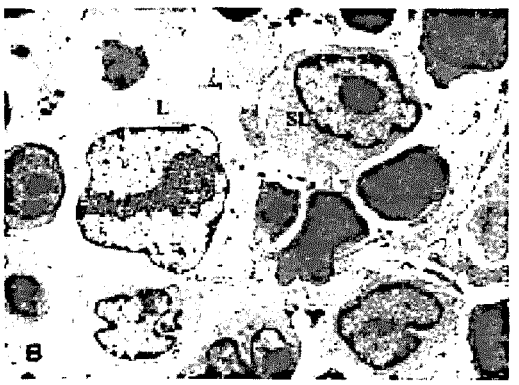

Figure 10: Transmission electron micrograph shows lymphocytes (L) during mitotic state in the 6 month-old calf. Note that the lymphoblast (L) and small lymphocytes (SL) are found in the germinal center during mitotic state. Bar= 2μm

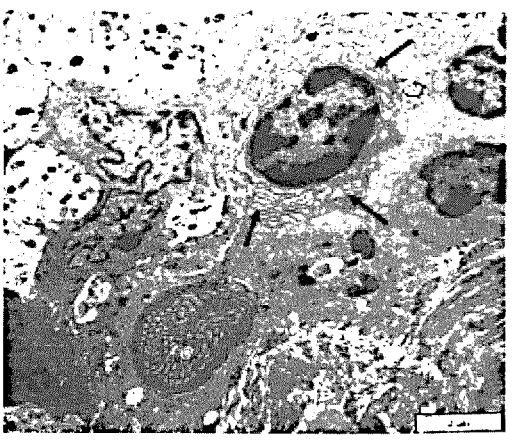

Figure 11: Transmission electron micrograph shows antibody producing plasma cells in the dome area of lymphoid nodules and lamina propria of lymphoid aggregates in BALT. Note that large quantities of rER (arrows) are dilated with a finely granular product that represents newly synthesis antibody. Bar= 2μm

reticulum, a few Golgi bodies, lysosomes, glycogens granules and a few of very large-sized vacuoles were also identified. These cells were connected to the adjacent cells by desmosomes. There was a great increase in the number of tonofilaments, but few cytoplasmic organelles were present.

TEM of the lymphoid cells compartments

The lymphoid nodules and the lymphoid aggregates comprised of lymphocytes, macrophages, follicular dendritic cells and plasma cells. The plasma cells of the dome area of lymphoid nodules were more in number than those in the lymphoid aggregates. The lymphocytes in the lymphoid nodules showed typical feature of small lymphocytes. The lymphoid nodules were heterogeneous in size and distribution. Lymphoblasts were found predominantly in the center of follicles and they were homogenous in appearances. A small number of lymphoid cells in the germinal centers of lymphoid nodules showed mitotic figures in the 6- and 8 month-old calves (Figure 10). Lymphocytes displayed scant peripheral cytoplasm containing few mitochondria and a small amount of Golgi apparatus and endoplasmic reticulum.

Occasionally macrophages were seen in the germinal centres and were surrounded by lymphocytes in some part of the follicles. The macrophages were also present in the follicle, dome area and lamina propria of lymphoid aggregates.

Plasma cells were abundant in the dome of lymphoid nodules and lamina propria of lymphoid aggregates. Plasma cells were round to oval and easily identified by their extensive rough and smooth endoplasmic reticulum (Figure 11). Their nuclei were very large, smooth and either centrally or peripherally located.

High endothelial venules (HEV) are specialised vessels that support active lymphocytes migration from peripheral blood to the secondary lymphoid organs. The HEV were found in the follicles with well-developed germinal centres. They were lined by high cuboidal

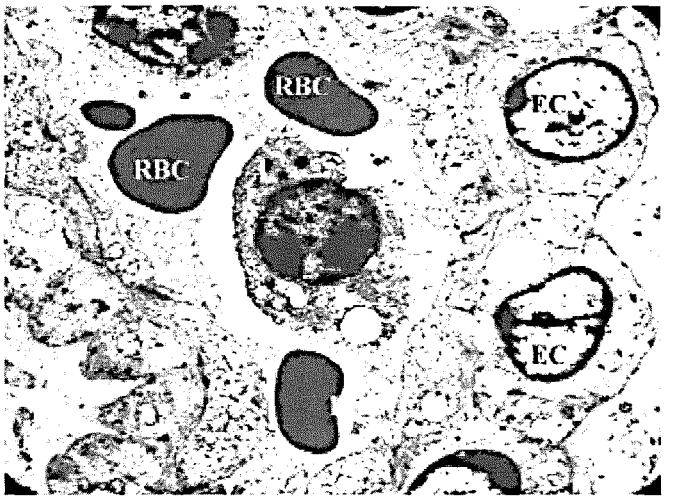

Figure 12: Transmission electron micrograph shows high endothelial venules (HEV) in parafollicular area of lymphoid nodule. Note that HEV is lined by simple cuboidal endothelial cells (EC). Lymphocyte (L) in the lumen of HEV is surrounded by the red blood cells (RBC). The nuclei of EC were irregular with peripheral condensation of chromatin towards the inner nuclear membrane. Bar= 2μm

endothelial cells, and closely-associated with lymphocytes and red blood cells. The lymphocytes were present within the luminal surface of HEV. The nuclei of the endothelial cells were irregular with peripheral condensation of chromatin towards the inner nuclear membrane (Figure 12).

Many lymphoid aggregates contained leukocytes-like cells with granules that were stained dark and had a homogeneous appearance. These cells were located superficial to the basement membrane of the epithelium adjacent to those follicles and formed clusters inside the lymphoid aggregates or occasionally on the luminal surface. Their granules resembled those of mast cells but were fewer than those of peripheral blood basophils. The nuclei of these leukocytes-like cells were always indented and had several lobes.

The IELs were individually and irregularly distributed among the epithelial cells. They were round or irregular in shape and generally located near the basement membrane. The IEL cytoplasm appeared denser than that of epithelial cells. It contained only a limited number of free ribosomes, a small amount of rough endoplasmic reticulum and round or oval mitochondria. Their nuclei were round, smooth and centrally located.

DISCUSSION

This study revealed clear differences between the epithelium of lymphoid aggregates and lymphoid nodules. SEM examinations showed the absence of cilia over the lymphoid nodules but present over lymphoid aggregates in all ages studied. The findings are similar to those described in ovine nasal tissue by Stanley *et al.* (2001). The non-ciliated cells in the calves were replaced by cells with short cytoplasmic projections similar to those seen in rabbit (Bienenstock and Johnston, 1976; Tenner-Racz *et al.*, 1979) and other mammals (Van der Brugge-Gamelkoorn and Kraal, 1985). In the present study, the flattened non-ciliated epithelial cells were found only in LPE of the 3-month-old calves and may be precursors of the M cells. However, in the 6- and 8-month-old calves, the non-ciliated epithelial cells were columnar in shape but in bronchioles, they were cuboidal. Regoli *et al.* (1994) speculated that the excessive invasion of lymphocytes into LPE might subsequently induce some subcellular changes in the non-ciliated cells. Thus, the shape of non-ciliated cells may depend on the age of the individual, and degree of invasion by lymphocytes. The expansion of LPE area in the 6- and 8-month-old calves was mainly due to the increase in number of the non-ciliated epithelial cells. Fagerland and Arp (1990) proposed that lack of goblet cells and the modification of microvillous cells in the LPE of lymphoid nodules may be mediated by lymphokines produced from infiltrating lymphocytes. This study has shown that the non-ciliated epithelial cells also contain variable-sized vacuoles and invaginated pits in their apical cytoplasm and enfold lymphocytes in that cytoplasm. Tingible-body macrophages were found in the follicles and dome area and may behave as antigen-presenting cells as described by Cunningham (1978). Plasma cells with extensive rough endoplasmic reticulum were found in the lamina propria of the bronchial mucosa. Basophils were found in and around the lymphoid nodules and their morphological structures were similar

to that observed in rabbits by Bienenstock and Johnston (1976). These cells may be involved in modulating resistance or susceptibility to disease.

The LPE can easily be distinguished from the adjacent epithelium, both topographically and ultrastructurally, and has been previously noted in related areas in many species. In this study, two types of M cells or non-ciliated cells (non-ciliated cuboidal cells and non-ciliated columnar cells) were observed in the LPE of BALT in the 3-, 6- and 8-month-old calves. This ultrastructural study clearly demonstrated that the morphology of non-ciliated cells appears to be related to age and number of IELs. This finding is similar to that of Kumar and Timoney (2005) who reported that two types of microvillous cells were found in follicle-associated epithelium of the tubal tonsil in the horse.

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