BOVINE FETAL LUNG DEVELOPMENT

by

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GENERAL INTRODUCTION

Most studies on pulmonary development have been done on rat or human fetal lungs. Other species studied include the dog, mouse, guinea pig, rabbit, pig, and sheep. Few studies have been reported on the large domestic animals which have a large number of respiratory disease problems, and economically important to animal industry.

Recently, a light and electron microscopic study of the development of the bovine lung was conducted in which three stages of fetal lung development were recognized: the glandular or pseudoglandular, the canalicular, and the alveolar or terminal sac period. However, light and electron microscopic studies of bovine fetal trachea, bronchus, bronchiole have not been reported. Further, information is lacking as to when alveolar macrophages and pulmonary surfactant appear in the fetal lung, nor do we know if Clara cells are present.

Information about respiratory system development may be important to our understanding of respiratory disease processes in the bovine species. It has been suggested that life is impossible without the development of Type II pneumocytes because these cells produce surfactant, which is necessary for lung function.

Gestational age alone does not provide adequate information about fetal maturity. Determination of lecithin levels in amniotic fluid are a sensitive indicator of fetal lung maturity . It has been established that the lecithin in amniotic fluid originate primarily from fetal lung.

Alveolar macrophages are responsible for the removal and distruction of small particles of approximately 1.0 micrometer or smaller found in the respiratory portion of the lung. Also alveolar macrophages have receptors for

lymphokines, chemoattractants, and histamine. Animal immune response requires the interaction of macrophage and T-lymphocytes.

The investigation reported on in this work was undertaken to investigate three areas: 1) to histochemically determine when alveolar macrophages appear in the fetal lung; 2) to correlate the morphologic characteristic of Type II pneumocytes with the appearance of pulmonary surfactant and, 3) to study using electron microscopic techniques the appearance of the fetal trachea, bronchus and bronchioles.

Part 1. TIME OF APPEARANCE OF ESTERASE - POSITIVE ALEVOLAR MICROPHAGES IN BOVINE FETAL LUNG

ABSTRACT

Fetal lung tissue obtained from abattoirs was examined histochemically using alpha-napthyl butyrate (nonspecific method), to defect the presence of pulmonary alveolar macrophages (PAM). Esterase-positive alveolar macrophages, which appear in the alveolar septa and lumina were first seen at 240 days of gestation. These macrophages were identified by the presence of numerous diffuse intensely red-stained granules in the cytoplasm. Macrophages at 240 days were 15.88 ± 1.67 Um (S.E.) (N=30) in size and in most cells the esterase-positive granules were sparsely distributed in the cytoplasm. Only four out of eight lungs examined at 240 days were positive for alveolar macrophages. However, all the lungs (N=10) examined from 250=day-old fetuses contained esterase-positive macrophages, which were distributed both in the alveolar septa and alveolar spaces. Compared to macrophages of the 240-day fetuses, those at 250 days were signi ficantly larger, 16.91 ± 1.33 µm (S.E.) (N=30) ($P<0.05$). Type II pneumocytes and T lymphocytes were not confused with PAM because in all lungs, T lymphocytes contained a single, small, esterase-positive granule compared with the numerous diffuse esterase-positive granules in PAM while type II pneumocytes reacted negatively to nonspecific esterase stain during lung development.

The time of appearance of alveolar macrophages coincides with the first appearance of surfactant at the alveolar surface-lining layer which suggests that macrophages could be involved with the clearing of excess surfactant being produced.

INTRODUCTION

The fully developed mammalian lung normally contains a resident population of pulmonary alveolar macrophages in the alveolar spaces and septa, which, according to recent studies[1], are derived primarily from circulating monocytes. Circulating mononuclear phagocytes not replication of cells of this resident population are responsible for any local increase seen in numbers of pulmonary alveolar macrophages. In the fetus, however Sorokin et al.[2] have demonstrated that a "resident, self-replicating population of macrophages becomes established at the outset of lung formation and is continued in evidence throughout much of the fetal period." In most instances studied, cells closely conforming to the microscopic appearance of macrophages are evident in the lungs of rabbit, rat, and hamster just prior to birth; however, cells "with the macrophages complete repertory of inclusions are seen only after birth"[2]. In the human, macrophages begin to populate the airspace surfaces during the saccular phase of lung development (± 168 days of gestation)[3]. The functional basis for the early appearance of such macrophage-like cells is not fully established but it has been suggested[4] that these cells are involved in clearance of alveolar surfactant.

Whether the appearance of histochemically identifiable pulmonary alveolar macrophages is associated with presence or appearance of surfactant in the bovine lung requires identification of the time of macrophage appearance. Therefore, the purpose of this study was to determine that time of first appearance of bovine pulmonary macrophages or macrophage-like cells.

MATERIALS AND METHODS

Tissue Collection:

Bovine lung tissues taken from the right caudal lobe of the lung of 83 Holstein-Friesian fetuses, were collected at nearby abattoirs.[a] The fetuses ranged in age from 80 - 270 days of gestation with age estimated using the criteria of Roberts[5]. Of the total number of fetal lungs collected, 10 each were taken from fetuses of 80, 120, 150, 200, and 250 days of gestation; 5 each from fetuses of 210, 220, 230, 260, and 270 days of gestation, and 8 from 240-day-old fetsuses. All lungs were removed from fetuses as soon as possible following death, consistent with practices of the abattoir. Tissues were fixed in cold (4C) formol-calcium at pH 7.0-7.2, and processed for histochemical analysis (nonspecific esterase method).

Histochemical Analysis:

Tissues were held in fixative at 4°C for 24 hours, washed in running tap water for 2-3 hours, placed in Tissue Tek II O.C.T. Compound (Miles Lab. Inc., Naperville, IL 60540) on small cork squares, frozen in 2-methyl butane cooled to the temperature of liquid nitrogen, sealed in plastic bags (Gemini Plastic Enterprises, Maywood, CA 90280), and stored at -85°C. Tissue sections were cut at 8.0 µm (-20°C) on a Slee Model HR Cryostate Microtome (Slee International, Tiverton, RI 02878), collected on coverslips, air dried for 2 hours, and incubated in alpha-napthyl butyrate-hexazonium pararosanilin solution (pH 7.2) for 5 minutes[6]. Sections were counterstained with an 0.5% aqueous solution of Methyl Green for 2 minutes, dehydrated in one change each of 95% ethanol and absolute ethanol for 15 seconds each, 45 second in absolute ethanol,

cleared for one minute each in two changes of toluol, and mounted with synthetic mounting medium on clean microslides.

Photomicrographs were taken using Kodak Panatomic X film in a Leitz Ortholux Microscope with Orthomat Camera attachment (Model PM-10-M). All measurements were made at a magnification of 1000X on a Zeiss RA Microscope using a calibrated eyepiece micrometer.

RESULTS

Lungs from fetal calves of 200 days gestation or less showed no positive nonspecific esterase reactions at the alveolar septa and alveolar space of the caudal lobe. In one of five lungs examined from 210 days old fetuses, cells with a diffuse distribution of esterase-positive granules typical of those seen in macrophages were found in the alveolar septa. The remaining four lungs at this stage showed no cells with diffuse esterase-positive reactions (Fig. 1). However, in all lungs at that stage cells were seen which contained a single, small, esterase-positive granule resembling that typical of an esterase-positive reaction of these T lymphocytes (Fig. 1). Other than T lymphocyte-like reactions in lungs from 220 and 230 day fetuses, no esterase-positive reacting cells were seen until 240 days of gestation. At 240 days, four of eight lungs examined contained numerous esterase-positive macrophages (Fig. 2). These macrophages were 15.88 ± 1.67 μm (S.E.) (N=30) in size and in most cells the esterase- positive granules were sparsely distributed in the cytoplasm (Fig. 1-Insert). While most of these cells were located in the alveolar septa (80%), some were seen in the alveolar spaces (20%).

All lungs (N-10) from 250 day old fetuses contained esterase-positive macrophages, which were distributed both in the alveolar septa and alveolar spaces. Compared to the macrophages of the 240 day fetuses, those at 250 days were significantly larger, 16.91 ± 1.33 (S.E.) (N=30) ($P<0.05$). Additionally, the esterase-positive granules of the macrophages were heavily distributed in the cytoplasm (Fig. 3). The five lungs each from calves of 260 and 270 days of gestation were identical to the 250-day fetal lungs in uniform presence, distribution, and size of the esterase- positive macrophages.

DISCUSSION

Many methods have been employed to aid in identification of macrophages. Among the more common techniques are those involving histochemical reactions that take advantage of the rich complement of lysosomal enzymes in macrophages. Nonspecific esterase identification of macrophages has been described by Koski et al.,[6]; nonspecific esterase identification of T Lymphocytes has been described by Knowles and Holck[7]. T lymphocytes display "a solitary red brown nodule of reaction product... which can be readily distinguished from the diffuse, cytoplasmic staining of monocytes..."[7]. In the macrophages, the nonspecific esterase reaction is distinguished by the presence of numerous diffuse and intensely red-stained granules in the cytoplasm against a green counterstain, which uniformly stains esterase-negative cells. Nonspecific esterase activity is strong in monocytes and macrophages and weak or absent in neutrophils, eosinophils, myeloblasts, promyelocytes, and erythroblasts.[8]

Several lines of evidence indicate that the pulmonary alveolar macrophage population is involved in the clearance of surfactant from the alveoli. The pulmonary alveolar macrophage is enveloped by surfactant in vivo[4], and the lysosomes of the pulmonary alveolar macrophage contain the hydrolytic enzymes necessary for surfactant degradation.[9,10]

This study has established that macrophage-like cells begin to appear in the alveoli of the lung between 240 and 250 days of gestation. The bovine lung, at this age, is in the alveolar stage of development.[11] Using tricomplex flocculation [$Pb(NO_3)_2$ and $K_3Fe(CN)_6$] and electron microscopy, Hambolu and Weinman (unpublished data) have shown surfactant at the alveolar surface-lining layer at 240 days of gestation. Macrophages, therefore, begin to appear at the same time as surfactant and thus could be directly involved with the clearing of excess surfactant being produced.

The appearance of esterase-positive cells in only one (210 days gestation) of the 55 lungs between 80 and 240 days of gestation used in this study cannot be readily explained. A possible explanation is that there was an error in the aging of the fetus.

Sorokin et al.[2] demonstrated that a resident, self-replicating population of macrophages becomes established at the outset of lung formation and remains in evidence throughout much of the fetal period in rabbit, rats, and hamsters. In humans, macrophages begin to populate the air-space surfaces during the saccular phase of lung development and this study has shown that macrophages populate the air space surfaces at 240-250 days (alveolar period) in the bovine. However, in both human and bovine, the pulmonary vascular supply forms during the earlier

canalicular period. These two factors suggest that pulmonary macrophages may originate from monocytes, an idea supported by van Furth,[1] who indicates that pulmonary alveolar macrophages are derived primarily from circulating monocytes.

A number of hydrolytic anzymes including nonspecific esterase[12] are present in type II pneumocytes. Esterase is present mainly in the lamellar inclusions, but with traces in the endoplasmic reticulum and mitochondria[12,13]. In the bovine, type II pneumocytes appeared at 200-210 days and lamellar bodies were seen using the electron microscope at 230 days (Hambolu and Weinman unpublished data). Esterase-positive, presumptive T lymphocytes were seen by the light microscope at 210 days and beyond. Type II pneumocytes never reacted to nonspecific esterase. Nonspecific esterase reaction in type II pneumocytes has only been documented in adult rats[14]. This suggests that during fetal life, type II pneumocytes are not reacting to nonspecific esterase and that the esterase-positive cells observed during the fetal periods are macrophage-like cells.

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Fig. 1. Bovine Fetal lung; 210 days gestation. Nonspecific esterase with methyl green counterstain. Cells showing large, multiple esterase-positive cytoplasmic granules are absent from alveoli and alveolar septa. Two cells (arrows) each show a single esterase positive granule similar to those seen in T Lymphocytes of mature lungs. Final mag. X275. Bar marker = 20 µm.

Fig. 2. Bovine Fetal Lung; 240 days gestation. Nonspecific esterase with methyl green counterstain. Multigranular, esterase-positive cells resembling macrophages (m) are present in alveoli and in alveolar septa. Final mag. X275. Bar marker = 20 µm. INSERT: High magnification of esterase-positive cells. Note the relative paucity of esterase-positive granules in cytoplasmic regions, compared with cells in Fig. 3, Insert. Final Mag. X550. Bar marker = 10 µm.

Fig. 3. Bovine Fetal Lung at 250 days gestation. Nonspecific esterase with methyl green counterstain. Numerous, macrophage-like cells (m) strongly reactive to nonspecific esterase stain are seen in alveoli and alveolar septa. Final mag. X275. Bar marker = 20 µm. INSERT: High magnification of esterase-positive cells; note size and distribution of cytoplasmic granules compared with cells at 240 days gestation (Fig. 2, Insert). Final mag. X550. Bar marker = 10 µm.

Fig. 1. Light photomicrophgraphs of fetal bronchioles. A. 230 days, B. 250 days, C. 260 days, D. 270 days of gestation. Note that at all stages of development all the mucosa cells of the bronchioles have nuclei at apical border (arrows) and the bronciole is surrounded by a layer of smooth muscle (a) (Harris hematoxylin and eosin stain. Paraffin embedded; glutaraldehyde fixed).

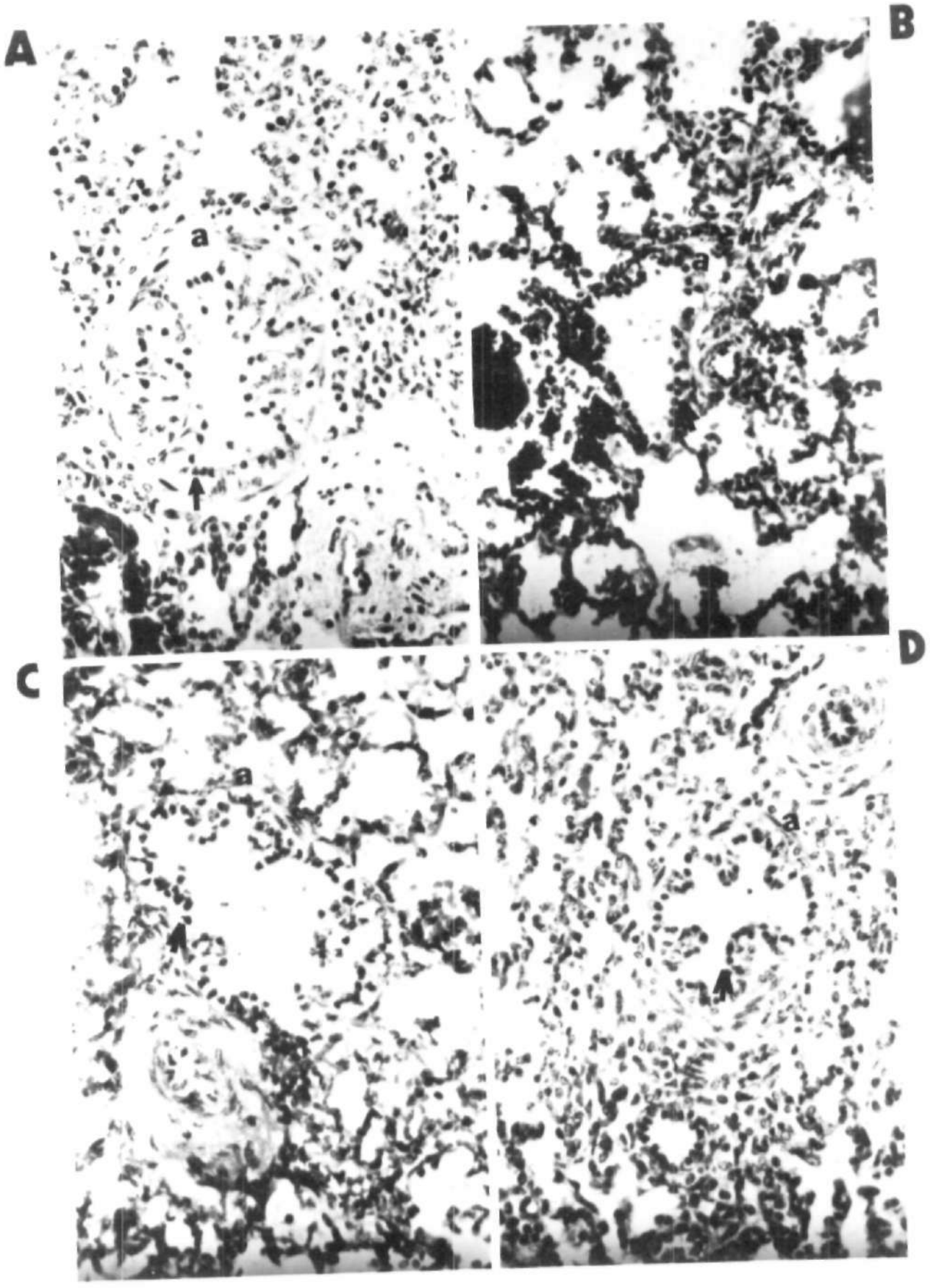

A
a
B
a
C
a
D
a

Fig. 2. Light photomicrographs of fetal bronchioles. A. 150 days, B. 180 days, C. 270 days of gestation. Note that at 150 and 180 days the nuclei of mucosa cells are apically located (arrows). (Toludine Blue X200). At 270 days some nuclei are centrally located (arrows) (Toludine Blue X200). D. Scanning micrgraph at 150 days showing bulbous aspices of the nonciliated cells (arrows) X4000.

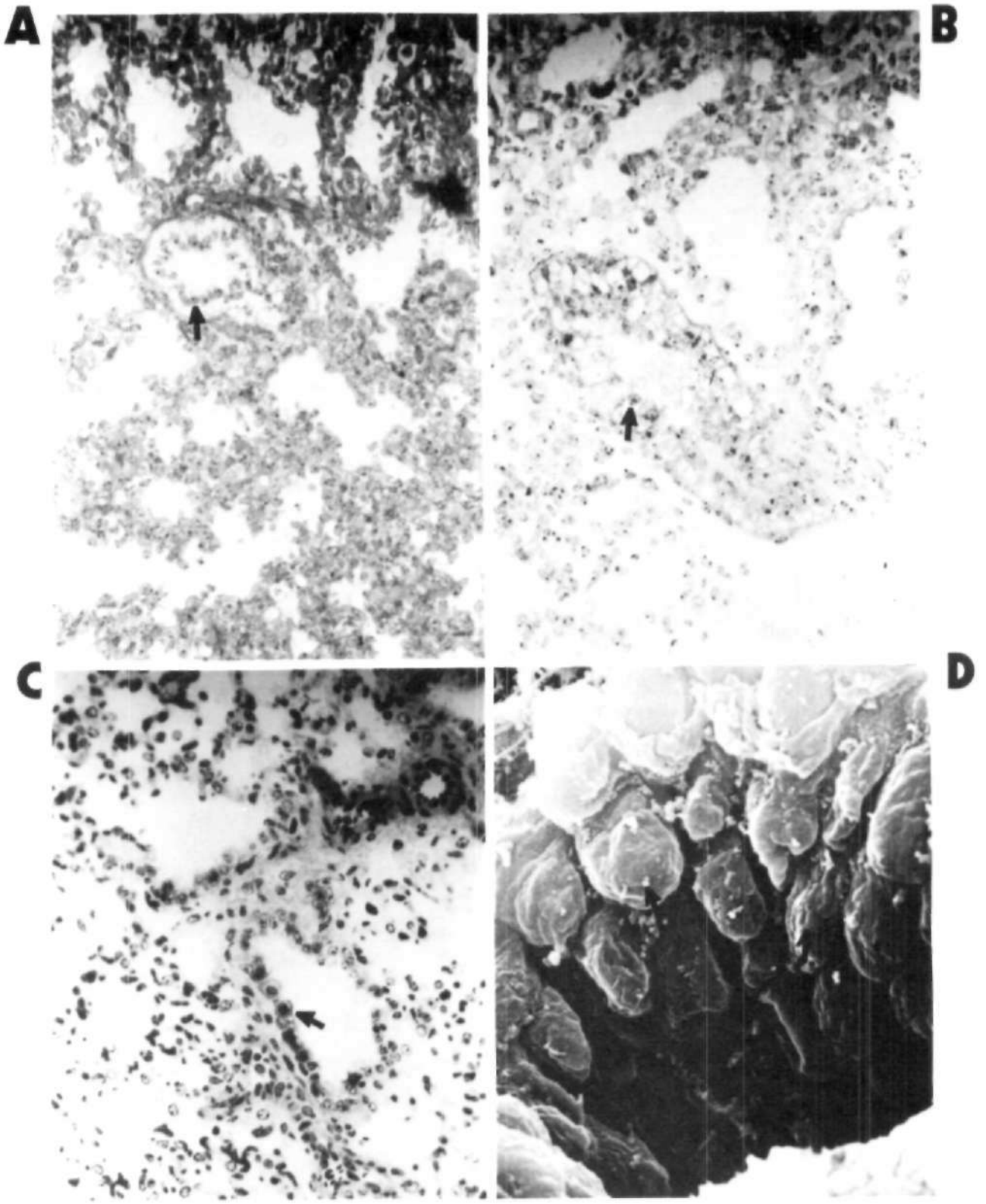

A
B
C
D

Fig. 3. Scanning electron micrographs of fetal bronchioles. A. 240 days, B. 250 days, C. 270 days, D. 275 days of gestation. Note the bulbous aspices of the primitive Clara cell (c) projecting above the ciliated cells (b). X4000.

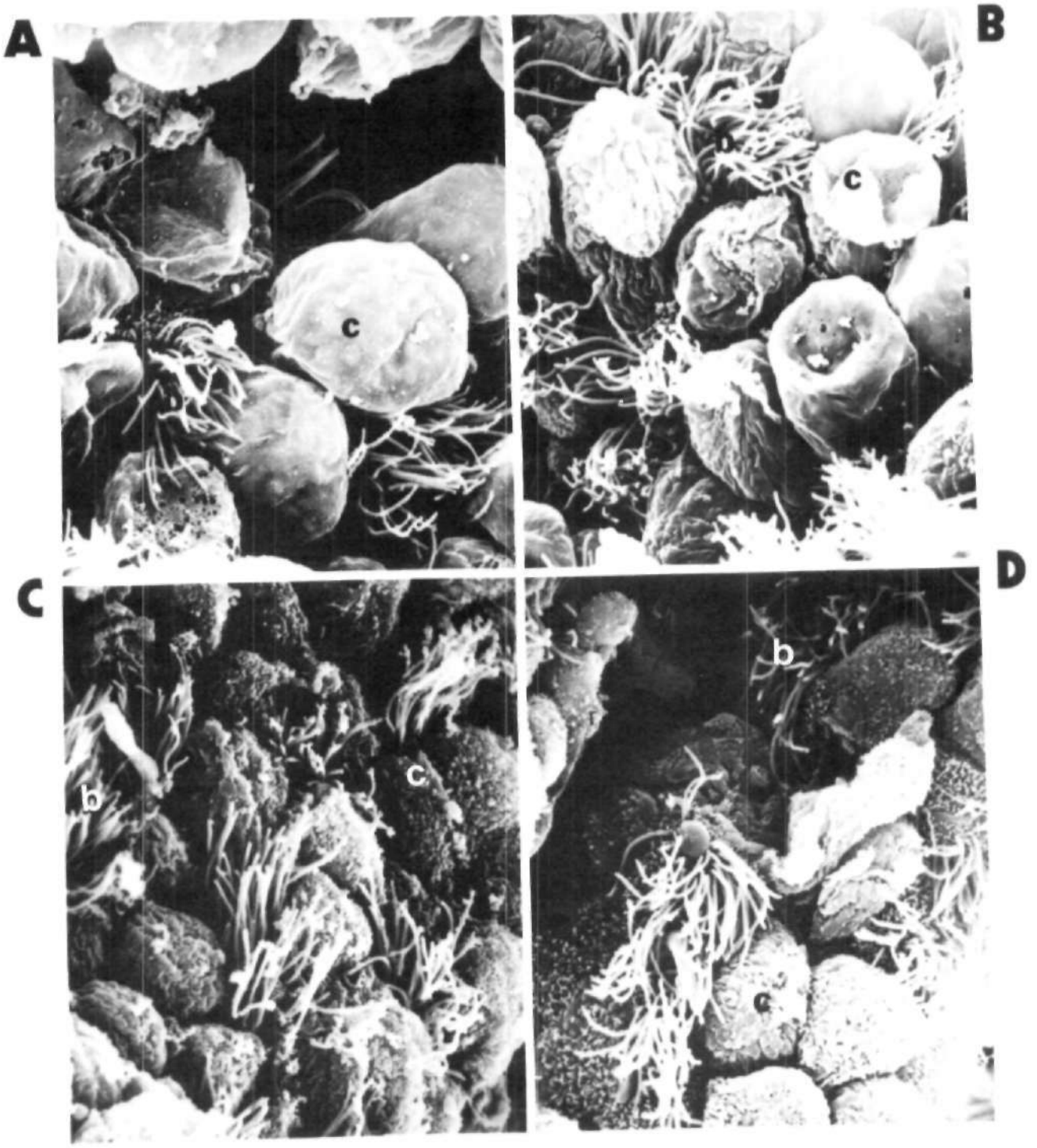

A
B
C
D
b
c
b
c
b
c

Fig. 4. Transmission electron micrographs of fetal terminal bronchioles: A. and B. at 150 days; C. and D. at 180 days. Note that the bronchiolar cells are all alike and cuboidal in shape with abundance of glycogen (g) and sparce cytoplasmic organelles, mitochondria (m), right junctions (t) and basal lamina (BM) (KV 60 X4760).

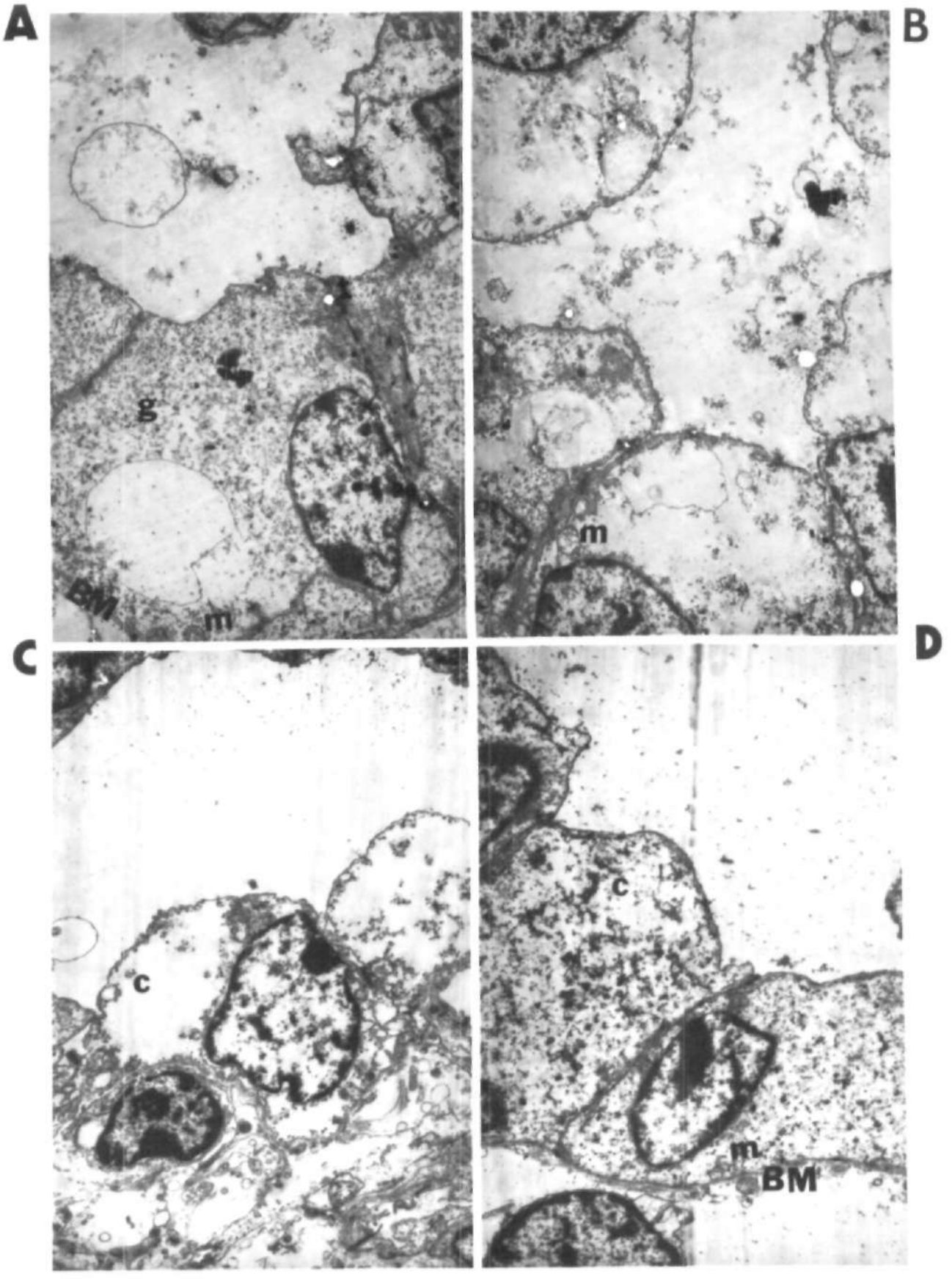

A
g
BM
m
B
m
C
c
D
c
m
BM

Fig. 5. Transmission electron micrographs of fetal respiratory bronchioles. A. and B. 180 days, C. and D. at 250 days of gestation. Note the primitive Clara cells (CL) with abundant glycogen (g), and deeply notch nuclei, ciliated cells with cilia (c), mitochondria (m), nucleus (N) and basal lamina (BM). (A, B and D KV 60 X3150) (C. KV 60 X4760).

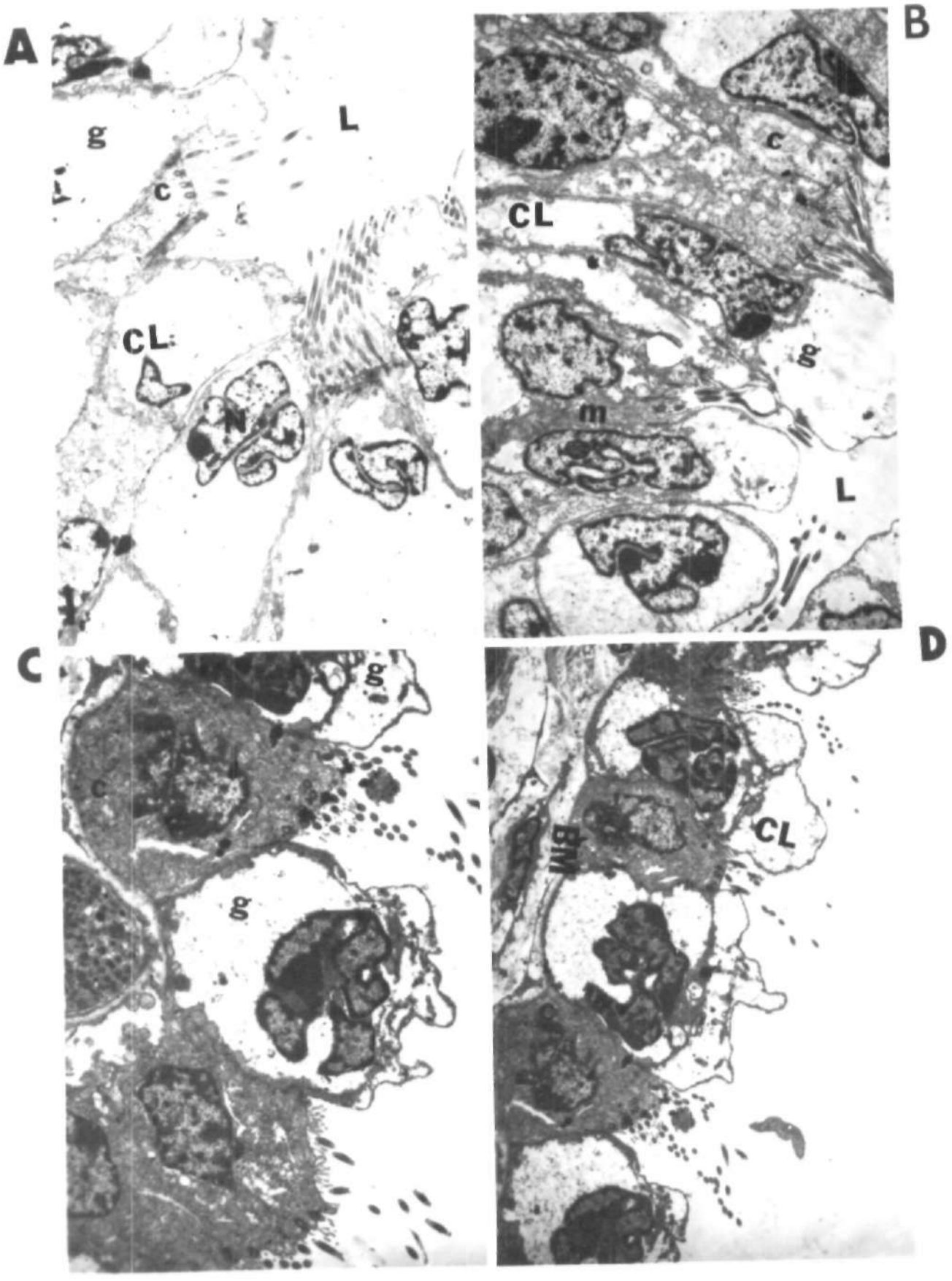

A
g
L
c
CL
N
B
c
CL
g
m
L
C
g
c
g
D
BM
CL

Fig. 6. Transmission electron micrographs of portion of fetal bronchioles. A. 250 days of gestation. Primitive Clara cells (CL) and ciliated cell (c) are present. (KV 60 X7020); Note that the nuclei of primitive Clara cells are deeply notched. B. 260 days. A nonciliated cell is shown which contain abundant glycogen (g). Nucleus (N) and basal lamina (BM) (KV 60 X3150). C. 260 days gestation. Ciliated cells (c) are evident. Lumen (L) and basal lamina BM) (KV 60 X3150).

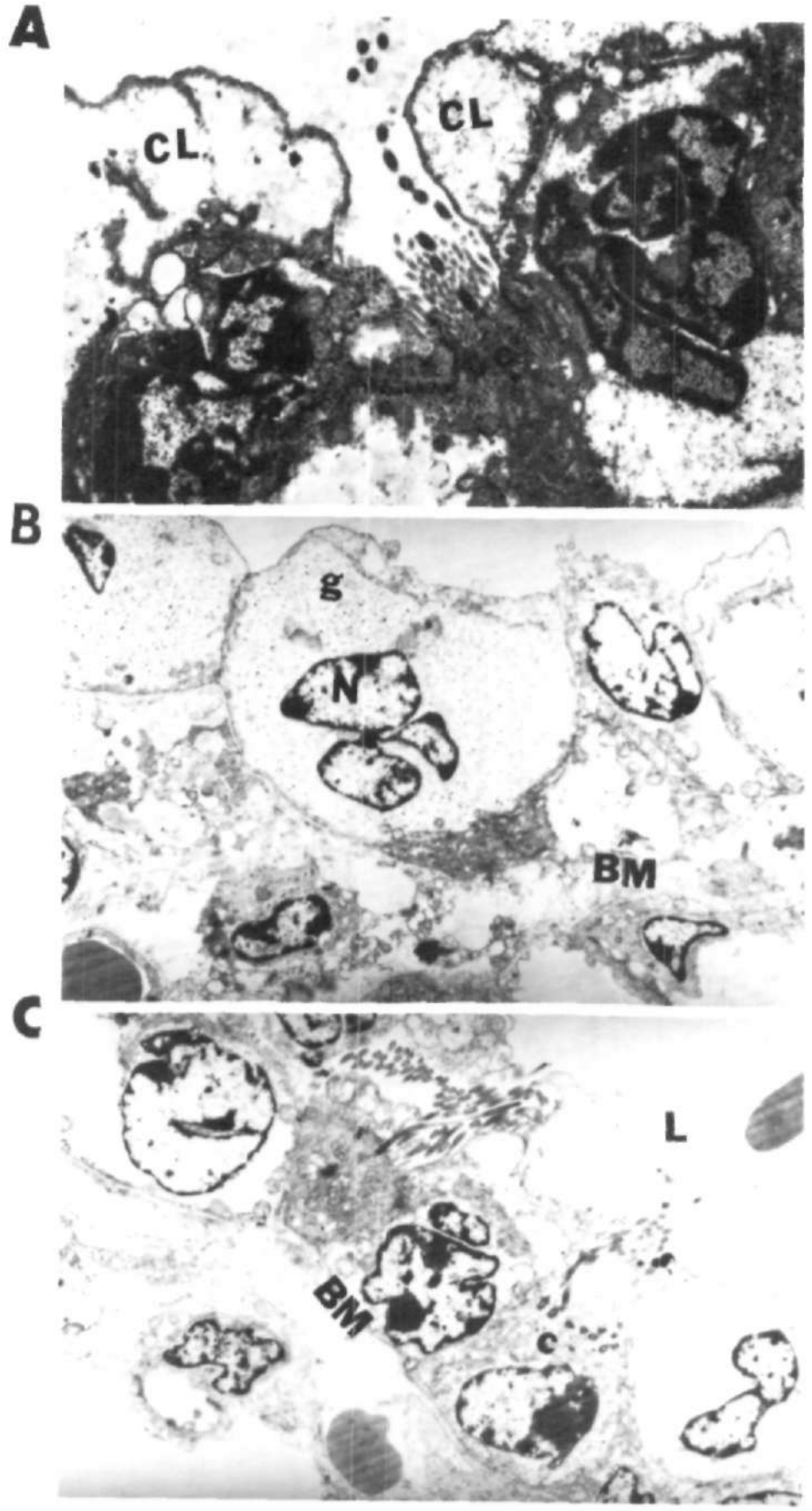
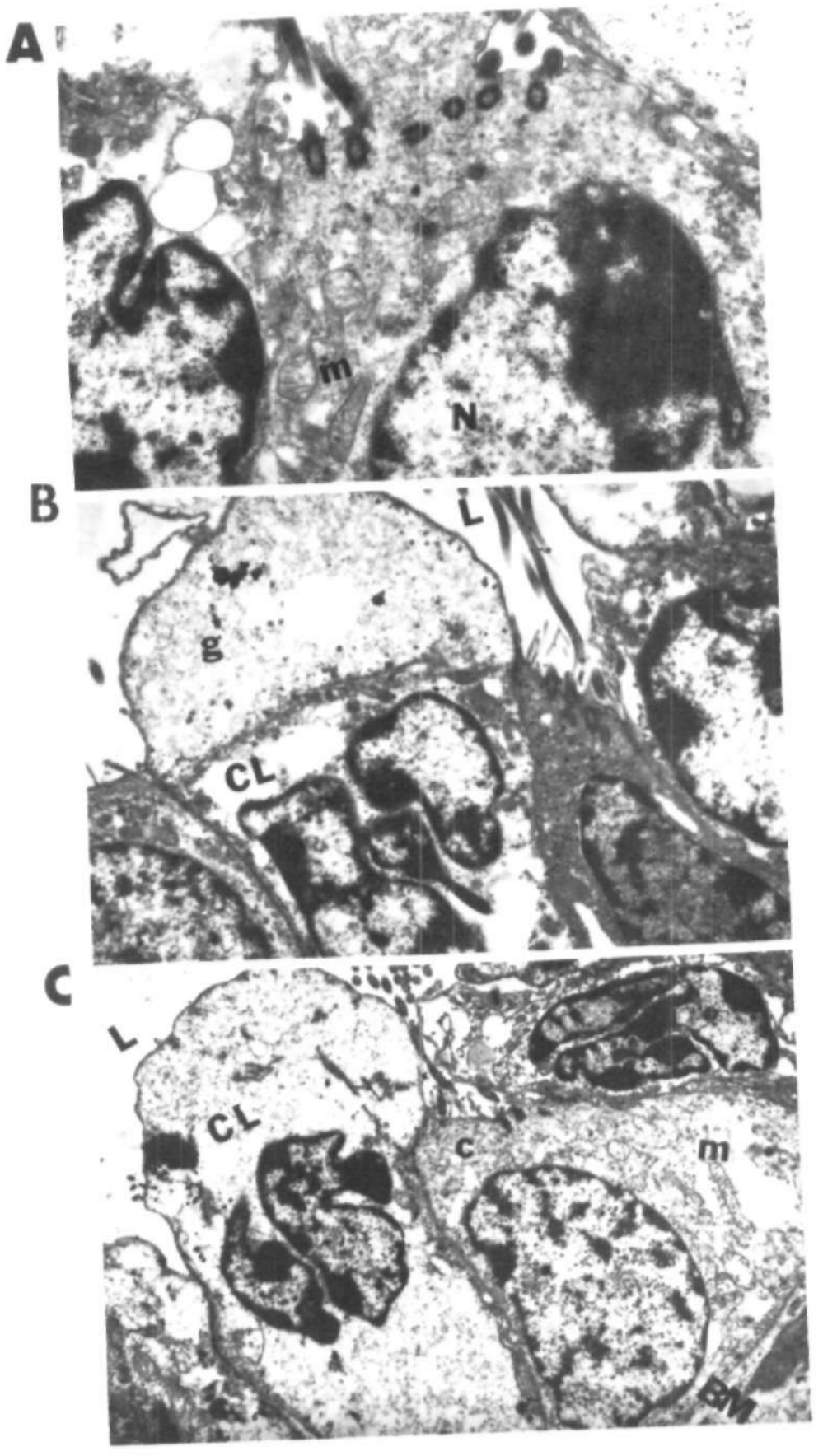

A
CL
CL
B
g
N
BM
C
L
BM
C

Fig. 7. Transmission electron micrographs of fetal bronchioles. A. 260 days; ciliated cell with numerous mitochondria (m) and nucleus of the cell (N). The cristae of the mitochondria are well developed. (KV 60 X7020). B. and C. 270 days of gestation; primitive Clara cells (CL) with abundance of glycogen (g) and deeply notched nuclei are visible. Ciliated cell (c) with numerous mitochondria (m); Lumen (L), basal lamina (BM). (B. KV 60 X7020) (C. KV 60 X4760).


A
m
N
B
L
g
CL
C
L
CL
m
BM

Part 4. TRACHEAL CELLS OF BOVINE FETUS FROM 80 TO 275 DAYS OF GESTATION

ABSTRACT

Forty fetal tracheas from 80-275 day old fetuses were obtained from an abattoir to study the cell types present during lung development. Five cell types, ciliated, secretory, brush, basal and neuroendocrine were identified. Ciliated cells had numerous mitochondria and were observed during pseudoglandular period. Brush cells with broad base and narrow apex had mitochondria, endoplasmic reticulum and microvilli at the apex. Basal cells had electron dense cytoplasm. Both brush and basal cells were identified during canalicular period. However, two types of secretory cells were observed, one with electron dense granules and the other electron lucent and neuroendocrine cells with large electron dense core granules were observed during alveolar period. Neuroendocrine cells were located at the basal layer and were seen as single cells. The function of neuroendocrine cells is not known.

INTRODUCTION

During the glandular phase, the respiratory epithelial lining in both man and rat is stratified and consists of vertically oriented cells.[1,2]

Cilia are first seen in trachea and then progressively at more distal levels of the respiratory tract.[3] Cilia appear at about gestation day 20 in the rats.[2]

Mucus-secretory cells are found in the rat fetus at 21 days gestation[2] while they are demonstrable by the thirteenth fetal week in human.[1,4]

Basal cells can be identified in the trachea[4] by 10 weeks of gestation in human, at about 21 days of gestation in rat[2,5] but are not present in the rabbit until term.

Brush cells have been described in the rabbit trachea at 22 days of gestation[6] while in the rat trachea possible precursors of this cell may be

found as early as 21 days of gestation.[2]

Neuroendocrine cells are found in the human fetus at 16 weeks of gestation at the basal layers in both distal and proximal airway epithelium. The neuroendocrine cells are ususally seen as single cells and occasionally reach the airway lumen. In contrast to those of adult, they typically have electron-dense cytoplasm and abundant glycogen.[7] Neuroendocrine cells have been found throughout the bronchial tree in the fetal rat and are present singly or in groups.[8] The granules of the human neuroendocrine cell appear to have a larger electron-dense core than in the rat.

Studies on fetal tracheal cells have not been reported in the bovine. This study reports on cell types observed in the bovine fetus during 80-275 days gestation.

MATERIALS AND METHODS

Tissue Collection

Bovine tracheas from 40 fetuses were collected from an abattoir (Wellington Quality Meat, Wellington, Kansas). Fetal ages were estimated by using published criteria by Roberts[9]. Tissues for sectioning were obtained from each trachea. Of the total number of fetal lungs collected, 5 each were taken from fetuses of 240, 250, 260 days of gestation; 4 each from fetuses of 150 and 180 days of gestation, 2 each from fetuses of 80, 120, 160 and 275 days of gestation; 3 from 230 days gestation and 6 from 270 day old fetuses.

Light Microscopy

Fetal tracheal tissues were fixed in 10% buffered neutral formalin (BNF), embedded in paraffin, sectioned at 6 micrometers, and stained with Harris hematoxylin and eosin and McManus. Photomicrographs were obtained with an Olympus photomicrographic system camera model PM-10-M, using Panatomic X film.

Scanning Electron Microscopy

Tracheal samples were fixed in cold 2.5% glutaraldehyde in 0.05M sodium

cacodylate, pH 7.3, containing 5mM KCl and 2.5mM $MgCl_2$, and rinsed three times in cool 0.1M sodium cacodylate buffer pH 7.3. Rinsed in distilled water two times, dehydrated in graded ethanol concentrations for about 10 minutes in each concentration, critical point dried with DCP-1 critical point Dryer, mounted on aluminum stub and gold palladium coated with Edwards sputter coater model S150A. Photomicrographs were taken with ETEC-Auto-Scan at accelerating voltage of 10 KV, using polaroid type 55 Kodak film.

Transmission Electron Microscopy

Tracheal samples of 1 mm^3 were fixed in cold 2.5% glutaraldehyde in 0.05M sodium cacodylate pH 7.3 containing 5mM KCl and 2.5 mM $MgCl_2$, rinsed three times in cold 0.1M sodium cacodylate buffer, pH 7.3, postfixed in 1% cacodylate buffered osmium tetroxide for 2 hours, rinsed in distilled water, and stained en bloc with 1% aqueous uranyl acetate for 2 hours before dehydration through a graded series of ethanol and acetone. Fixation and prestain procedures were in ice buckets.

Tissues were embedded in Epon/Araldite, trimmed, and sectioned with glass knives in a Reichert OM-U2 ultramicrotone. Thin sections (60-150 nanometers) were stained for 10-15 minutes with 5% uranyl acetate-50% ethanol and with Reynolds lead citrate for 5-7 minutes.

All sections were examined in a Philips 201 transmission electron microscope at 60 KV. Micrographs were taken on Cronar Ortho S Litho film. Thick sections were cut and stained with toludine blue, photomicrographs were taken with an Olympus photomicrographic system camera model PM-10-M, using Kodak Panatomic X film.

RESULTS

Light microscopy

Light photomicrographic pictures show that from 80 days of gestation (pseudoglandular phase of lung development) the epithelia of the trachea are

pseudostratified and cilia are present (Figs. 1A-D, and 2A). From the canalicular phase (120th to 180th day of gestation) glands were seen in the submucosa (Figs. 1B-D). Tracheal epithelium reacted positively to McManus stain indicating the presence of glycogen (Figs. 2A,C,D and 3B). Toludine blue stained sections at 240 days of gestation showed numerous submucosal glands (Fig. 3A), while at 275 days, the epithelium is well organized (pseudostratified) with numerous ciliated cells (Fig. 3C).

Scanning Electron Microscopy

During the pseudoglandular period (120 days) (Fig. 4A) and canalicular period (Figs. 4B-C) nonciliated cells and ciliated cells were seen. However, from 230 days (Terminal Sac period) onwards the ciliated cells were much more numerous with only a few nonciliated cells between the ciliated cells (Figs. 5A-C).

Transmission Electron Microscopy

Transmission electron micrographs at 150 days (canalicular period) showed ciliated cells with numerous mitochondria and Golgi complex (Fig. 6A). The cristae of the mitochondria are poorly developed (Fig. 6A). Brush cells which are flask-shaped in outline, broader at their base than at the apex, with mitochondria, endoplasmic reticulum, and microvilli were observed at 150 days of gestation (Fig. 6B). At 150 days of gestation (canalicular period) the basal cells have mitochondria but are devoid of other organelles (Fig. 6C). However from 240 days of gestation, the basal cells had electron dense cytoplasm (Figs. 6D, 8A and 9B).

The secretory cells identified from 250 days (Fig. 7A) were of two types, one with electron dense (Figs. 7A, 8C and 9A) and the other electron lucent granules (Fig. 7A). The ciliated cells from 250 to 275 days of gestation had numerous mitochondria and vesicles in the cytoplasm (Figs. 7B, 7C, 8C, 9C).

Neuroendocrine cells were identified at the basal layer with electron dense

cytoplasm (Fig. 8B). The neuroendocrine cells have large electron-dense core granules in the cytoplasm (Fig. 8B). The cytoplasm of the neuroendocrine cell contained mitochondria (Fig. 8B). Neuroendocrine cells were seen as single cells.

DISCUSSION

Bovine lung development has been classified into five periods, the embryonic (30th to 50th day of gestation), the pseudoglandular (50th to 120th day of gestation), the canalicular (120th to 180th day of gestation), terminal sac (180th to 240th day of gestation) and alveolar (240th to 260th day of gestation).[10]

Five cell types were recognized in the tracheal epithelium during fetal development. They are ciliated, brush, secretory, basal and neuroendocrine cells.

Ciliated cells were observed during the pseudoglandular period in this study. However, in the rat, cilia did not appear in the trachea until at (canalicular period) about day 20 of gestation.[2] Cilia were first seen in the trachea and then progressively at more distal levels of the respiratory tract in mammals.[3] The ciliated cell had microvilli and cilia.

Brush cells observed during the canalicular period in this study, had a broad base, a narrow apex with microvilli, and cytoplasm with scant endoplasmic reticulum and mitochondria. The microvilli formed a brush border. Study of the rabbit trachea has shown brush cells to be present at 22 days of gestation[6] (pseudoglandular period) while in the rat trachea, possible precursor of this cell may occur as early as 21 day of gestation(terminal sac period).

Basal cells have been identified in the trachea[4] by 10 weeks of gestation in human(pseudoglandular period), at about day 21 of gestation(terminal sac period) in rat,[2,5] and at term in rabbits.[5] In this study, basal cells,

identified at 150 days (canalicular period), had electron lucent cytoplasm with mitochondria. However, from 240 days of gestation(alveolar period), basal cells cytoplasm was electron dense.

Neuroendocrine cells, observed at 260 days in bovine(alveolar period) had large electron-dense core granules, and occurred as single cells located in the basal region. Human fetal airway epithelium show neuroendocrine cells at 16 weeks of gestation(Canalicular period) with large electron dense core granules similar to those observed in this study[2]. Neuroendocrine cells have been observed in trachea of fetal rat.[8]

Neuroendocrine cell function is in despute. Rosan and Lauweryns (1972)[7] suggest an influence on lobule growth and differentiation. Lauweryns and Cokelaere (1973)[11] suggest that the neurosecretory cell might play a role in the response of the young lung to hypoxia in the rat.

In this study, two types of secretory cells were recognized from 250 days of gestation(alveolar period), one had electron-dense and the other electron-lucent granules. Secretory cells appear in the rat fetus at 21 days of gestation[2](terminal sac) and in the human featus at the thirteenth week(pseudoglandular period) of development.[1,4]

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Fig 1. Light photomicrographs of bovine fetal trachea: A. 80 days B. 180 days C. 230 days D. 260 days of gestation. Note that the trachea epithelium is pseudostratified. Cilia are present (arrows) and the submucosal glands are developed (s) from 180 days until term. H ∞ E. X200.

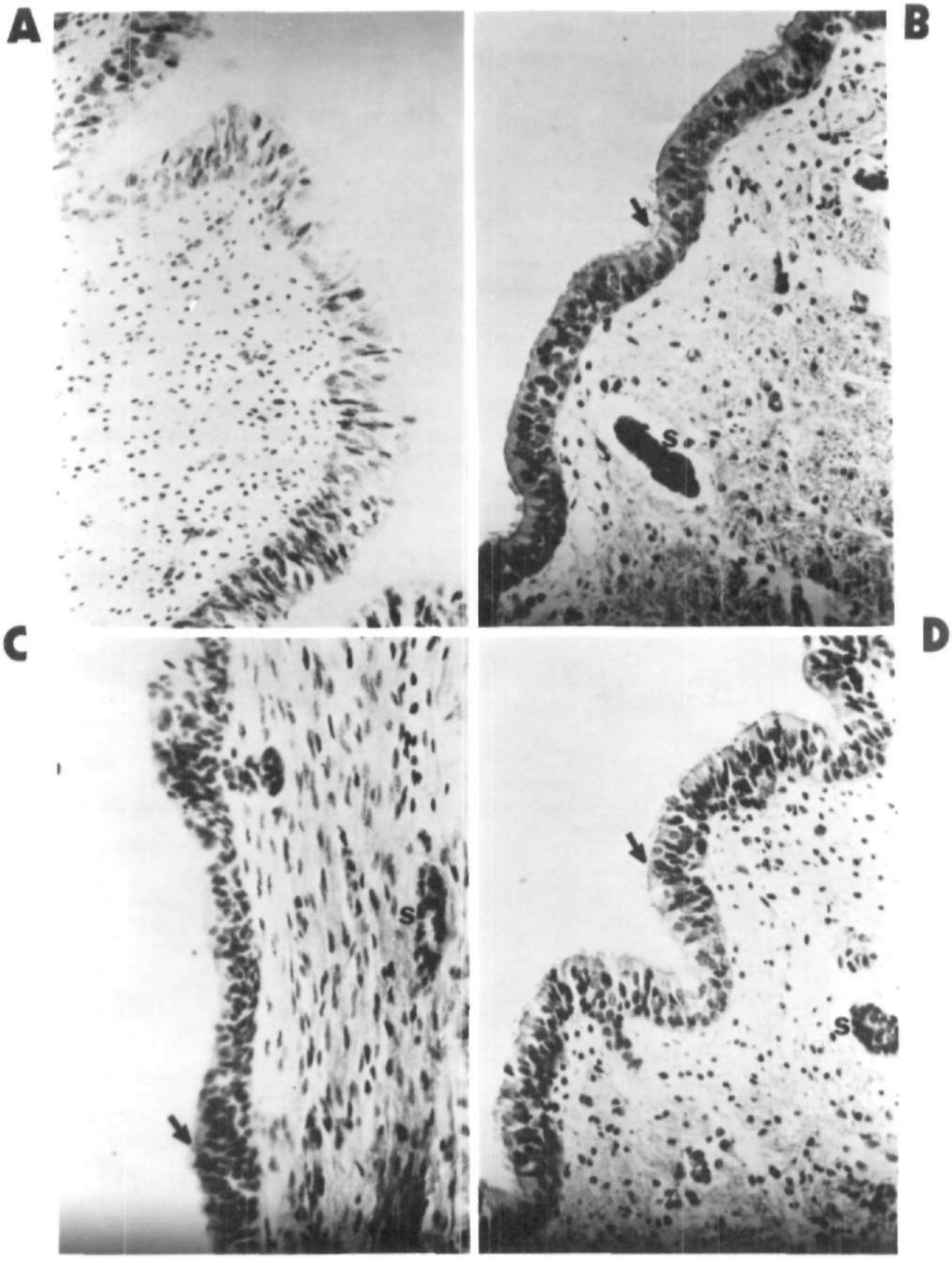

A
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S
C
S
D
S

Fig 2. Light photomicrographs of fetal trachea: A. 120 days B. and C. 250 days D. 260 days of gestation. Note that the epithelium was stained for glycogen (arrows). Pseudostratified epithelium with cilia (arrows) and lumen (L). (A, C and D McManus X200) (B. H ∞ E. X200.)

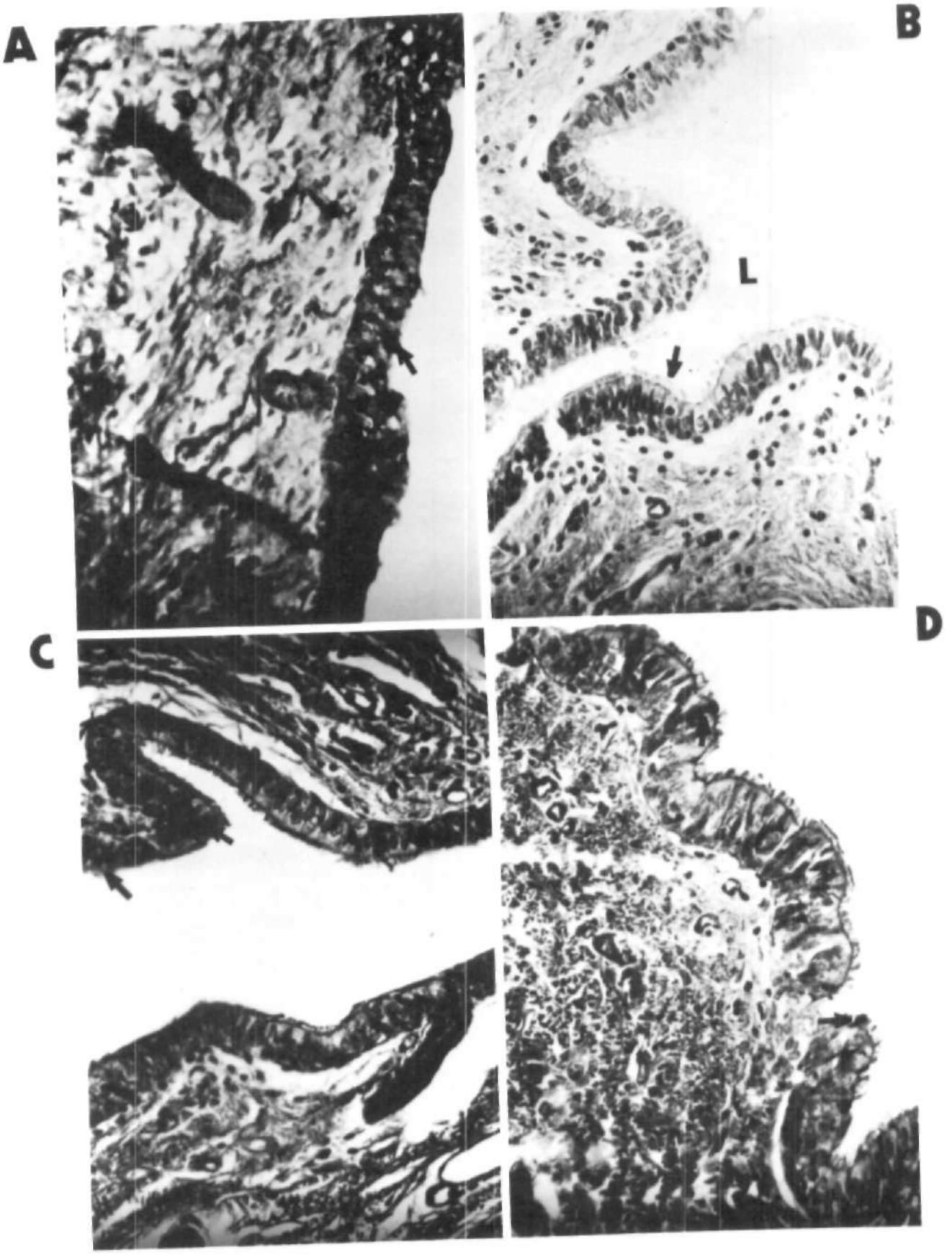

A
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Fig 3. Light photomicrographs of fetal trachea: A. 240 days B. 270 days C. 275 days of gestation. Note the submucosal glands (S), glycogen stain (arrows) and a well organized pseudostratified epithelium with cilia (arrows). (A. Toludine Blue X200) (B. McManus X200) (C. Toludine Blue X400).

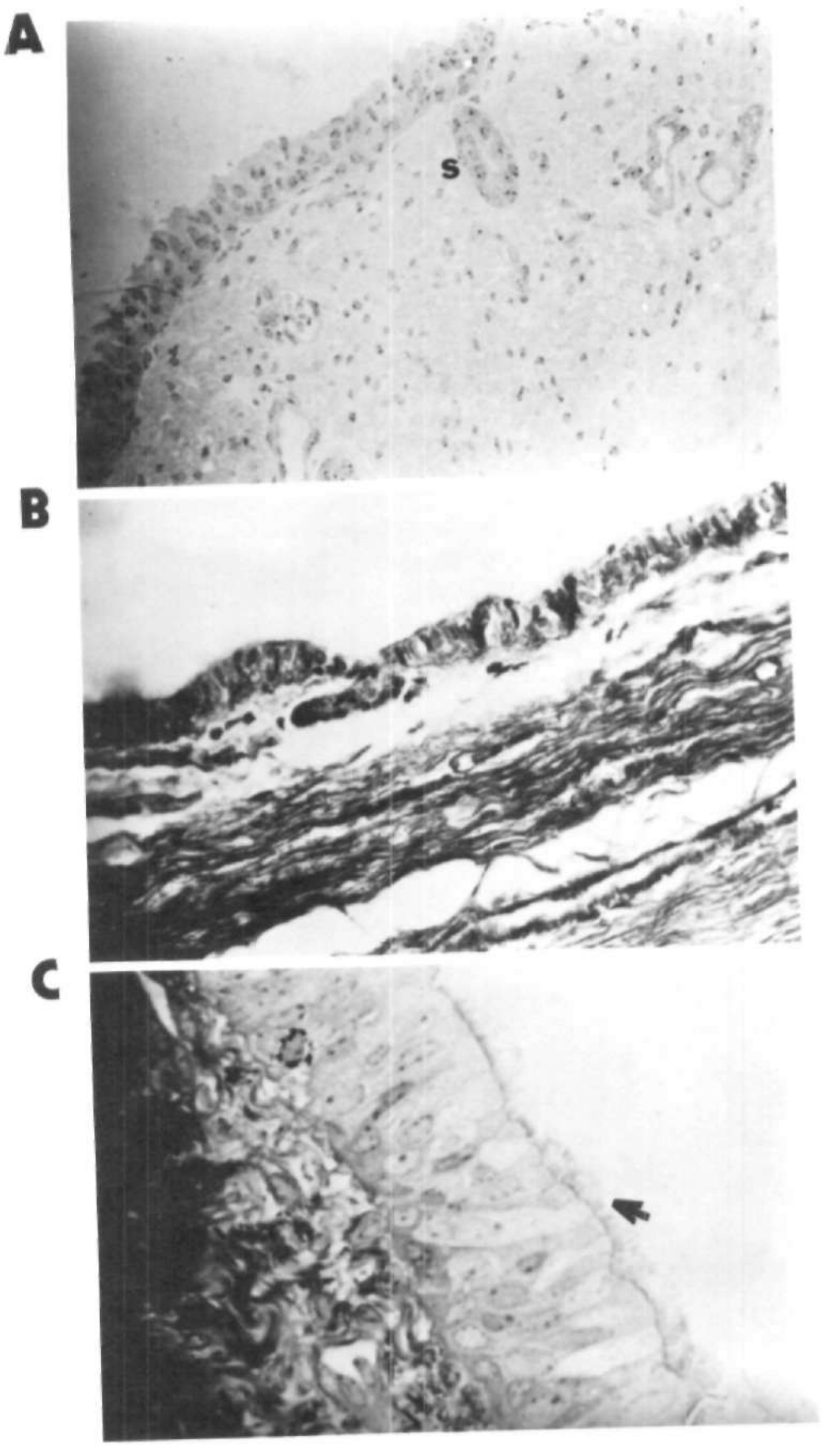

A
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Fig 4. Scanning electron micrographs of bovine fetal trachea: A. 120 days B. 150 days C. 160 days of gestation, showing ciliated cell (c) and nonciliated cells (b). X4000.

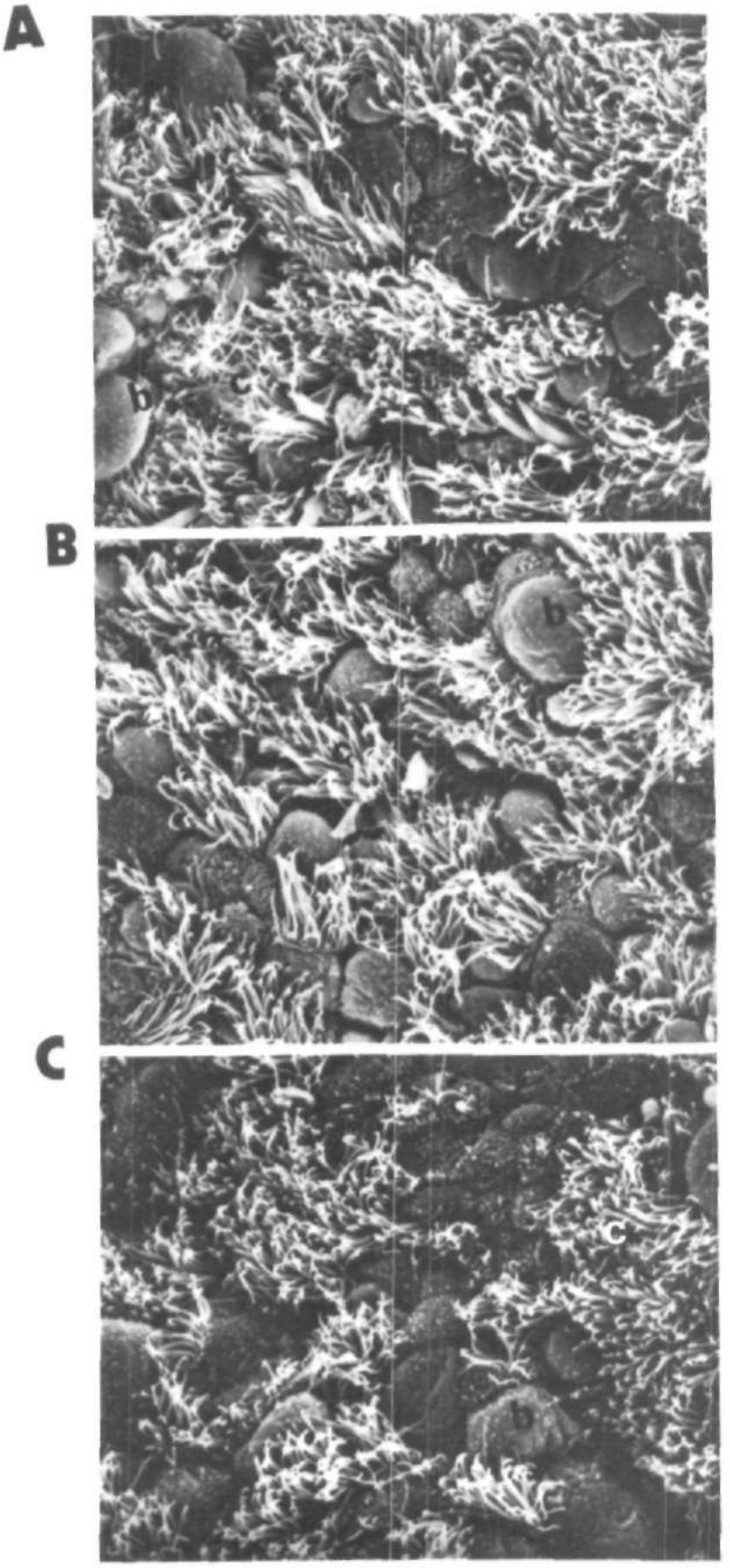

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B
b
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b

Fig 5. Scanning electron micrographs of fetal trachea: A. 230 days B. 250 days C. 270 days of gestation. Note the nonciliated cell (b) in between ciliated cells (c). At 270 days the ciliated cells were more relative to nonciliated cells. X4000.

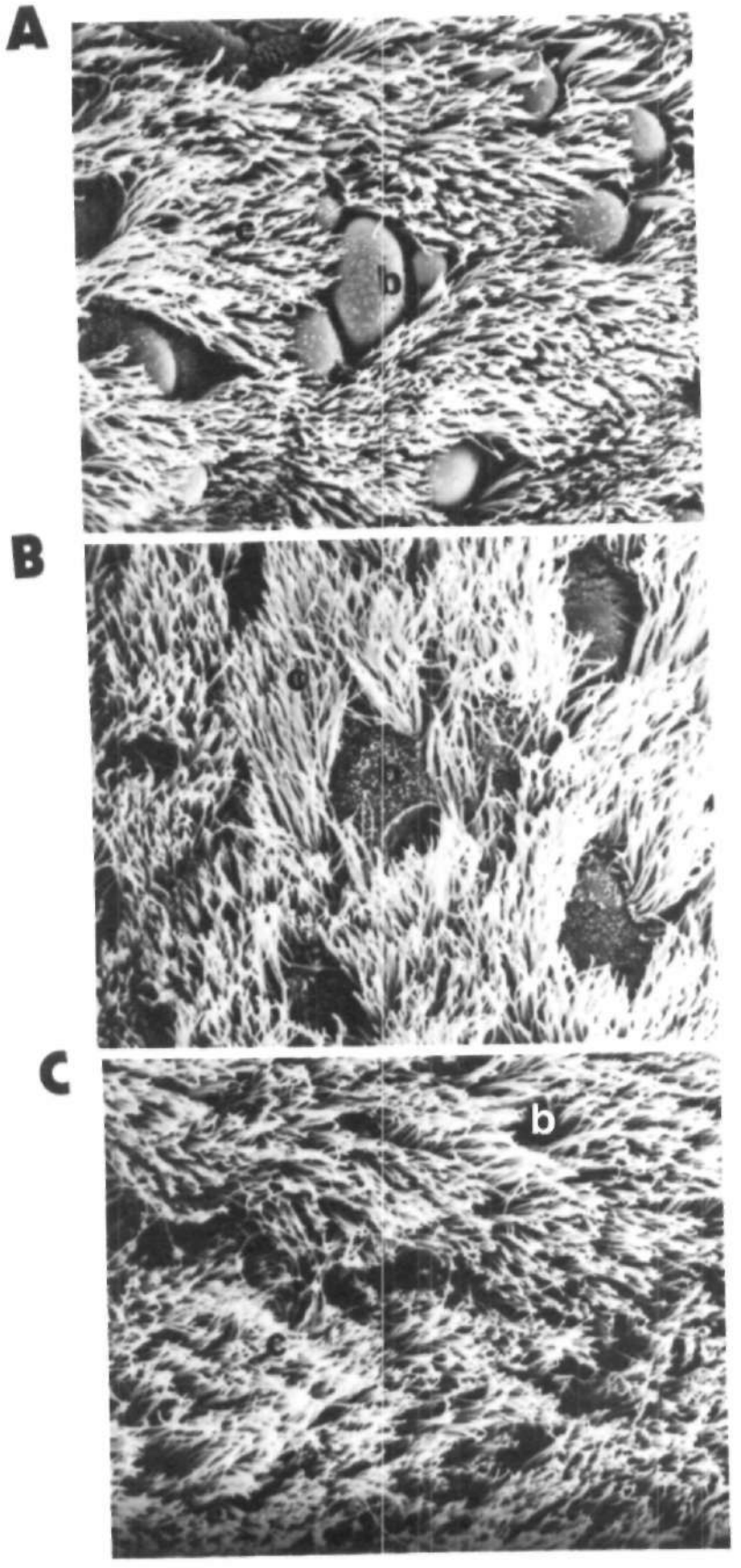

A
B
C
b

Fig 6. Transmission electron micrographs of bovine fetal trachea: A. and B. 150 days of gestation. Note ciliated cell (c) with numerous mitochondria (m), Golgi complex (G), lumen (L), brush cell (B) with endoplasmic reticulum (ER) and microvilli (mi). (A. KV 60 X7020) (B. KV 60 X4760). Basal cell at C. 150 days D. 240 days of gestation. Note the electron dense cytoplasm (CT) nucleus (N) and basal lamina (BM) KV 60 X10,020.

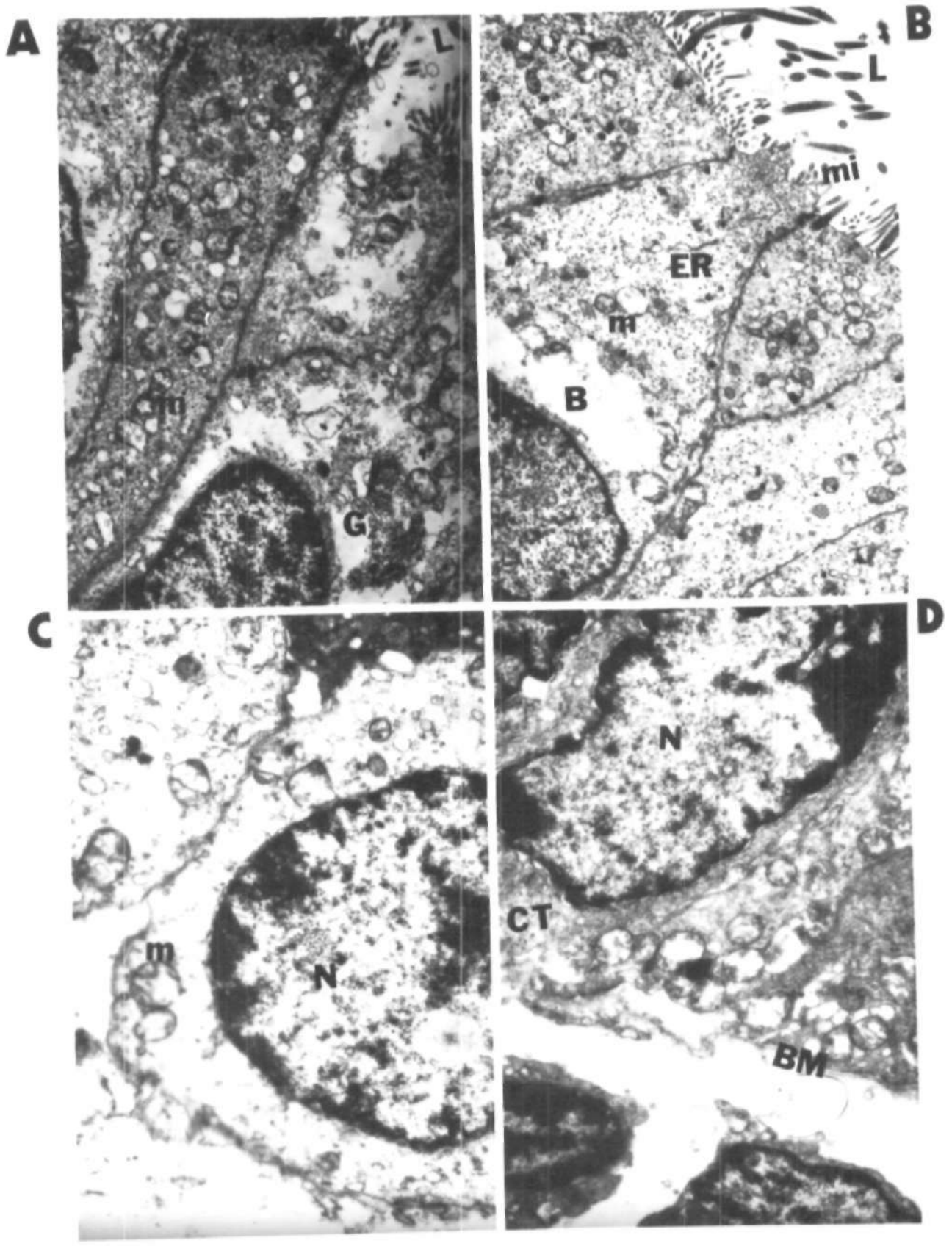

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Fig 7. Transmission electron micrographs of bovine fetal trachea: A. 250 days B. and C. 260 days of gestation. Note ciliated cell (c), secretory cells with electron dense granules (SC), electron lucent granules (g), numerous mitochondria (m). lumen (L), nucleus (N) and vesicles (v). (A. KV 60 X3150) (B. KV 60 X4760) (C. KV 60 X7020).

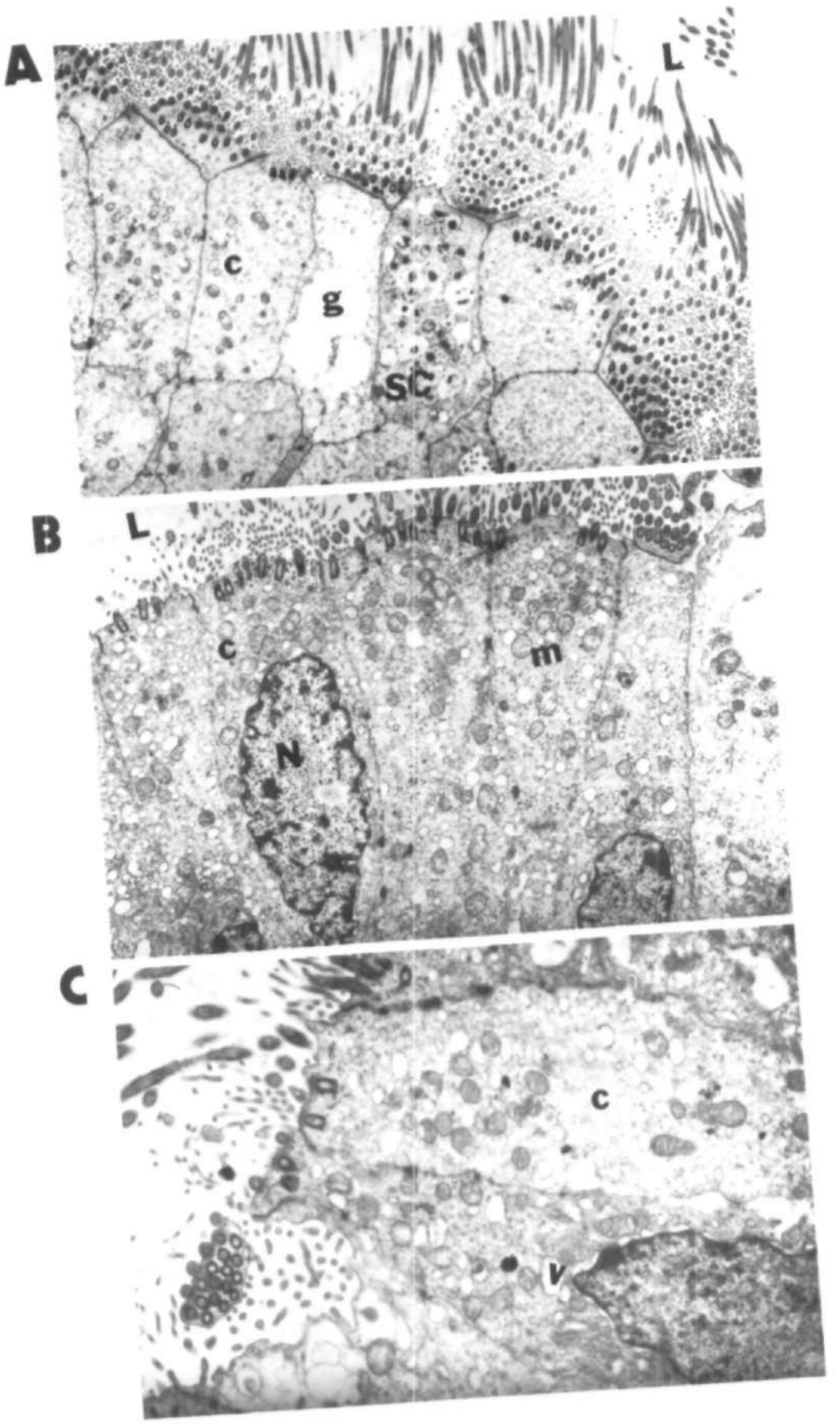

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Fig 8. Transmission electron micrographs of bovine fetal trachea: A. Basal cell at 260 days of gestation with electron dense cytoplasm (CT), mitochondria (m), nucleus (N) and basal lamina (BM). KV 60 X4760. B. Neuroendocrine cell at 260 days showing electron dense granules (G), mitochondria (m), connective tissue (t) and basal lamina (BM). KV 60 X7020. C. Fetal trachea at 275 days, showing secretory cells (S), ciliated cell (c), nucleus (N), and lumen (L). KV 60 X2150.

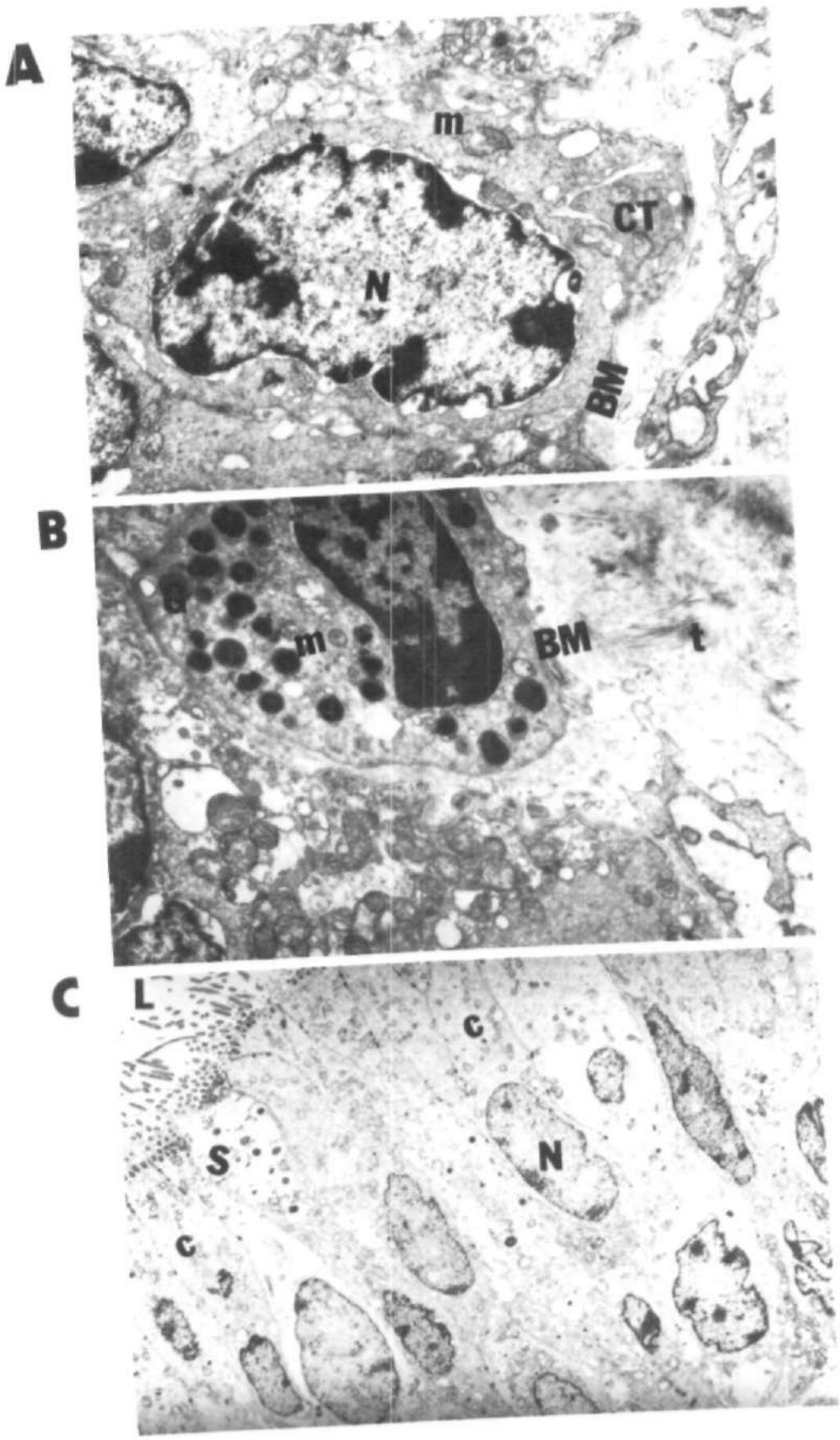
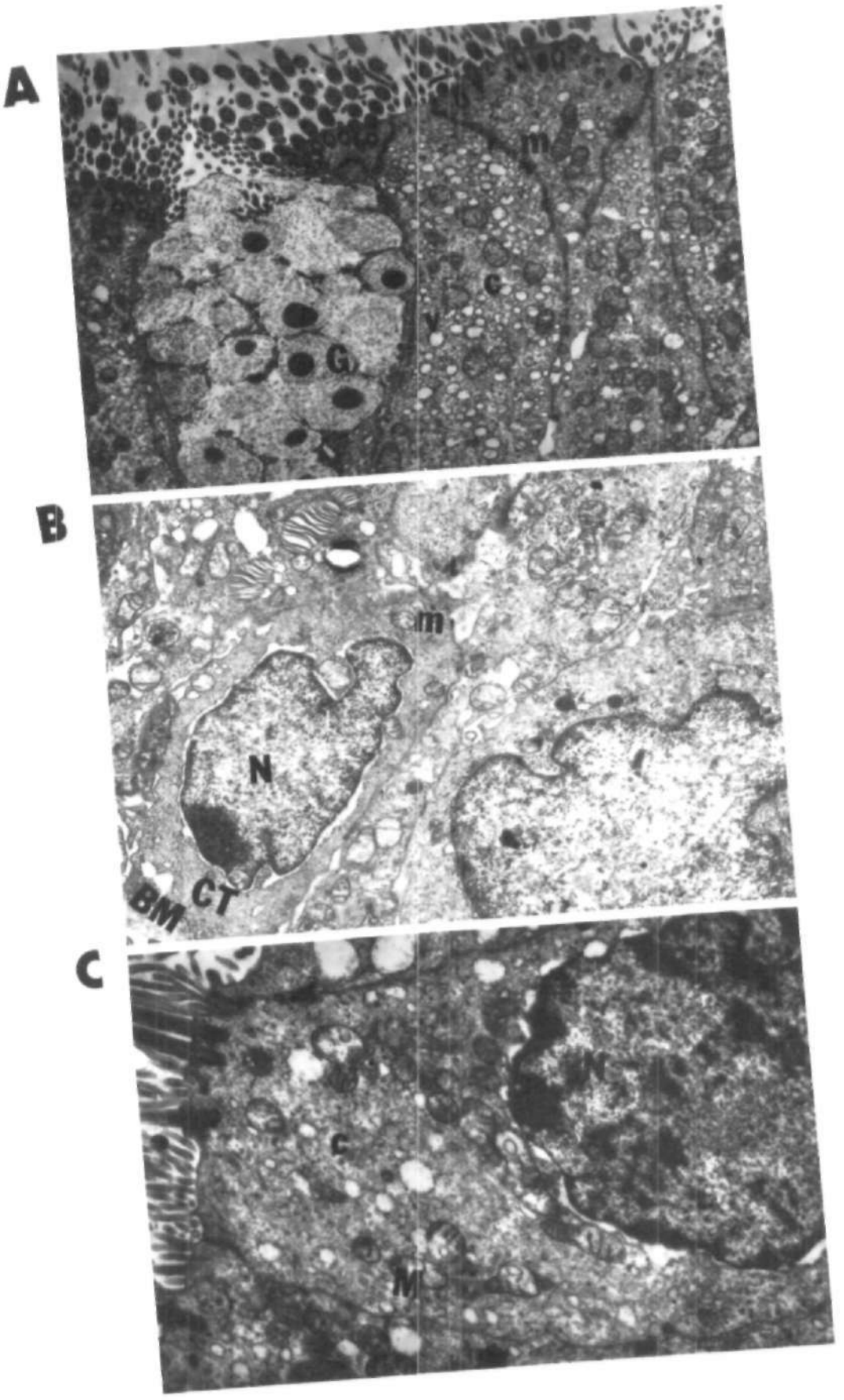

A
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Fig 9. Transmission electron micrographs of bovine fetal trachea at 275 days of gestation. A. A higher magnification, showing secretory cell with electron dense granules (G) and ciliated cell (c) with numerous vesicles (v), mitochondria (m), KV 60 X7020. B. Basal cell with mitochondria (m), nucleus (N), electron dense cytoplasm (CT) and basal lamina (BM). KV 60 X 7020. C. Higher magnification of ciliated cell (c), mitochondria (M) and nucleus (N). KV 60 X10,020.


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Part 5. DEVELOPMENT OF BOVINE FETAL BRONCHIAL CELLS FROM 80 to 275 DAYS OF GESTATION

ABSTRACT

Forty bovine fetuses were obtained from an abattoir to study the epithelial cells of the bronchi during lung development. During the pseudoglandular and canalicular periods electron microscopy showed three cell types, ciliated, secretory and basal. Secretory cells with electron dense granules were observed during canalicular period. Neither the neuroendocrine cell described in human and rat fetal lung nor the brush cell described in rabbit and rat fetal lung were identified in the bovine fetal bronchi.

INTRODUCTION

In the human, four cell types, ciliated, mucus-secreting, intermediate and basal have been described in the proximal airways using light and electron microscopy.[2] The clear cell seen by light microscopy has been referred to as bronchial neuroendocrine cell in man[3] and the rat[4]. The most complete electron microscopic studies have been made using the laboratory rat.[4,5]

During the pseudoglandular phase of lung development, all cells of the bronchial epithelium are ultrastructurally similar and resemble those of the adjacent developing esophagus.[6,7,8]

In man, cilia are found throughout the bronchial tree by the thirteenth fetal week[6] (pseudoglandular) while cilia appear late in fetal life at about day 20 of gestation in the rat[8].

Lung development in the bovine has been classified into embryonic, pseudoglandular, canalicular, terminal sac and alveolar periods.[9]

There is no information on the cell types of bovine bronchus during lung development. This study reports on the cell types of the bronchus from 80-275 days of gestation using light and electron microscopy.

MATERIALS AND METHODS

Tissue Collection

Bovine bronchi from 40 fetuses were collected from an abattoir (Wellington Quality Meat, Wellington, Kansas). Fetal ages were estimated by using published criteria.[10] Tissues for sectioning were obtained from each bronchus. Of the total number of fetal lungs collected, 5 each were taken from fetuses of 240, 250, 260 days of gestation; 4 each from fetuses of 150 and 180 days of gestation; 2 each from fetuses of 80, 120, 160, and 275 days of gestation; 3 from 230 days of gestation and 6 from 270 day old fetuses.

Light Microscopy

Fetal bronchial tissues were fixed in 10% buffered neutral formalin (BNF), embedded in paraffin, sectioned at 6 micrometers, and stained with Harris hemotoxylin and eosin and McManus. Photomicrographs were obtained with an Olympus photomicrographic system camera Model PM-10-M, using Kodak panatomic X film.

Scanning Electron Microscopy

Bronchial samples were fixed in cold 2.5% glutaraldehyde in 0.05M sodium cacodylate, pH 7.3, containing 5mM KCl and 2.5 mM $MgCl_2$, and rinsed three times in cool 0.1M sodium cacodylate buffer pH 7.3. Rinsed in distilled water two times, dehydrated in graded ethanol concentrations for about 10 minutes in each concentration, critical point dried with DCP-1 critical point dryer, mounted on aluminum stub and gold palladium coated with Edwards sputter coater model S150A. Photomicrographs were taken with ETEC-Auto-Scan at accelerating voltage of 10 KV, using poloraid type 55 Kodak film.

Transmission Electron Microscopy

Bronchial samples of $1mm^3$ were fixed in cold 2.5% glutaraldehyde in 0.05M sodium cacodylate, pH 7.3 and containing 5mM KCl and 2.5 mM $MgCl_2$, rinsed three times in cold 0.1 M sodium cacodylate buffer, pH 7.3, postfixed in 1%

cacodylate buffered osmium tetroxide for 2 hours, rinsed in distilled water, and stained en bloc with 1% aqueous uranyl acetate for 2 hours before dehydration through a graded series of ethanol and acetone. Fixation and prestain procedures were in ice buckets.

Tissues were embedded in Epon/Araldite, trimmed and sectioned with glass knives in a Reichert OM-U2 ultramicrotome. Thin sections (60-150 nanometers) were stained for 10-15 minutes with 5% uranyl acetate-50% ethanol and with Reynolds lead citrate for 5-7 minutes.

All sections were examined in a Philips 201 transmission electron microscope at 60 KV. Micrographs were taken on cronar ortho S Litho film.

RESULTS

Light Microscopy

Bronchial epithelium is pseudostratified from 80 to 270 days of gestation (Figs. 1A-D, 2A-C, 3A-C). Cilia were observed from 80 days (pseudoglandular period) of gestation (Fig. 1A). From 80 days onwards the bronchial epithelium reacted positively to McManus stain indicating presence of glycogen (Figs. 1A, 1B, 1D, 2B, 3A and 3C).

Scanning Electron Microscopy

From pseudoglandular period, the bronchial epithelium showed ciliated and nonciliated cells (Figs. 4A-C, 5A-C, 6A-C). However, during alveolar period (250-260 days) the bronchial epithelium showed numerous ciliated cells with a few nonciliated cells.

Transmission Electron Microscopy

Transmission electron micrographs show that from late canalicular periods (180 days) the bronchial epithelium have both ciliated and secretory cells (Figs. 7A-B, 8A-C, 9A-C). The secretory cells have electron dense granules and mitochondria with poorly developed cristae. From late canalicular period, the basal cells have electron dense cytoplasm containing mitochondria with poorly developed cristae (Fig. 7C,D). Well developed basal lamina can

also be identified during canalicular period.

DISCUSSION

Bovine lung development has been classified into five periods, the embryonic (30th to 50th day of gestation), the pseudoglandiular (50th to 120th day of gestation), the canalicular (120th to 180th day of gestation), the terminal sac (180th to 240th day of gestation) and alveolar (240th to 260th day of gestation).[9]

This study shows that from the pseudoglandular period the bronchial epithelium contains cilia. The epithelium is pseudostratified and reacted positively to McManus stain which indicates the presence of glycogen. In man cilia are found throughout the bronchial tree by the thirteenth fetal week[6] (pseudoglandular period) while cilia appear late in fetal life in the rat[8]. DeZabala and Weinman[9] have also shown glycogen to be present in the epithelium of future airways during lung development.

Three cell types were identified by means of electron microscopy. The ciliated cells had numerous mitochondria. Secretory cells with electron dense granules appeared from 180 days while at this same period the basal cells were identified with electron dense cytoplasm.

Neither the Kultschitsky (neuroendocrine) cell, described in the human fetal lung[11] and throughout the bronchial tree in the fetal rat[12] nor the brush cell described in rabbit[13] and rat[8] were identified in the bovine fetal bronchus.

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Fig. 1. Light photomicrographs of bovine fetal bronchial epithelium: A. 80 days (Pseudoglandular period) B. 180 days (Canalicular period) C. and D. 230 days (Terminal sac period) of gestation. Note that the epithelium reacted positively to McManus stain indicating presence of glycogen (arrows). From 80 days the epithelium was pseudostratified and cilia were present (arrows). (A. McManus stain X200) (B. McManus stain X400) (C. H ∞ E X200) (D. McManus X200).

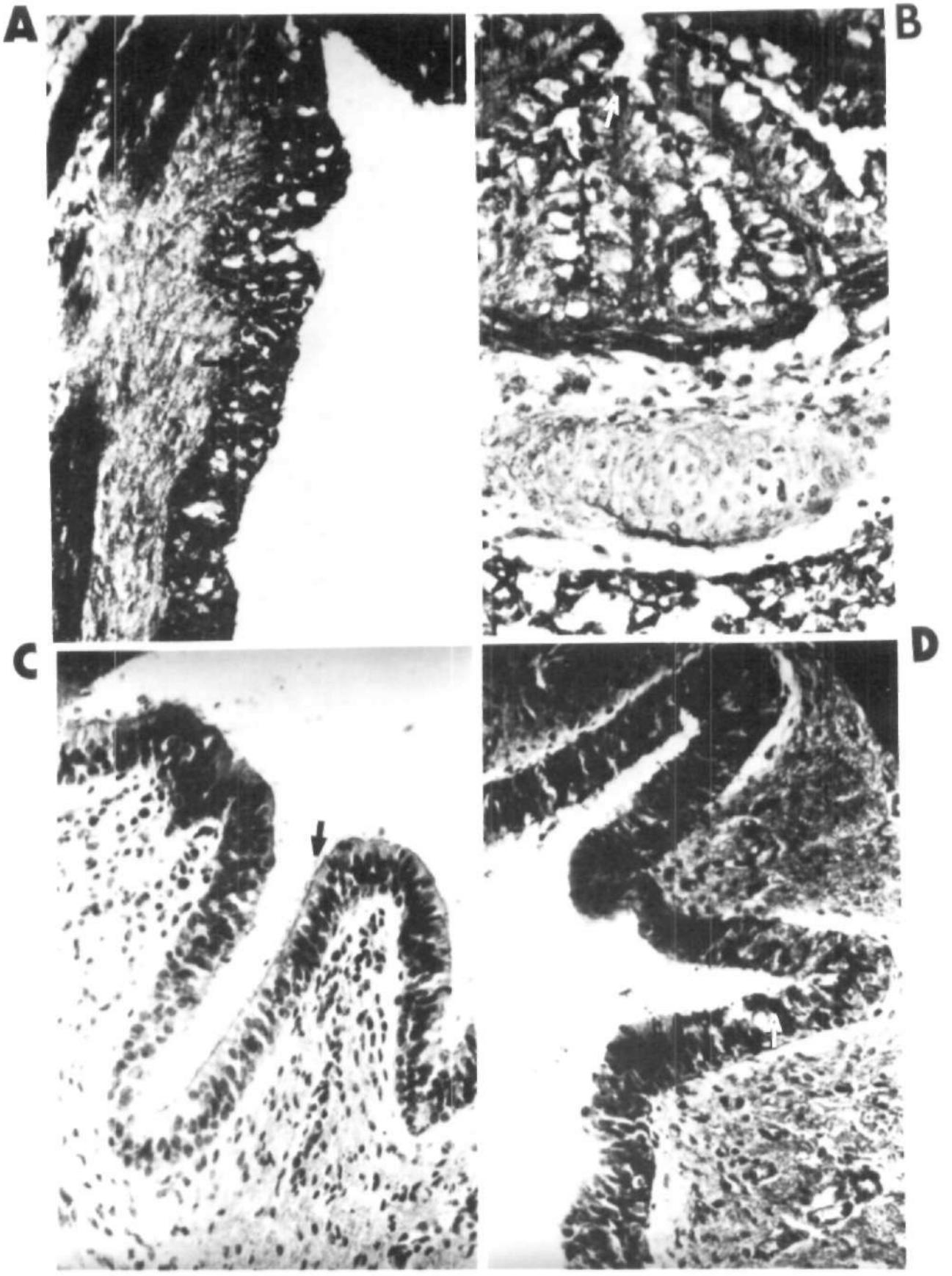

A
B
C
D

Fig. 2. Light photomicrogaphs of bovine fetal bronchial epithelium: A. and B. 250 days (Alveolar period), C. 260 days (Alveolar period) of gestation. Note the pseudostratified epithelium and presence of cilia (arrows). Bronchus epithelium at 250 days was positive for glycogen stain (arrows). (A. H ∞ E X200). (B. McManus stain X400). (C. H ∞ E X200).

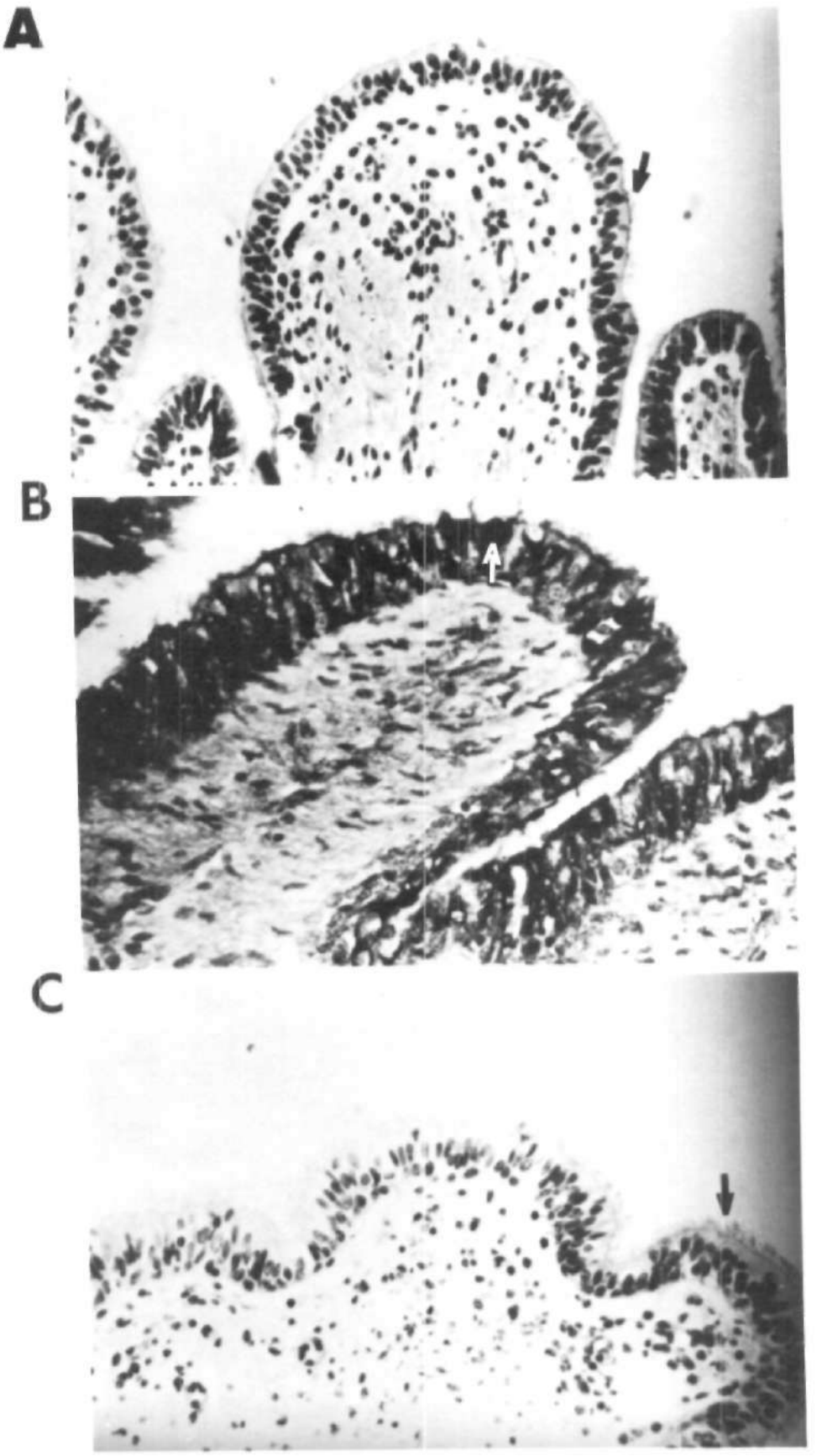
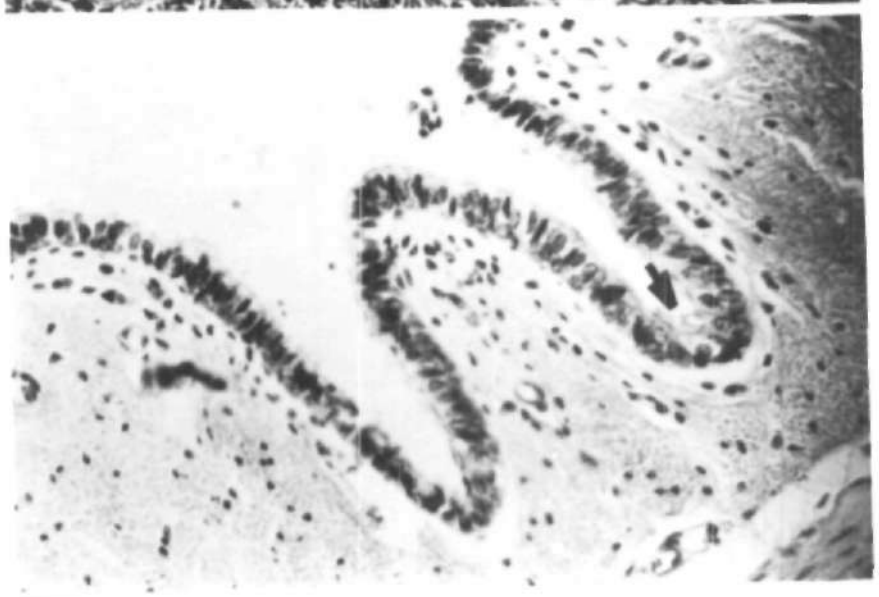

A
B
C

Fig. 3. Light photomicrographs of bovine fetal bronchial epithelium: A. 260 days (Alveolar period), B. and C. 270 days (Alveolar period). Note that the bronchial epithelium reacted positively to McManus stain indicating presence of glycogen (arrows). The epithelium is pseudostratified and cilia are present (arrows) (A. and C. McManus X400). (B. H ∞ E X200).

A



B



C

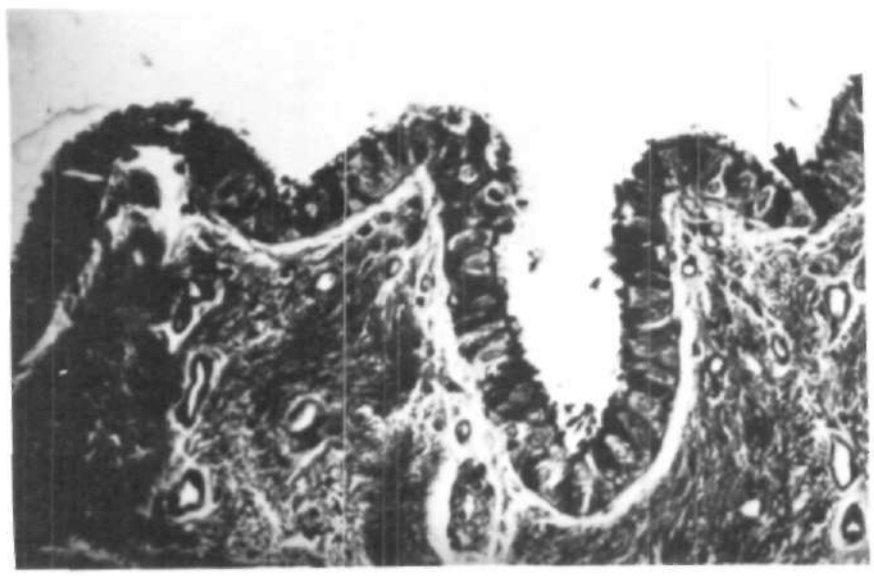
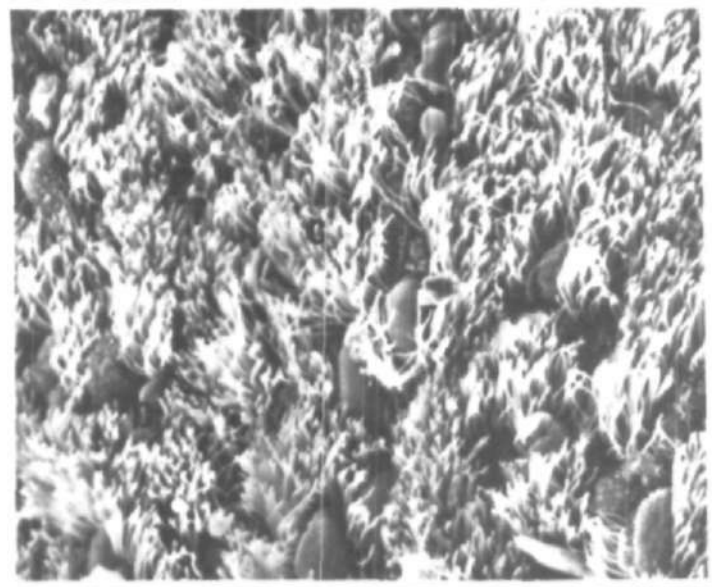


Fig. 4. Scanning electron micrographs of bovine fetal bronchi: A. and B. 120 days (Pseudoglandular period). C. 150 days (Canalicular period) of gestation. Note that both ciliated cell (c) and nonciliated cells (b) are present during these periods. X4000.

A



B



C

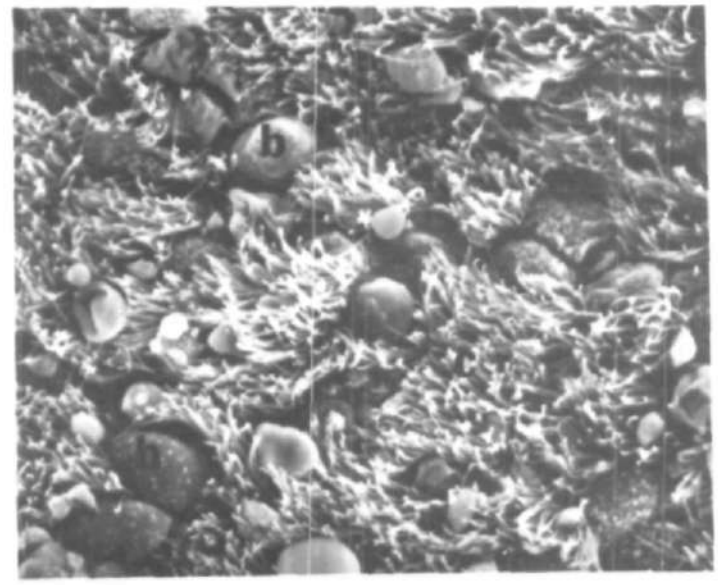


Fig. 5. Scanning electron micrographs of bovine fetal bronchi: A. 160 days (Canalicular period) B. 180 days (Canalicular period) C. 230 days (Terminal sac period) of gestation. Note that the epithelium contained both ciliated cell (c) and nonciliated cell (b) just as was shown during canalicular periods. X4000.

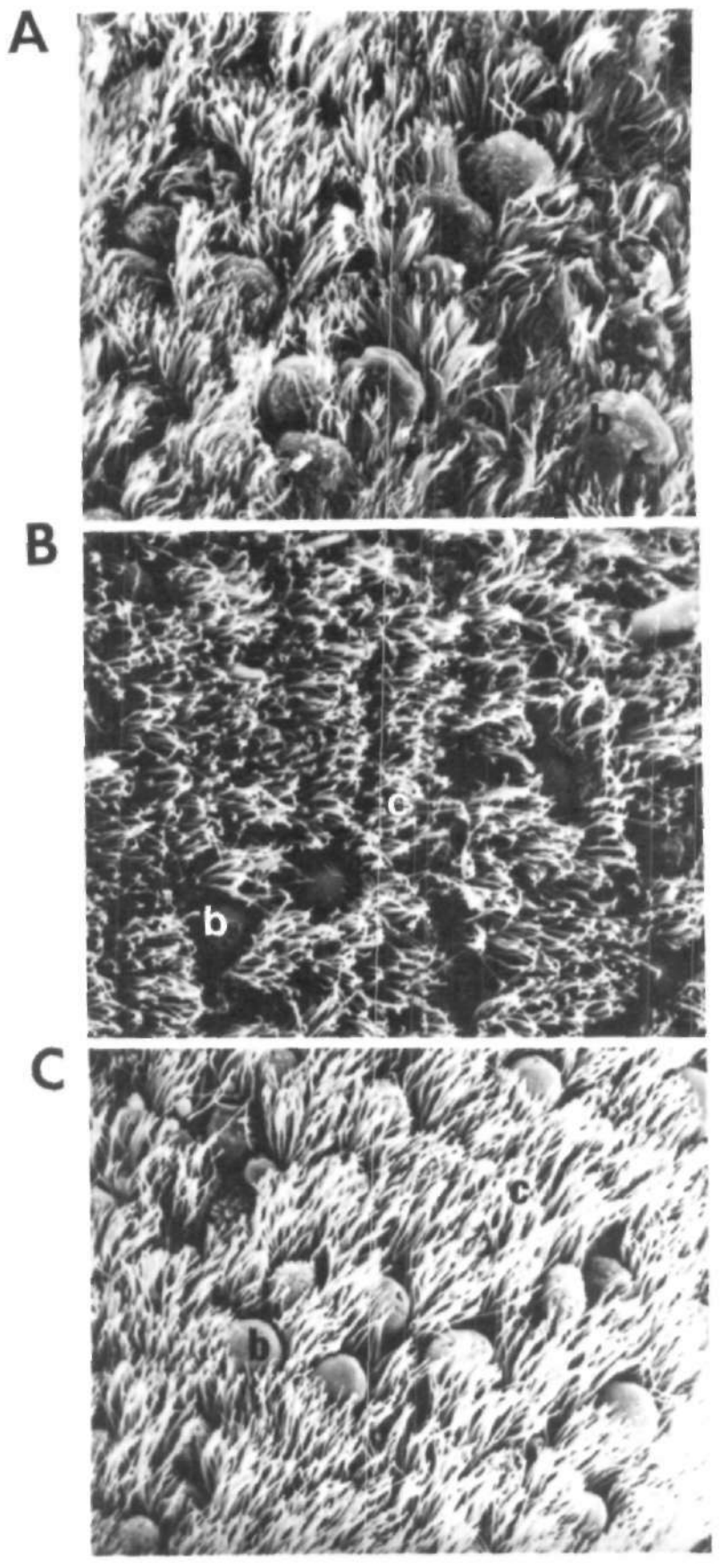

A
b
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c

Fig. 6. Scanning electron micrographs of bovine fetal bronchi: A. 230 days (Terminal sac) B. 250 days (Alveolar period); C. 260 days (Alveolar period) of gestation. Note that during alveolar period the epithelium is mainly covered with cilia with nonciliated cell in between in some places. X4000.

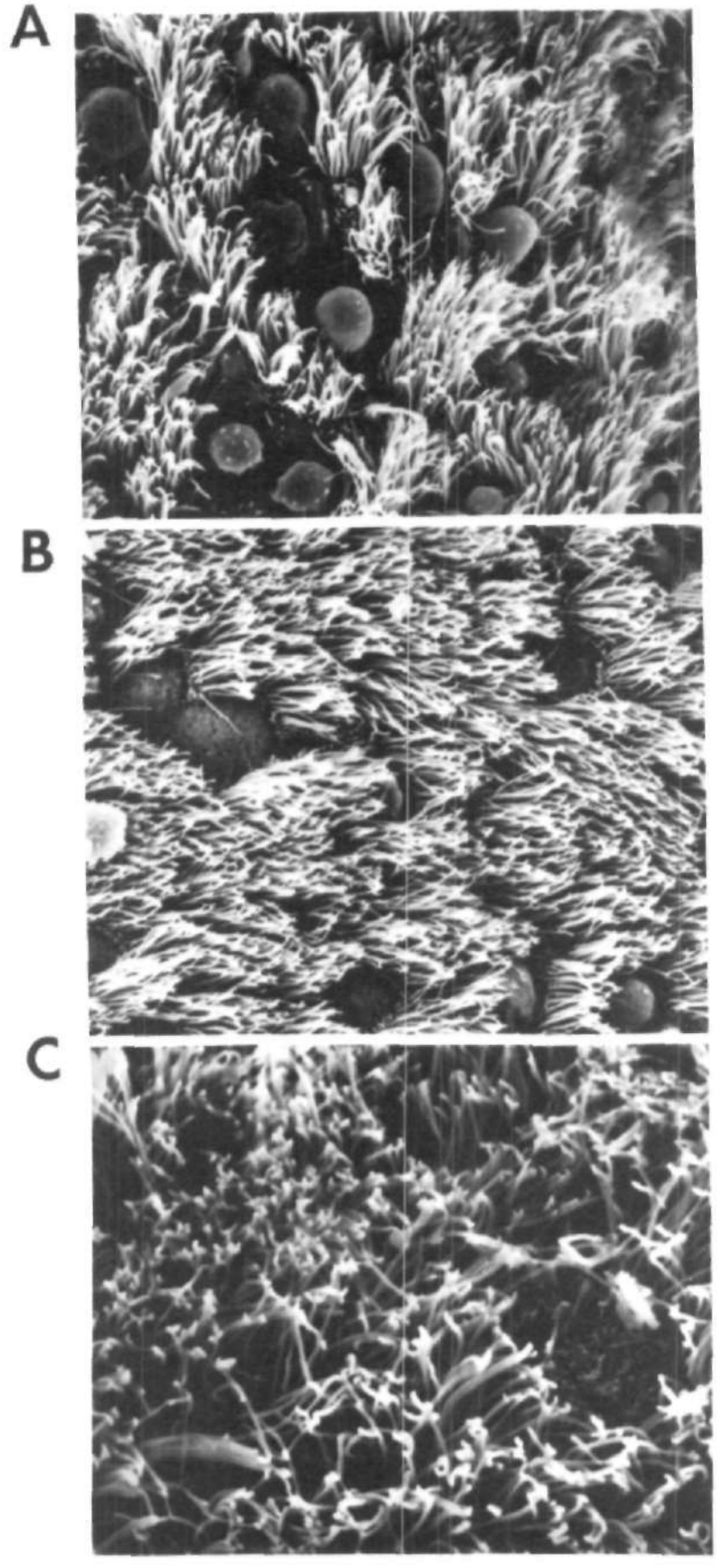

A
B
C

Fig. 7. Transmission electron micrographs of bovine fetal bronchi: A. B. and D. 180 days, C. 150 days of gestation. Note the presence of ciliated cell with cilia (c), secretory cell (s), mitochondria (m), lumen (L). Secretory cells had electron dense core granules (g). Basal cells at 150 and 180 days had electron dense cytoplasm (CT); connective tissue (t) and fibroblast (F). (A. KV 60 X3150) (B. and D. KV 60 X7020). (C. KV 60 X4760).

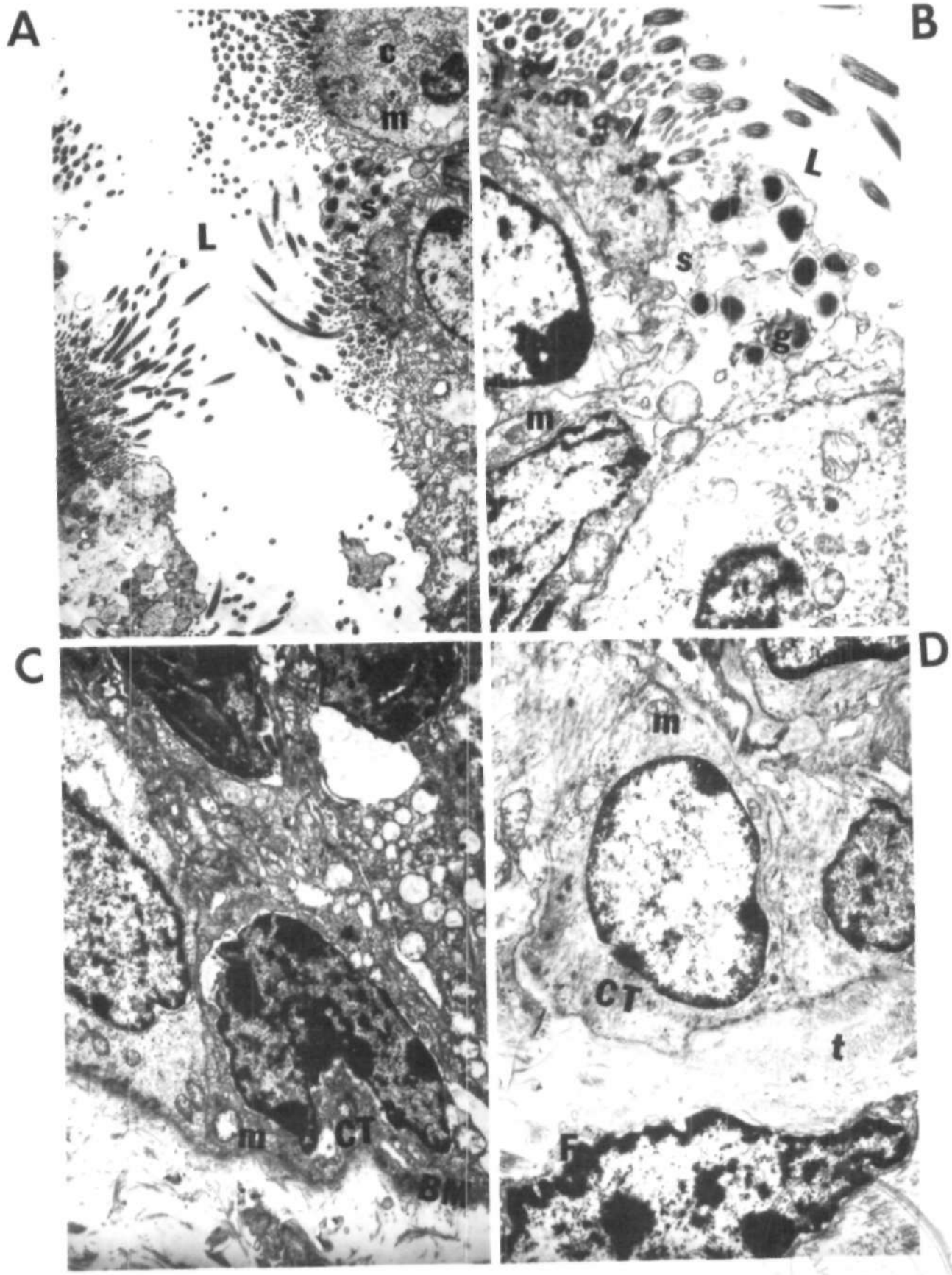

A
c
m
L
s
B
L
s
g
m
C
m
CT
BM
D
m
CT
t
F

Fig. 8. Transmission electron micrographs of bovine bronchi: A. 240 days (Alveolar periods), B. and C. 260 days (Alveolar period) of gestation. Note the presence of ciliated (c), mitochondria (m), nucleus (N), lumen (L) and secretory cells (s) with electron dense core granules (g). (A. KV 60 X3150) (B. KV 60 X2150) (C. KV 60 X4760).

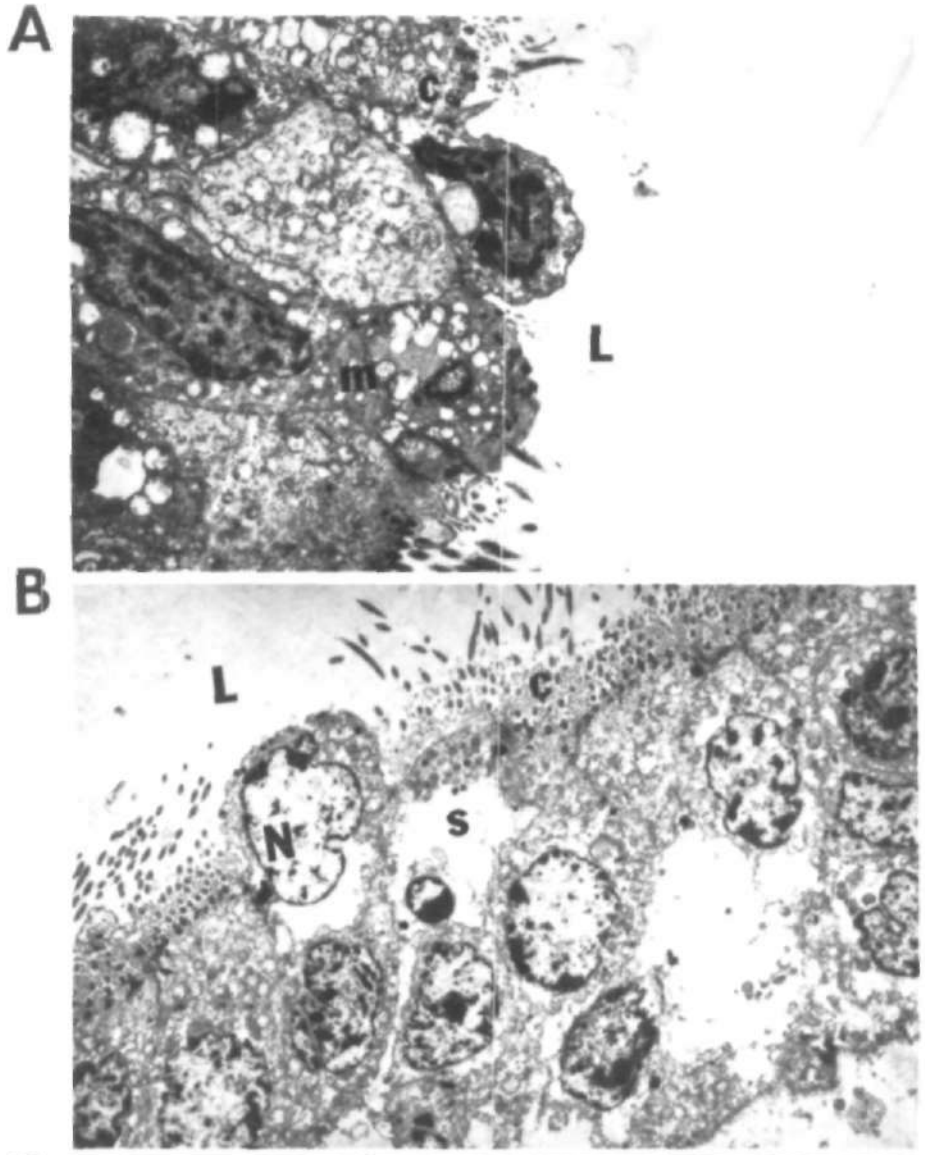
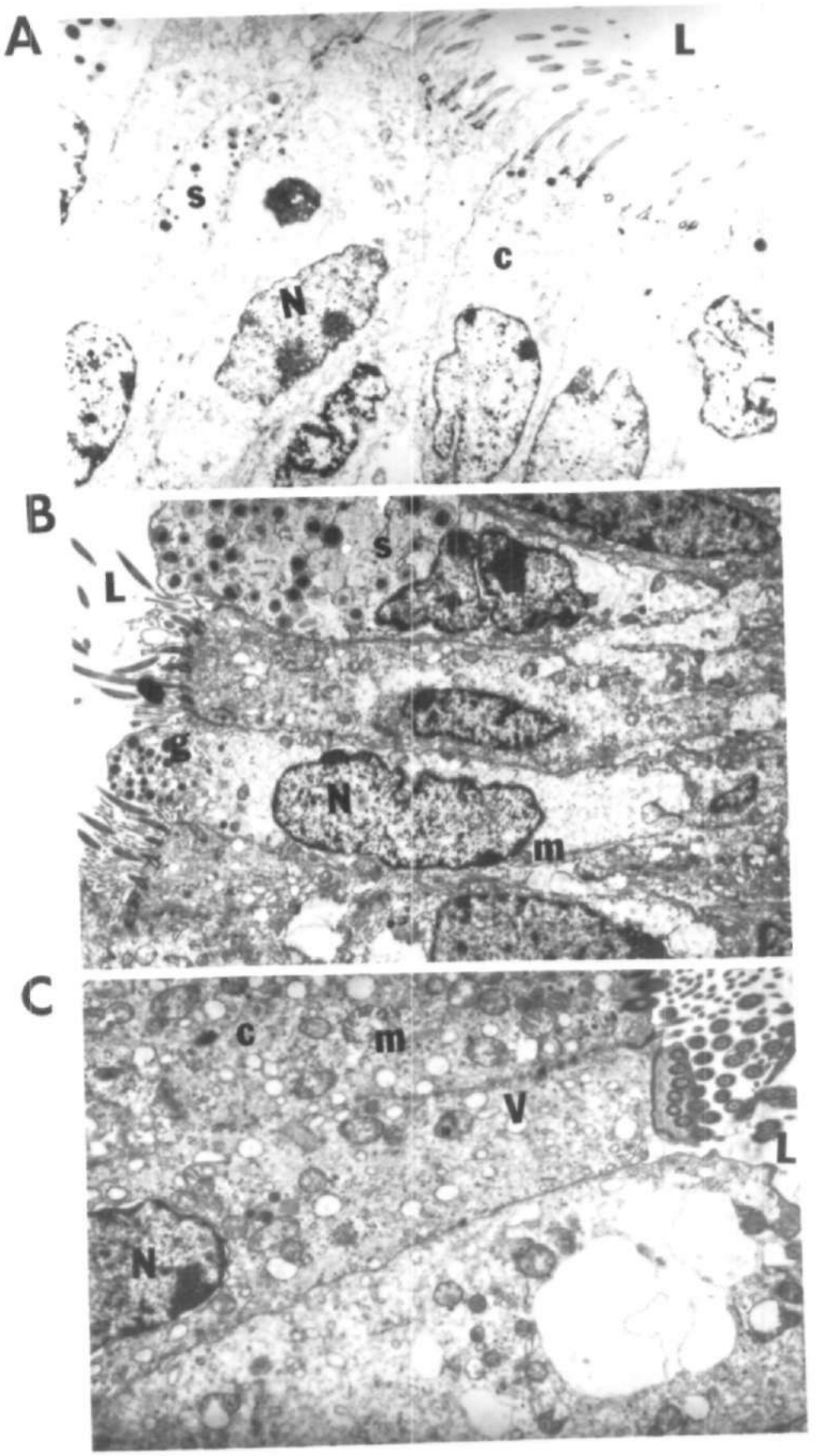

A
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L
m
B
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g
S
L
c

Fig. 9. Transmission electron micrographs of bovine fetal bronchus at 275 days (Alveolar period) gestation. Note the presence of ciliated cell (c), secretory cells (s) with electron dense granules (g), nucleus (N), mitochondria (m), vesicles (V) and lumen (L). (A. KV 60 X3150) (B. KV 60 X4760) (C. KV 60 X7020).


A
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APPENDIX

LITERATURE REVIEW

Origin of Alveolar Macrophages:

It is now known that macrophage do not derive from lymphocytes or mesenchymal cells[1]. Tissue macrophages originate from bone marrow cells as obtained from chimera studies, which demonstrate donor origin of peritoneal macrophages, liver macrophage, and lung macrophages after x-irradiation and bone-marrow transplantation[1]. Studies by van Furth[1], using ^{3}H-thymidine in mice show that only monoblasts and promonocytes divide actively during the steady state and that monocytes and macrophages are in essence non-dividing cells. The same conclusions have been reached for human mononuclear phagocytes[2]

In vivo labeling studies, in normal adult mice and in irradiated mice with partial bone-marrow shielding, showed that the monocytes migrate into the tissues , where they become macrophages[1,3]. Recent information indicating that pulmonary macrophages derive mainly from circulating monocytes, exclude the existence of an interstitial pool of dividing precursors cells , and provide evidence that the limited local production of pulmonary macrophages is the result of mononuclear phagocytes recently derived from the circulation[4]. In general quantitative kinetic studies in normal mice have shown that of the monocytes leaving the circulation, about 56% become Kupffer cells[5], about 15% become pulmonary macrophages[3], and about 8% become peritoneal macrophages[6].

However, Sorokin et al.,[5] using a variety of morphological and functional markers have shown that cells evidently in a macrophage line development are present in fetal lung of rabbit , rats, and hamsters for a considerable time before birth. These macrophage-like cells are established before bone marrow is formed and, in rats, before monocytes have appeared

in the circulation. Further, as they could not identify monocytes in either the pulmonary stroma or the airways in the fetus, they concluded that a replicating population of macrophage-like cells resides in the fetal lungs.

A new mathematical approach of the kinetics of pulmonary macrophages made it possible to evaluate the relative contribution of locally dividing cells and the influx of circulating monocytes to the total population of macrophages in the lung[8]. These calculations showed that for mice in the normal healthy state the maintenance of the normal lung population of pulmonary macrophages depends for at least 70% on monocyte influx and, at a maximum, 30% on local production of macrophages. These locally produced macrophages orginate from dividing precursor cells that have very recently come from bone marrow and most probably divide only once. The calculated mean turnover time of the population of pulmonary macrophages is slightly less than 6 days, while that of peritoneal macrophages and Kupffer cells showed a mean turnover time of about 19 and 4 days respectively[3].

The constant influx of monocytes into tissues, where they become macrophages, implies a constant efflux of cells from the tissue compartment and/or a constant cell death in the tissues[4]. Very little information is available at this point, but various studies have shown that macrophages from liver, lung, skin and gut arrive in the local lymph nodes[9,10]. The absence of monocytes and macrophages in the efferent lymph from these nodes suggest that these macrophages are destroyed in them[11]. Lung macrophages leave the body via the air space as well[4]. Whether macrophages in other tissues migrate to other sites is not known, nor is there firm information concerning recirculation of macrophages via the peripheral blood[12].

During acute inflammatory response, the numer of macrophages occurring in normal tissues is generally too small to deal effectively with either

microorganisms or non-infectious substances that cause tissue injury. A kinetic study of pulmonary macrophages and their direct precursors during an acute inflamatory reaction caused by an intravenous injection or aerosol of heat-killed Bacillus Calmette Guerin (BCG) indicated that the number of circulating monocytes doubled in the first 48 hours and stayed close to that level for the next 6 days[13]. The total number of pulmonary macrophages increased about 2 1/2 fold at about the 4th day and decreased thereafter[13]. Quantitative studies using ^{3}H-thymidine show that the increase of the pulmonary macrophage population during an acute inflamation is brought about mainly by monocytes and to a minor extent by a temporarily increased local production of macrophages[13].

Appearance of Pulmonary Macrophages

During the glandular and the canalicular phases of lung development in human, no intraluminal free cells are observed[14]. This suggests that macrophages must develop in the saccular phase or rather that they begin to populate the airspace surfaces in this period only. They are definitely present in the rat on fetal day 21 or just prior to birth. Sorokin et al.,[7] found that in rats, free cells ranging in appearance from rather undifferentiated leukocytes to typical macrophages were seen a day or two before birth, but cells with the macrophage's complete repertory of inclusions were seen only after birth.

Sorokin et al.,[7] used a variety of morphological and functional markers have shown that cells evidently in a macrophage line of

development are present in fetal lung of rabbits, rats and hamsters for a considerable time before birth.

Function of Alveolar Macrophage

Particles greater than 10.0um are usually cleared from the lung on the mucociliary escalator. Small particles of approximately 1.0um or smaller may reach the respiratory portion of the lung[15] where the alveolar macrophage is thought to be responsible for their removal and destruction[16]. The membrane of the lysosomes fuse with the membrane surrounding the ingested particle and the hydrolytic enzymes[15,17] of the lysosome digest the ingested foreign particles. Alveolar macrophages have also been shown to express receptors for various factors, including lymphokines,chemoattractants, antiproteases, and histamine. They also secrete a large number of other substances having biologic significance[18].

Macrophages have been implicated in the etiology of fibrotic diseases caused by inhaled particles. In man, rats, and mice, Kessel et al.,[19] have shown that silica is toxic to the macrophage. It has been proposed that a substance released by the macrophage, in an attempt to digest the foreign inhaled particles, may be a stimulus to the formation of fibrosis characteristic of silicosis[20]. Allison and coworkers[21] showed that silica ingested by the macrophage caused the release of lysosomal enzymes into its cytoplasm, with the resultant death of the cell. The dead macrophages release a substance that attracts fibroblasts which respond by producing a fibrosis.

The development of an animal immune response requires the interaction of macrophages and antigen-specific T-lymphocytes[22]. Not only do

macrophages process and present antigen to lymphocytes[23] but they also release a variety of monokines, such as interleukin which potentiate lymphocyte functions.

Histochemical Identification of Monocytes and Macrophages

The identification of monocytes and macrophages in mixed lymphoid cell population presents problems for investigators particularly those who are not experienced morphologist[24]. Many methods have been employed to aid identification of these cells including phagocytosis of easily visualised particles and various histochemical reactions that take advantage of macrophage rich complement of lysosomal enzymes[24]. A nonspecific esterase stain has been described by Koski et al.,[24] is a modification of a staining technique for nonspecific esterase originally described by Yam et al.,[25]. Koski et al.,[24] used alpha-naphthyl butyrate esterase activity to identify macrophages. The nonspecific esterase containing cells were distinguished by the presence of intensely red-stained granules in the cytoplasm compared with green counterstain of the esterase-negative cells. Nonspecific esterase activity is strong in monocytes, macrophages, histocytes and megakaryocytes and weak or absent in neutrophils, eosinophils, myeloblasts, promyolocytes and erythroblasts[25]. It is also usually absent from lymphocytes and plasma cells, although occasionally these cells contain a single intensely stained granule. The technique has been successfully applied in studies of human, monkey, mouse, rat, and guinea pig cells[24]. Ennist and Kenneth[26] used acid alpha-naphthyl acetate esterase to rapidly identify macrophages which showed intense red-brown granules after 5 minutes incubation. Dhingra et al.,[27] demonstrated a nonspecific acid alpha naphthyl acetate esterase activity in bovine

lymphocytes and monocytes-macrophages. Lymphocytes exhibited a solitary reddish brown globular reaction adjacent to the cell membrane (T-pattern) whereas the monocytes-macrophages revealed a diffuse reddish brown cytoplasm reaction (M-pattern) products.

Other methods used to identify macrophages include immunocytochemical tests for cell surface antigens[28], phagocytic assays, or phase contrast microscopy[29] which are expensive, take much time, or require considerable experience by the observer to perform as a routine preparation step for most experiments.

Relation Between The Pulmonary Alveolar Macrophages, Surfactant and Phospholipid Metabolism.

The metabolism of phospholipid in the lung and its surfactant physiology has recently been reviwed[30]. The principal component of surfactant is dipalmitoyl lecithin[30,31] and studies performed with labeled precursors have established that the Type II alveolar cell is the primary site for synthesis of surfactant.

The Type II alveolar cell is characterized morphologically by the presence of membrane-bound lamellar bodies in the cytoplasm[32,33]. These lamellar bodies are believed to be an intracellular site of surfactant storage[30,34,35,36]. Tubular mylein, a complex lattice of membrane-like structures has been described within the extracellular alveolar-lining layer[31,37] and is believed to represent an an extracellular storage form[34] or a breakdown product of surfactant[38]. Irregular concentric whorls, known as myelin figures[34], have also been identified in the extracellular space. Tubular myelin and myelin figures can be demonstrated in bronchopulmonary lavage fluid[31,39].

Several lines of evidence indicate that the pulmonary alveolar macrophage population is involved in the clearance of surfactant from the alveoli. The pulmonary alveolar macrophage is enveloped by surfactant in vivo[37], and the lysosomes of the pulmonary alveolar macrophage contain the hydrolytic enzymes necessary for surfactant degradation[40,41].

Bronchoalveolar-lavage fluid obtained from smokers, which contains a larger number of macrophages than does fluid from nonsmokers, has comparatively less surfactant[42,43]. Pulmonary alveolar macrophages have been shown to ingest tubular myelin[39] and both myelin and meylin figures have been identified within the phagolysosome of pulmonary alveolar macrophages[40,44]. Finally, studies with radioactively labeled precursors indicate that surfactant is catabolized within pulmonary alveolar macrophages[45,46].

Surfactant is crucial to the respiratory function of lungs. Derangements in surfactant production or degradation are important in several pathologic conditions, including neonatal-respiratory-distress syndrome, oxygen toxicity, atelectasis, radiation pneumonitis, paraquat poisoning and pulmonary-alveolar proteinosis[47]. The pulmonary alveolar macrophage probably has a major role in clearance of the surfactant material, and quantitative or functional changes in the alveolar macrophage may contribute to the pathogenesis of some lung diseases by altering the amount or composition of the alveolar lining material[47]. In addition, alterations in surfactant may lead to secondary dysfunction of the pulmonary alveolar macrophage.

Lung Maturity

The need for estimation of fetal maturity in human by amniocentesis is

especially indicated when early termination of pregnancy is required in cases of diabetes, Rh sensitization, hypertensive disorders, poor obstetrical history, or onset of premature labor where the decision to attempt to stop labor must be made[48].

Gestational age alone does not provide adequate information about fetal maturity. Several methods have been used for the estimation of maturity, including creatinine levels in amniotic fluid, spectrophotometric peak for unconjugated bilirubin and Nile Blue staining for cytologic evidence of maturity[49].

Evidence shows that infants who develop respiratory distress syndrome lack an adequate surface active alveolar lining which normally stabilizes the fine alveolar air spaces. Alveolar surface activity is attributed primarily to the presence of the phospholipid, lecithin[50].

Lung lecithin is synthesized by two pathways in the human fetus[51].

1. methylation of phosphatidylethanolamine produces α-palmitic β-myristic surface active lecithin which is identifiable beginning week 22 to 24 in fetal lung and amniotic fluid.

2. Cytidine diphosphocholine + D-α-β-diglyceride --> lecithin (dipalmitoyl). Generally this pathway becomes active during week 34 to 36 at which time lecithin levels in lung and amniotic fluid rise sharply.

The fact that the fetus can synthesize lecithin by the methylation pathway enables some premature babies to survive, however, this pathway is easily inhibited by stresses such as hypothermia, hypoxia-acidosis and hypercapnea. Such inhibition leads to respiratory distress syndrome[50].

Determination of lecithin level in amniotic fluid is a sensitive indicator of fetal lung maturity[51]. It has been established that the lecithin in amniotic fluid originates primarily from the fetal lung[51].

The basis for the L/S Ratio Test is the determination of the ratio of two phospholipids, lecithin and sphingomyelin. In normal development, sphingomyelin concentrations are greater than those of lecithin until about gestation week 26. After this , lecithin levels are equal to or slightly greater than those of sphingomyelin in amniotic fluid. At week 34 to 36, a rapid surge of lecithin levels indicates a mature lung and respiratory distress syndrome should not occur. At this time lecithin may be 4 times greater than sphingomyelin[51].

Appearance of Surfactant

Lamellar bodies are related to the production of phospholipids[36,52,53,54]. There are many observations that the development of lamellar inclusions in Type 11 pneumocytes shortly before birth coincides with the first appearance of surface-active material in lung extracts. This temporal coincidence has been demonstrated on day 18 of fetal development in mice[55,56], around day 20 in rat[57], between days 120 and 130 in lambs[58,59], 5 to 6 months or 160 days[60] of age in human and at 92 days of gestation in the pig[61].

AIRWAY LINING EPITHELIUM

Light microscopic appearance

In the human, using hematoxylin and eosin and specific stains for mucus, four cell types have been described in the epithelium of proximal airways: (a) ciliated, (b) mucus-secreting goblet, (c) intermediate and (d) basal cells[62]. In the terminal brochioles a fifth cell, the clara cell has been described[63].

Using a combined Alcin blue and periodic acid-schiff technique, more than one type of mucous cell has been found, and in man three types of mucus-secreting cells can be distinguished[64].

Other cell types reported by light microscopy in the airway epithelium of man and other species include lymphocytes[62], mast cells[65], and clear or argyrophilic cells[66].

The epithelial lining in both man and rat is pseudostratified and consists of vertically orientated cells with their thinner ends extending to more basally situated cells during the glandular phase[67].

Ciliated cells are found throughout the bronchial tree by the thirteenth fetal week in man[68]. In the rat, cilia appear late in fetal life at about day 20 of gestation[67]. Cilia first appear in the trachea and then progressively at other more distal level of the respiratory tract[69].

Human mucus is histologically demonstrable by the thirteenth week of gestation[70]. At this time both neutral and acidic mucus-secreting cells are present in similar number. In the rat fetus, at 21 days' gestation mucus-secreting cells are found in the trachea[67].

In man a basal layer of polygonal cells can be identified by 10 weeks of gestation, at which time a few spindle-shaped intermediate cells can be found[68]. Clear cells are present in the epithelium of newborn infants at all levels of airway[71]. Similar cells have been reported in the young rabbit[72].

Electron Microscopic Appearance

All cell types found by light microscopy have been seen by electron microscopy except that two types of acidic-secreting goblet cells cannot be distinguished with the staining techniques available for electron

microscopy[73]. The most complete electron microscopic studies made used the laboratory rat[74]. Less is known about the ultrastructure of the airways of man and other species[73].

During the glandular phase of development of the lung, all cells of the bronchial epithelium are ultrastructurally similar and resemble those of the adjacent developing esophagus[70]. In man[75], and rat[67], each cell has electron-lucent cytoplasm and a nucleus of smooth outline with one or two nucleoli. Cell organelles are sparse, consisting of a few polysomes, mitochondria with well developed cristae, an occasional profile of rough endoplasmic reticulum and glycogen. Smooth endoplasmic reticulum and Golgi material are absent at this stage[73] but a few microvilli, are present and occasionally, a single primary cilium, tight junctions and desmosomes are seen.

CELL TYPES

Type II Pneumocytes

The Type II pneumocyte is cuboidal in shape, measuring approximately 9 micrometers in diameter, has microvilli along its free alveolar edge, contains more organelles than the Type I and contains osmiophilic lamellated bodies thought to be associated with the production of pulmonary surfactant[36,52].

Type I and II pneumocytes are present in the alveoli in the proportion of 40-60%, repectively. However, approximately 95% of the alveolus is covered by Type I pneumocytes and only 5% by the Type II[76].

Type II pneumocytes are capable of proliferation in response to damage to the epithelial lining[77-79]. Proliferating Type II cells are later thought to be transformed into Type I pneumocytes possibly after

mitosis[78,80].

Osmiophilic inclusion bodies first appear at about 121 days of gestation in lambs[58]. In the rat fetal lung, lamellar bodies and their potential precursors appear for the first time on the twentieth gestational day which is 2 days before birth[57,81]. Wang et al.,[82] have shown them to become detectable in 26 day-old rabbit fetuses. Although the appearance in human fetal lungs is not clearly established yet, there is some indication that osmiophilic granules may be formed at a relatively earlier prenatal stage[60], namely during the sixth month.

Ciliated Cells

Primary cilia are first seen during early fetal life and in rat they appear by day 16 of fetal life[67]. The primary cilium develops from a centriole formed by division of a centriole of the diplosome[69].

Secretory Cells

The secretory cells of the proximal airways of man and other species may be classified, according to their type of secretion and ultrastructure into glycoprotein-secreting cells and clara cells[73].

By 16 weeks' gestation, the airway epithelium of human fetus contains secretory cells[67]. Two types of secretory granules dense and electron lucent[67] may be identified. However, after birth, only goblet cells with electron-lucent granules are seen in adult trachea[83]. Goblet cells develop later in gestation in rat and rabbit than in man[67,84]. In rat, the early undifferentiated cell becomes secretory by formation of rough endoplasmic reticulum and granules during the canalicular phase[67]. The endoplasmic reticulum becomes abundant and it is dilated by amorphous content. At this stage, intracellular glycogen is reduced and electron dense granules

develop at the apex of the cell[67]. At birth, secretory cells still have rough endoplasmic reticulum dilated by amorphous content but by 5 days of age, such distension is no longer visible. Cireli[85] believes these cells secrete a protein but the function of this secretion is unknown.

Clara Cells

Information on the developing Clara, or nonciliated bronchiolar cell, in lung is scarce. According to Jeffery and Reid[75] the nonciliated cell populations comprises four cell types, the Clara cells, serous cells, intermediate and brush cells. Clara and serous cells comprise approximately 20 per cent of the nonciliated cell population and intermediate and brush cells comprise approximately 15 per cent. Therefore, 35 per cent of the bronchiolar cell population is nonciliated cell while ciliated cell is 65 per cent. Jeffery and Reid[67] observed Clara cells in the 16-week human fetus. The Clara cell at this time has an abundance of intracellular glycogen with occasional profiles of rough endoplasmic reticulum and polysomes as does the nonciliated cell of the prematurely born infant of 22 weeks. By 23 weeks some smooth endoplasmic reticulum has ribosomes associated with it[67]. Clara cells of prematurely born humans have fewer secretory granules and even these have a different morphology from those of the adult[86].

Ultrastructural studies of the Clara cells in different mammals such as the mouse[87], rabbit[88], pig[89], rat[74], monkey[90], calf[91], and man[92] have shown morphological species differences. Smith and coworkers[93] compared the Clara cells in the terminal bronchioles of different mammals by light microscopy and by transmission and scanning electron microscopy. In the rat, clara cells are high columnar, nonciliated cells projecting high above the luminal surface of the ciliated cells into the bronchiolar lumen. The

smooth endoplasmic reticulum, mostly arranged in small vesicles and many membrane-bound granules. Rough endoplasmic reticulum is found especially around the oval appearing nucleus and is closely associated with mitochondria. The well developed Golgi apparatus lies in the proximity of the nucleus on its apical and lateral sides[93].

The intracytoplasmic granules which are usually thought to be secretory[94] show a great variability in size, shape and electron density. The exact biochemical nature of the granules remains to be resolved. As shown by pepsin digestion studies[95], the secretory granules most probably contain a protein, the nature and the actual function of which is not known.

The function of the Clara cells is not known. Most morphological and cytochemical features are compatible with a secretory function[96]. Some experimental studies with tracer particules show the Clara cells in neonatal rabbit to be capable of phagocytizing foreign particles[97]. Clara cells have also been proposed as the cells of origin for bronchioloalveolar carcinoma of the lungs[98]. From studies on the renewal of the bronchiolar epithelium, Evans and co-workers[99] concluded that Clara cells were progenitor cells for the bronchiolar epithelium.

In the fetal rabbit, Clara cells lack secretory granules and have large amounts of glycogen and few organelles. In the rat the primitive epithelial cell has developed into a nonciliated cell with supranuclear glycogen and some rough endoplasmic reticulum by 16 to 17 days gestation[74]. By 21 days' gestation, small electron-dense granules are seen at the Clara cell apex but the cell is immature and lacks the abundance of smooth endoplasmic reticulum seen in the mature Clara cell.

Kultschitsky Cells

Cells containing large numbers of small dense-cored granules are present at all levels of the tracheobronchial tree[100] and have recently been recognized at alveolar level in the rat[72]. The cells occur individually interspersed among other epithelial cells of the airways and grouped into organized structures termed neuroepithelial bodies[72,101].

In humans, using electron microscopy these cells are found in the basal layers of both the distal and proximal airway epithelium at 16 weeks gestation[67]. In prematurely born infants, Kultschitsky cells are present and, as in the adult, are usually seen as single cells, occasionally reaching the airway lumen. In contrast to those of the adult, they typically have electron dense cytoplasm and abundant glycogen[102].

Brush Cells

At 22 days' gestation, brush cells have been described in trachea cells of rabbit[84]. In rat trachea, possible precursors of this cell may be found as early as 21 days gestation but a brush border is clearly identified at 12 days of age and by 19 days the brush cell has its adult appearance and is present at all airway levels[67].

Basal Cells

Basal cells can be identified in the human trachea[68] by 10 weeks gestation. In man, there is no electron-microscopic description of these cells in the developing fetus[73]. In rat trachea, basal cells appear at about 21 days gestation[67,85]. At this age, the cells have electron-lucent cytoplasm which gradually increases in density with age, presumably by an

rabbit, basal cells are not present until term. Basal cells have been described in the bronchi of pigs 4 to 18 weeks old[103], but no change with age has been reported.

EMBRYOLOGY

The human lung begins as an epithelial bud, arising from the caudal end of the laryngo-tracheal groove. The lung bud appears on day 26 after ovulation[104]. It divides, then elongates into two lung buds. These grow dorsally on either side of the esophagus[105]. Meanwhile, upper, middle, and lower buds have developed which give rise to primary buds of the ten bronchopulmonary segments and become the future airways[106].

Spooner and Wessells[107] described the initial lung bud formation as follows. *The first indication of the lung rudiments is the appearance of* two laterally-placed endodermal buds at the 25-27 somite stage. The buds appear during embryogenesis in response to some initiating induction in the foregut. The two lung buds elongate in a caudo-ventral direction and at the same time the ventral endoderm, immediately caudal and between the bases of the buds, moves dorsally in a relative sense. This results in a ridge of tissue forming that connects the posterior surfaces of the lung buds. By this process, the primary branch point of the lung primordium is established. The two buds now constitute the primary bronchi, structures traditionally considered to rise by bifurcation of the posterior end of the trachea. Trachea formation begins at this posterior branch point, and *extends in an anterior direction. Tracheal development is independent of* lung bud formation[107]. As Hooper and Hart[108] explained, the nature and source of the inducing stimulus involved in fetal lung development, remains unknown. It has been suspected for a long time that the stimulus deals with

a epithelio-mesenchymal interaction and in 1971 Alescio and Dani[109] made three distinctions about all the previous research done in this area. They explained how the wide variety of data obtained from other studies emphasized the idea of multiplicity of mesenchymal factors operating in this epithelio-mesenchymal interaction. They considered that there were three different types of epithelio-mesenchymal effects that were experimentally distinguishable: control of growth rate, control of morphogenetic patterns, and regulation of specific metabolic patterns.

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BOVINE FETAL LUNG DEVELOPMENT

by

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AN ABSTRACT OF A DOCTOR'S DISSERTATION

submitted in partial fulfillment of the
requirements for the degree

DOCTOR OF PHILOSOPHY

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Manhattan, Kansas

1985

Bovine fetal lungs were collected from abattoirs, (Jack Polen Beef Co., Kansas City, Kansas and Wellington Quality Meat, Wellington, Kansas) for three purposes: 1) to histochemically determine when alveolar macrophages appear in the fetal lung; 2) to correlate the morphologic characteristic of Type II pneumocytes with appearance of pulmonary surfactant and, 3) to study, using electron microscopic techniques, the appearance of the fetal trachea, bronchus and bronchioles.

Esterase-positive alveolar macrophages which appear in the alveolar septa and lumina were first seen at 240 days of gestation. These macrophages were identified by the presence of numerous diffuse intensely red-stained granules in the cytoplasm. The time of appearance of alveolar macrophages coincides with the first apppearance of surfactant at the alveolar surface-lining layer which suggests that macrophages could be involved with the clearing of excess surfactant being produced.

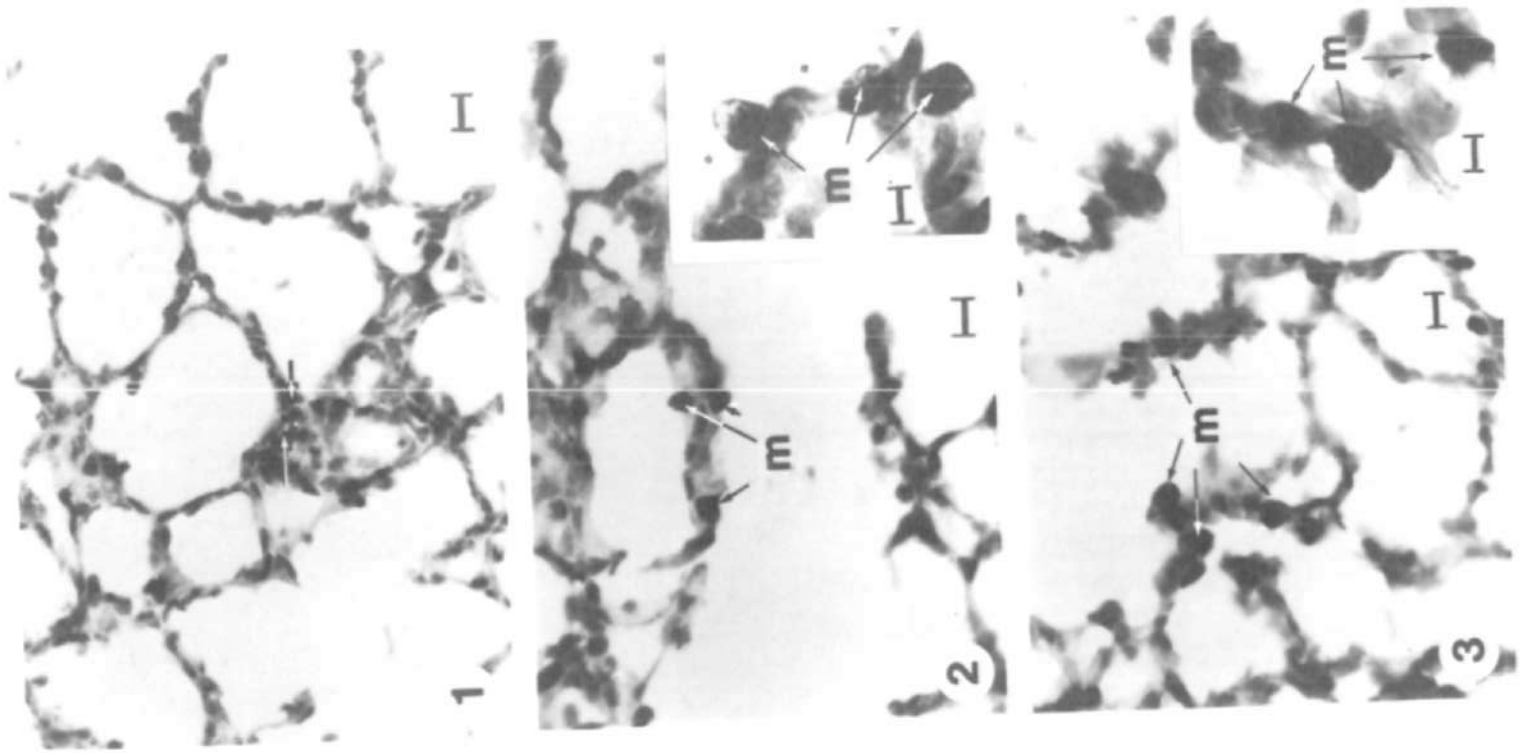
Type II pneumocytes without lamellar bodies appeared at 200 - 210 gestation days. Type II pneumocytes with lamellar bodies appeared at 230 days which coincides with the appearance of lecithin in amniotic fluid from the fetal lung. The aleveolar surface lining layer was covered with surfactant at 240 gestation days.

Five cell types, ciliated, secretory, brush, basal and neuroendocrine were identified in the fetal trachea. Neuroendocrine cells of unknown function had granules with large electron-dense cores. Two types of secretory cells were observed, one with electron dense granules and the other electron lucent. Basal cells had electron dense

cytoplasm. Brush and basal cells were identified during the canalicular period while secretory and neuroendocrine cells were observed during the alveolar period.

Neither the neuroendocrine cell described in human and rat fetal lung nor the brush cell described in rabbit and rat fetal lung were identified in the bovine fetal bronchi. During the pseudoglandular period electron microscopy showed three cell types, ciliated, secretory and basal. Secretory cells with electron dense granules were observed during canalicular period.

Two bronchiolar cell types were identified, the ciliated and the primitive Clara cells. However, Clara cells were seen earlier than ciliated cells which may suggest that the other bronchiolar cells were derived from Clara cells. Neurosecretory and the intermediary cells described in other species were not observed in this study.


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Part 2. CORRELATION OF MORPHOLOGIC CHARACTERISTICS OF TYPE II PNEUMOCYTES WITH THE APPEARANCE OF PULMONARY SURFACTANT IN BOVINE FETUS

ABSTRACT

Bovine fetal lungs from 80 fetuses age range 120-270 days were studied to correlate the morphologic characteristics of type II pneumocytes with the appearance of surfactant. Type II pneumocytes without lamellar bodies appeared at 200-210 gestation days. Type II pneumocytes with lamellar bodies appeared at 230 days which coincides with the appearance of lecithin in amniotic fluid from the fetal lung. The alveolar surface lining layer was covered with surfactant at 240 days of gestation.

INTRODUCTION

There are many observations of lamellar inclusion development in type II pneumocytes shortly before birth which coincide with the first appearance of surface active material in the lung extracts. This temporal coincidence has been demonstrated on day 18 of fetal development in mice,[1,2] around day 20 in rat,[3] between days 120 and 130 in lambs,[4,5] 5 to 6 months or 160 days[6] of age in human and at 92 days gestation in the pigs.[7]

The principal component of surfactant is dipalmitoyl lecithin[8,9] and studies performed with labeled precursors have established that the type II alveolar cell is the primary site for synthesis of surfactant.

Lung lecithin is synthesized by two pathways in the human fetus.[10] One involves methylation of phosphatidylethanolamine to produce α-palmitic β-myristic surface active lecithin identifiable beginning week 22 to 24 in fetal lung and amniotic fluid. The other pathway uses cytidine diphosphocholine and D-α-β-diglyceride to form lecithin (dipalmitoyl). Generally the latter pathway becomes active during week 34 to 36 at which time lecithin levels in lung and amniotic fluid rise sharply.

Surfactant is crucial to the respiratory function of the lungs. Derangements in surfactant production or degradation are important in several pathologic conditions including neonatal-respiratory-distress syndrome.[11]

Children with respiratory distress syndrome are born without surfactant and their lungs remain collapsed.

Transmission electron microscopy has been used to identify phospholipid of surfactant by tricomplex flocculation technique in the guinea pig.[12]

There are no reports indicating when surfactant appears in bovine fetal lung. The purpose of this study is to determine when surfactant appears in the fetal lung and to correlate this appearance with the morphologic characteristics of type II pneumocytes.

MATERIALS AND METHODS

Tissue collection

Bovine lung tissues from 80 Holstein Friesian fetuses were collected from abattiors (Jack Polen Beef Co., Kansas City, Kansas and Wellington Quality Meat, Wellington, Kansas). Fetal ages were estimated by using published criteria.[13] Of the total number of fetal lung collected, 10 each were taken from fetuses of 120, 150, 250 and 250 days gestation; 5 each from fetuses of 210, 220, 230, 260 and 270 days of gestation, 8 from 240 day old fetuses and 7 from 190 day old fetuses. All lungs were processed and used for transmission electron microscopy study. From the same fetuses, using sterile needles and screw cap glass test tubes amniotic fluids were obtained from 3 fetuses at 190 days of gestation and from 5 fetuses each at 210, 230, 240, 260 and 270 days of gestation. Amniotic fluids were kept in ice bucket for a few hours and later put in the freezer. Amniotic fluid were processed for lecithin-sphingomyelin ratio at Petterson's Commercial Pathology Laboratory. Strips approximately one centimeter were cut from the caudal lobe and divided into one millimeter blocks.

Transmission Electron Microscopy

Lung samples of 1 mm^3 were fixed in cold 2.5% glutaraldehyde in 0.05M sodium cacodylate pH 7.3 containing 5mM KCl and 2.5mM $MgCl_2$, rinsed three times in cold 0.1M sodium cacodylate buffer, pH 7.3, postfixed in 1% cacodylate

buffered osmium tetroxide for 2 hours, rinsed in distilled water, and stained en bloc with 1% aqueous uranyl acetate for 2 hours before dehydration through a graded series of ethanol and acetone. Other samples were fixed in cold 2.5% glutaraldehyde in 0.05M PIPES, pH 7.3 containing 5mM KCl and 2.5mM $MgCl_2$. The samples were treated like those in sodium cacodylate buffer. Fixation and prestain procedures were in ice buckets.

Fixation of Pulmonary Surfactant for Electron Microscopy

Some samples were fixed in cold 2.5% glutaraldehyde in 0.05M soidum cacodylate, pH 7.3 containing 5mM KCl and 2.5mM $MgCl_2$ for 1 hour. The tissues were rinsed briefly in buffer and treated by means of tricomplex flocculation approximately 30 minutes by placing the tissue in a solution made 0.05N in $Pb(NO_3)_2$ and $K_3Fe(CN)_6$. The tissues were rinsed again with buffer and postfixed in a 1% osmium tetroxide in cacodylate buffer for 1 hour. Other tissues were fixed in glutaraldehyde and tricomplex flocculation. The tissues were then dehydrated quickly in graded acetone.

All tissues were embedded in Epon/Araldite. The blocks were trimmed and sectioned with glass knives in a Reichert OM-U2 ultramicrotome. The thin sections (60-150 nanometers) were stained for 10-15 minutes with 5% uranyl acetate-50% ethanol and with Reynolds lead citrate for 5-7 minutes. Thin sections from samples treated with tricomplex flocculation were not stained with uranyl acetate and Reynolds lead citrate.

All sections were examined in a Philips 201 transmission electron microscope at 60 KV. Pictures were taken on Cronar Ortho S Litho film.

Surfactant Analysis - Lecithin/Sphingomyelin Ratio

Fetal amniotic fluid was obtained by sterile needles and placed into a screw cap glass test tube. Immediately the amniotic fluid was obtained, it was centrifuged at slow speed (1000 RPM) for 3 minutes and the supernatant removed. Specimens were kept in ice bucket for a few hours. Each sample received 2ml of methanol, mixed thoroughly on a vortex type mixer for 10-15

seconds, followed by 2ml of chloroform and mixed for at least 20 seconds on the mixer. Samples were centrifuged at high speed (3000 RPM) for at least 10 minutes. The tubes were tilted at 45° angle and a 9 inch Pasteur pipette was used to separate the chloroform layers from the methanol layers. Lecithin and sphingomyelin were extracted from the sample with a chloroform-methanol mixture and spotted on a thin layer chromatography (TLC) plate. The two extracts were separated using chloroform-methanol-ammonium hydroxide solvent system and made visible using a dilute cupric acetate-phosphoric acid reagent. The lecithin and sphingomyelin were identified by comparison to a standard. The relative intensity of the two spots was expressed as a ratio.

RESULTS

Surfactant Analysis - Lecithin/sphingomyelin Ratio

Results show no production of lecithin at 190-210 days of gestation (Fig. 1A). However some sphingomyelin was detected in the amniotic fluid during this period (Fig. 1A). From 230 days of gestation, both Lecithin and Sphingomyelin were detected in the amniotic fluid (Figs 1A, B). However, the intensities of sphingomyelin were more than that of lecithin during 230-270 days of gestation. Intensity of sphingomyelin at 270 days of gestation was less than that at 240-260 days of gestation (Fig 1B). At 240 days of gestation, the ratio of sphingomyelin to Lecithin was approximately 2:1 (Fig 1B). However, from 260 days of gestation the ratio of lecithin to sphingomyelins was approximately 1:1 when compared with the standard control of 2:1 lecithin to sphingomyelin ratio.

Type II pneumocytes

Type II pneumocytes begin to appear in the lung at 200-210 days of gestation (terminal sac period) (Fig. 1C). Lamellar bodies typically found in type II pneumocytes with osmiophilic inclusions were absent during this period (Fig.1C). Also the cytoplasm of the type II pneumocyte was devoid of organelles. By 230 days, lamellar bodies with osmiophlic inclusions appeared in the type II pneumocytes (Fig. 1D). Type II pneumocytes with numerous

lamellar bodies were observed from 240 to 270 days of gestation (Figs. 2A-D, 3A-C). Mitochondria were observed in the cytoplasm of the type II pneumocytes at 260 days of gestation (Fig. 2D).

Observation of Pulmonary Surfactant by Electron Microscopy

The alveolar surface lining layer contained electron dense globules showing that surfactant was present from 240 days of gestation (alveolar period) (Fig. 3D). In Figs. 4A-D electron dense globules were seen forming in some places an almost continuous epithelial lining layer. In other places, the alveolar surface lining layer was devoid of any dense material during 260-270 days of gestation.

DISCUSSION

This study shows that type II pneumocytes contain lamellar bodies at 230 days of gestation. The appearance of osmiophilic inclusions of the lamellar bodies at 230 days of gestation coincides with the appearance of lecithin in amniotic fluid. The principal component of surfactant is dipalmitoyl lecithin[8,9] and studies performed with labeled precursors have established that the type II pneumocytes is the primary site for synthesis of surfactant. Also, it was shown by electron microscope using tricomplex flocculation that the alveolar surface lining layer contained surfactant at 240 days. This temporal coincidence has been demonstrated on day 18 of fetal development in mice,[1,2] around day 20 in rat,[3] between days 120 and 130 in lambs,[4,5], 5 to 6 months or 160 days[6] of age in human and at 92 days of gestation in the pig.[7]

The ratio of sphingomyelin relative to lecithin decreased at 260-270 days of gestation, indicating that more lecithin is produced during this period. Since the ratio of lecithin to sphingomyelin is still less than the control ratio 2:1, it implies that the lung during this period is still not mature and that lung maturity occurs at the time of birth or shortly after in bovine. In normal human development, sphingomyelin concentrations are greater than those of lecithin until about gestation week 26. After this, lecithin

levels are equal or slightly greater than those of sphingomyelin in amniotic fluid.[10] At week 34 to 36, a rapid surge of lecithin levels indicates a mature lung and is a period when the threat of respiratory distress syndrome is decreased. At this time the concentration of lecithin may be 4 times greater than sphingomyelin.[10] Similar work had not been reported in bovine. However, preliminary work reported in this study indicates that by 260 days of gestation the lecithin level rises relative to that of sphingomyelin.

Embryo transfer and multiple pregnancies are becoming common in domestic animals particularly in the valuable females. In order to save potential valuable animals and the fetus there may be a need to terminate the pregnancy. This may require the determination of the maturity of the fetus. Hence it will be necessary to determine lecithin-sphingomyelin ratio in domestic animals to evaluate fetal maturity.

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Fig. 1. A. Thin layer chromatograph showing the lack of production of lecithin at 190-210 days and an increase in production of both lecithin and sphingomyelin from 230 days of gestation. B. Thin layer chromatograph demonstrating the increase in lecithin production from 240-270 days. At 270 days of gestation the sphingomyelin intensity was lowest compared with the intensities at 240-260 days of gestation. C. Transmission electron micrograph taken at 200-210 days of gestation showing type II pneumocyte (EP2). Typical lamellar bodies have not yet developed in the type II cell. Tight junction complexes are present between type 1 and type II pneumocytes (arrows) (X7020). D. Transmission electron micrograph of bovine fetal lung at 230 days of gestation showing type II pneumocyte with lamellar bodies containing osmiophilic inclusion (b) and erythrocytes (ER) in capillary. X 13,940. PG = Phosphatidylglycerol, PI = phosphatidylinositol, PS = phosphatidylserine.

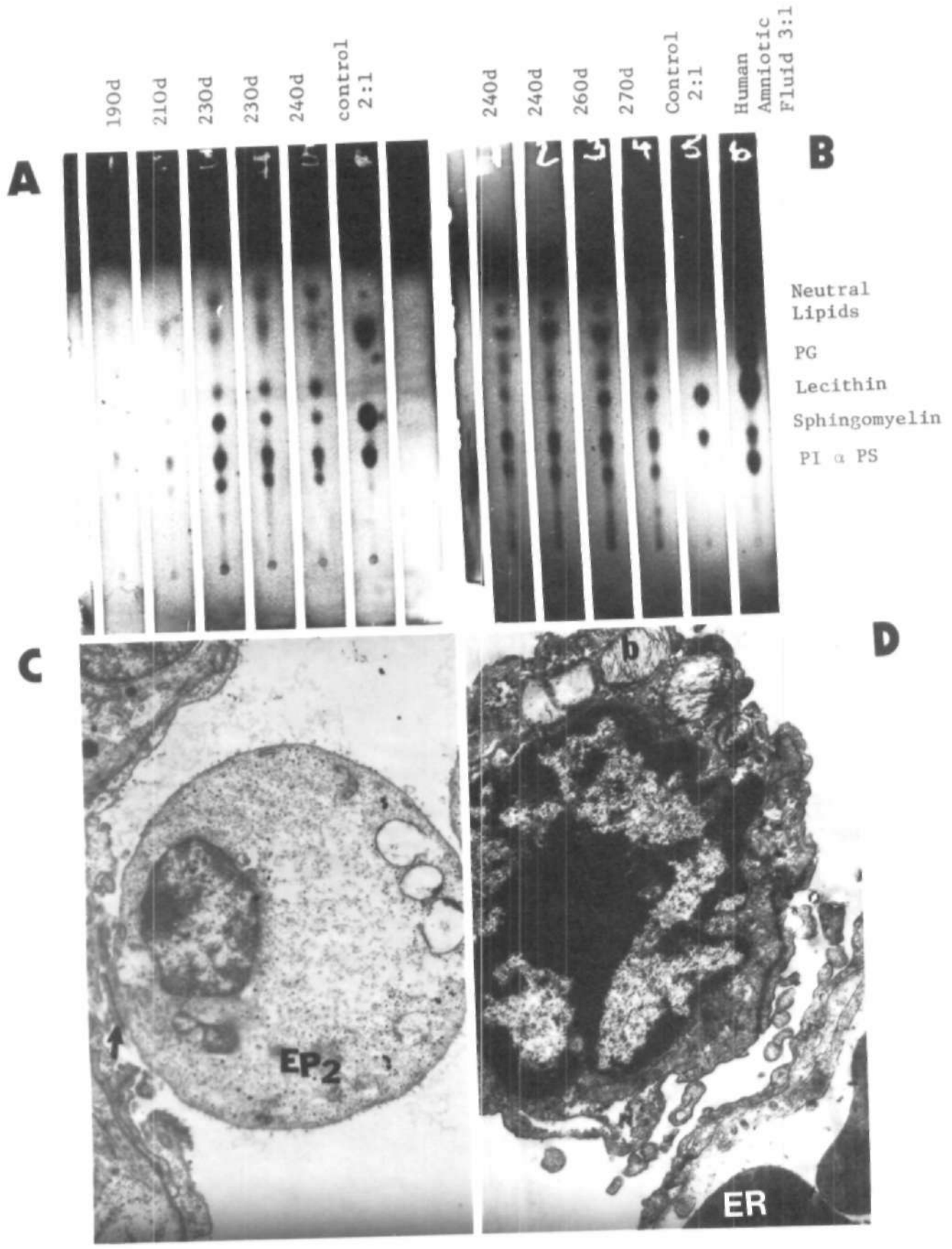

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C
EP2
D
b
ER

Fig. 2. Transmission electron micrographs of bovine fetal lung at 240 and 260 days of gestational development showing typical appearance of type II pneumocytes with lamellar bodies. A. 240 day (PIPES buffer) X7020; B. 260 day (PIPES buffer) X3150; C. 260 day (PIPES buffer) - higher magnification showing details of lamellar bodies (b) X7020; D. 260 day (Cacodylate buffer); X7020 Note mitochondria (m) and presence of tight junction (arrows) between a type I and type II pneumocyte. X7020.

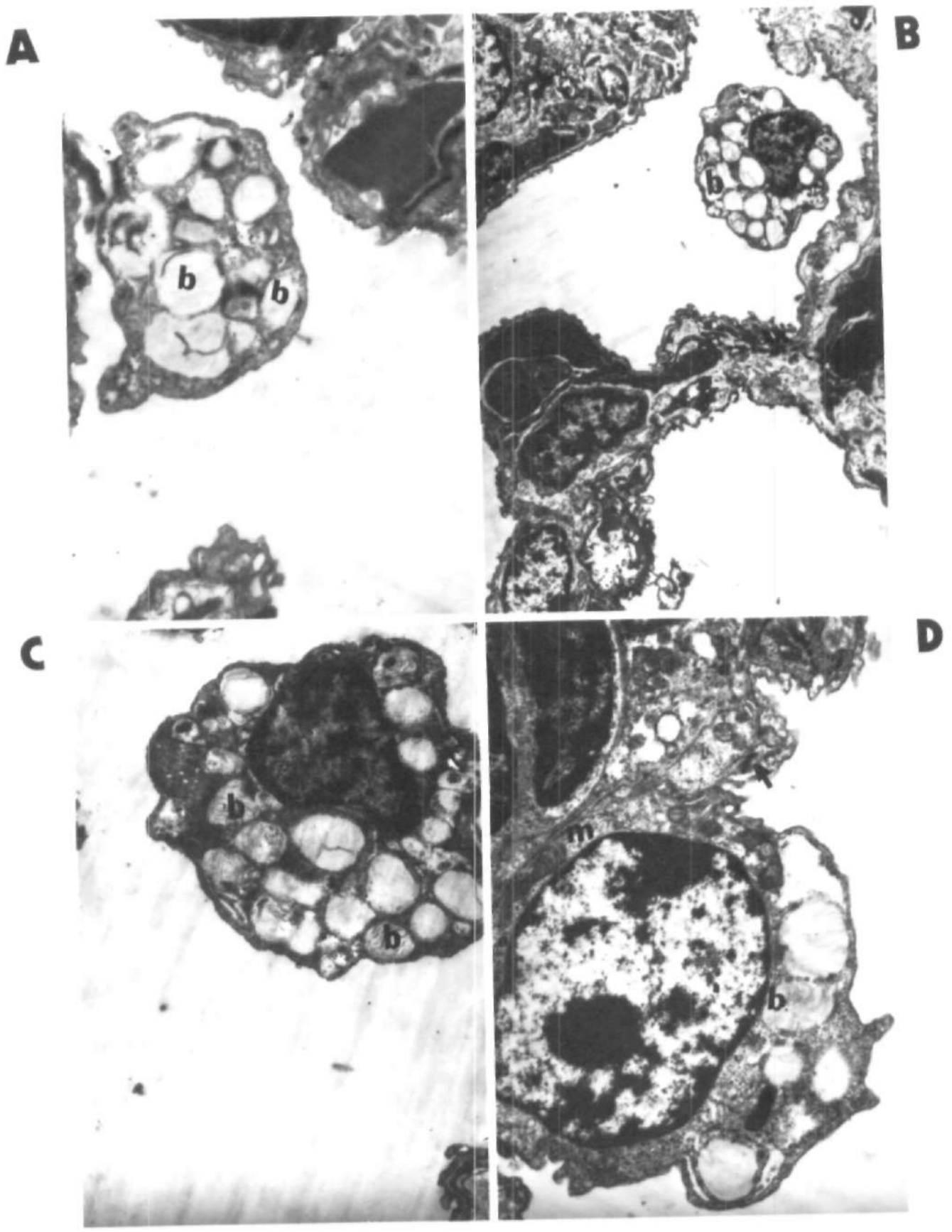

A
b
b
B
b
C
b
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m
b

Fig. 3. Transmission electron micrographs of bovine fetal lung at 270 days showing type II pneumocyte with lamellar bodies (b). A. 270 days (cacodylate buffer) X7020; B 270 days (cacodylate buffer) X4760. C. 270 days (cacodylate buffer) X19,000; Note capillary (c), tight junction complexes between type I and type II pneumocytes (arrows), nucleus of type I pneumocyte (N), erythrocyte in capillary (E) and collagen fibers (t) in alveolar septa. D. Transmission electron micrograph of lung tissue at 240 days of gestation prefixed in glutaraldehyde with tricomplex flocculation and postfixed in osmium tetroxide. Note the electron opaque globules (surfactant) forming in epithelial lining layer (arrows). Endothelium (EN), erythrocyte (E) and alveolar space (AS). Unstained section X4760.

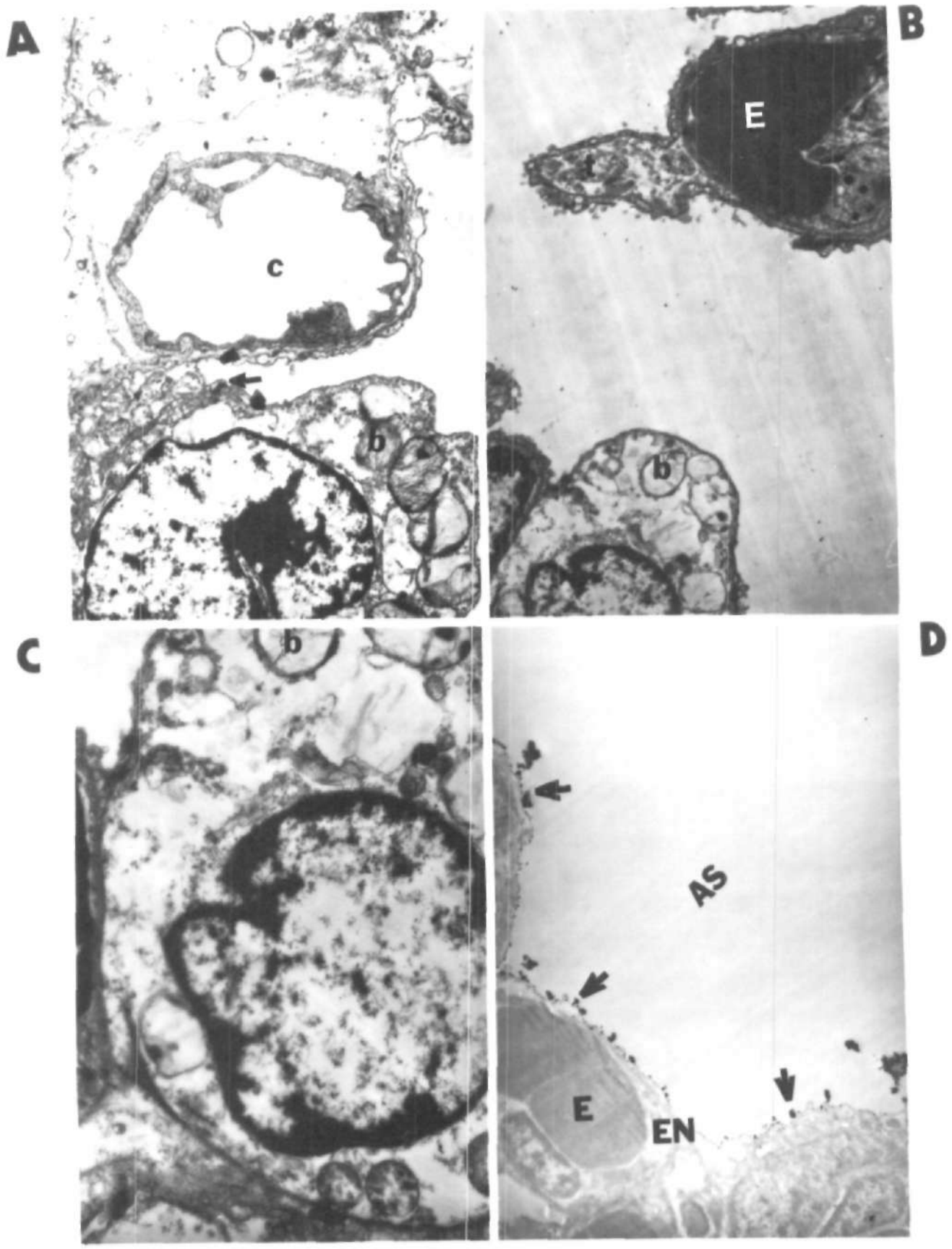
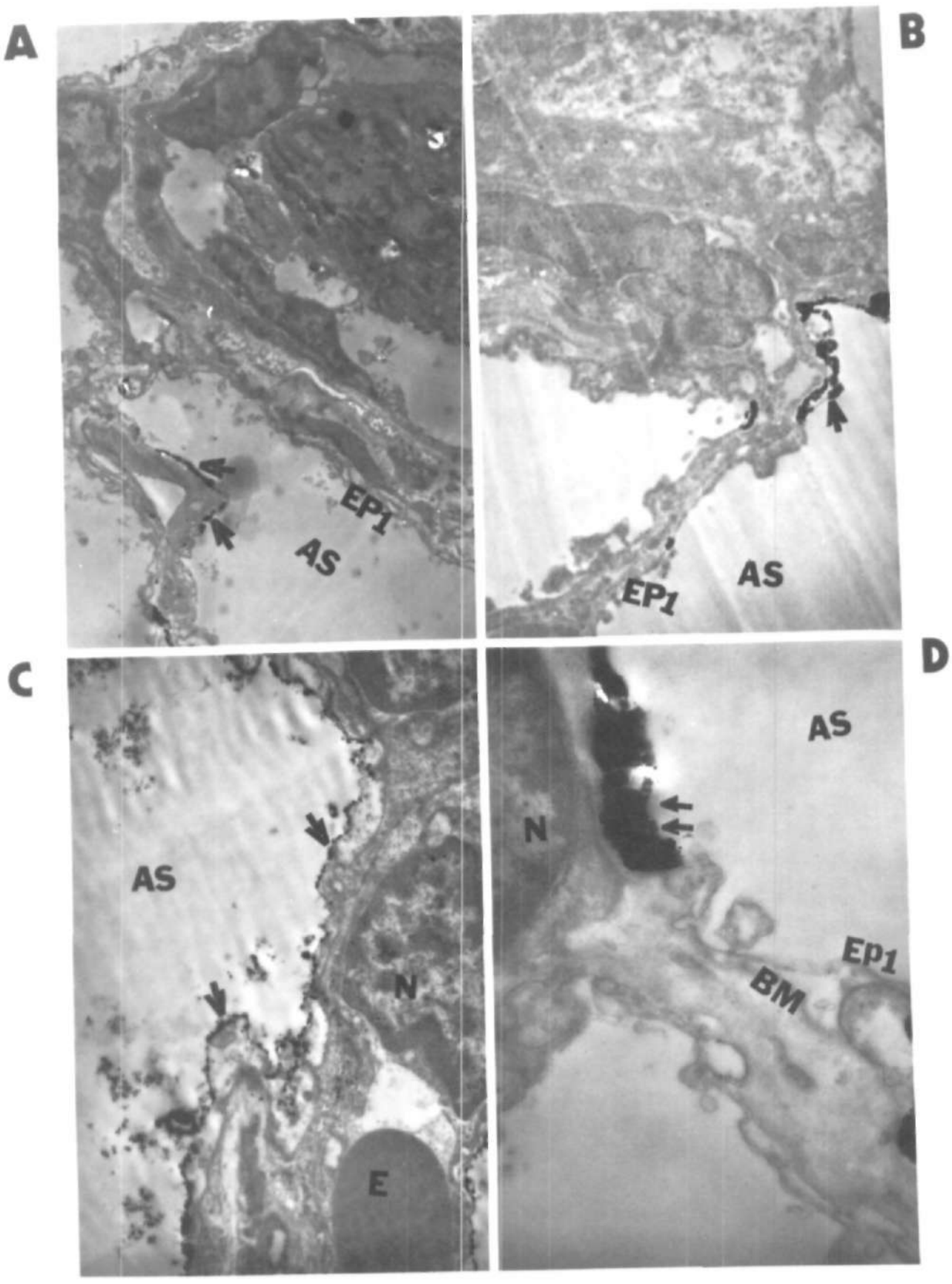

A
c
b
B
E
b
C
b
D
AS
E
EN

Fig. 4 *Transmission electron micrographs of unstained lung tissues:* A. 260 days B. 270 days of gestation. Tissues were prefixed in glutaraldehyde and treated with tricomplex flocculation. Note the electron opaque globules (surfactant) in the epithelial layer. EPI = type I pneumocyte; AS = alveolar space. A. X4760. B X 13940. C and D. Transmission electron micrographs of lung tissues at 270 days of gestation. Tissues were prefixed in glutaraldehyde, treated with tricomplex flocculation and postfixed in OsO_4. Note: electron opaque globules (surfactant) forming in the epithelial layer (arrows). E = erythrocyte in capillary; N = nucleus of endothelial cell; AS= alveolar space; EPI = type I pneumocyte; BM = Basal lamina. C X7020. D X19000.


A
EP1
AS
B
EP1
AS
C
AS
N
E
D
AS
N
Ep1
BM

Part 3. HISTOLOGICAL AND ULTRASTRUCTURAL OBSERVATIONS OF BOVINE FETAL BRONCHIOLAR CELLS

ABSTRACT

Bovine lung tissues from 40 fetuses ranging from 150 to 275 days of gestation were collected from abattoirs to study the development of bronchiolar cells. Two cell types were identified, the ciliated and the primitive Clara cells. Neurosecretory and the intermediary cells described in other species of animals were not observed in this study. Clara cells were seen earlier during canalicular period then ciliated cells which might suggest that ciliated cells were derived from Clara cells.

INTRODUCTION

Using the electron microscope, four cell types have been indentified in the lining epithelium of the bronchioles in the adult rat: (i) "Clara" non-ciliated; (ii) brush; (iii) Kulchitsky and; (iv) ciliated cells.[1] The Clara cell has only been found in the terminal bronchioles.[2]

Dechelotte et al., 1983[3], studying bronchiolar cells of human fetus from fifteen to twenty six weeks, found ciliated, Clara, neurosecretory and intermediary cells. Clara cells also were identified in the 16-week human fetus by Jeffery and Reid, 1977.[4]

Smith et al., 1979[5], studied the terminal broncioles of calf 4 months old and another 1 year old and found nonciliated Clara cells deeply embedded among cilia of neighboring cells. Intracytoplasmic clusters of glycogen granuels were a prominent feature in the Clara cells[5]. Nuclei were deeply notched and mitochondria were small and elongated, measuring 0.2 µm in diameter[5]. Sparse secretory granules were seen[5].

Information on the developing Clara or nonciliated bronchiolar cell of bovine fetus is lacking. Study on other cell types of the fetal bronchiole has not been reported.

This study reports the histological and ultrastructural observations of

bovine fetal bronchiolar cells from 150 to 275 days of gestation.

MATERIALS AND METHODS

Tissue Collection

Bovine lung tissues from 40 fetuses ranging from 150 to 275 days of gestation obtained from nearby abattoirs (Jack Polen Beef Company, Kansas City, Kansas and Wellington Quality Meat, Wellington, Kansas) were used in this study. Fetal ages were estimated using criteria published in Roberts, 1971[6]. All lungs were removed from fetuses after standard abattoir protocol and were fixed in 10% buffered neutral formalin (BNF) or in 2.5% glutaraldehyde. Of the total number of fetal lungs collected, 5 each were taken from fetuses of 180, 230, 240, and 260 days of gestation; 6 each from fetuses of 150, 250 and 270 days of gestation, and 2 from 275 day old fetuses.

Light Microscopy

Fetal lungs were fixed either in 10% buffered neutral formalin (BNF) or in 2.5% glutaraldehyde in 0.05M sodium cacodylate, pH 7.3, 350 mOsm and dehydrated in graded concentrations of ethanol. Tissue samples were embedded in paraffin, sectioned at 6 micrometers, and stained with Harris hematoxylin and eosin. Photomicrographs were obtained with an Olympus Photomicrographic system camera model PM-10-M, using Kodak Panatomic X film and black and white prints were obtained.

Scanning Electron Microscopy

Fetal lungs were fixed in cold 2.5% glutaraldehyde in 0.05M sodium cacodylate, pH 7.3, containing 5mM KCl and 2.5 mM $MgCl_2$, rinsed three times in cool 0.1M sodium cacodylate buffer pH 7.3. Rinsed in distilled water two times and dehydrated in graded ethanol concentrations for about 10 minutes in each concentration, critical point dried with DCP-1 critical point Dryer, mounted on aluminium stub and gold palladium coated with Edwards sputter coater model S150A. Photomicrographs were taken with ETEC-Auto-Scan at accelerating voltage

of 10 KV using polaroid type 55 Kodak film.

Transmission Electron Microscopy

Lung samples of $1mm^3$ were fixed in cold 2.5% glutaraldehyde in 0.05M sodium cacodylate pH 7.3 containing 5mM KCl and 2.5mM $MgCl_2$, rinsed three times in cold 0.1M sodium cacodylate buffer, pH 7.3, postfixed in 1% cacodylate buffered osmium tetroxide for 2 hours, rinsed in distilled water, and stained en bloc with 1% aqueous uranyl acetate for 2 hours before dehydration through a graded series of ethanol and acetone. Fixation and prestain procedures were in ice buckets.

All tissues were embedded in Epon/Araldite. The blocks were trimmed and sectioned with glass knives in a Reichert OM-U2 ultramicrotome. The thin sections (60-150 nanometers) were stained for 10-15 minutes with 5% uranyl acetate-50% ethanol and with Reynolds lead citrate for 5-7 minutes.

All sections were examined in a Philips 201 transmission electron microscope at 60 KV. Pictures were taken on Cronar Ortho S Litho film. Thick sections were cut and stained with toludine blue, photomicrographs were taken with an Olympus photomicrographic system camera model PM-10-M, using Kodak Panatomic X film and black and white prints obtained.

RESULTS

Light Microscopy

From 150 days of gestation (Canalicular period) the broncioles had folded mucosa and the mucosal cell nulcei were located at the apical border (Figs. 1A-D, and 2A-C). The folded mucosa was surrounded by a layer of smooth muscle cells.

Scanning Electron Microscopy

Scanning electron micrograph at 150 days of gestation (Canalicular period) showed bulbous aspices of primitive Clara or nonciliated cells. At 150 days, ciliated cells were not seen (Fig. 2D). From 240 to 275 days of gestation,

(Alveolar period), scanning electron micrographs showed aspices of primitive Clara cells which were higher than the ciliated cells (Fig. 3A-D).

Transmission Electron Microscopy

During the canalicular period (150 days) of lung development, the cells of the bronchiolar epithelium were ultrastructurally similar and cuboidal in shape (Fig. 4A-D). Cell types could not be identified at this period. Cell organelles were sparse, a few mitochondria with poorly developed cristae, tight junctions and desmosomes were seen. From 180 days of gestation (late canalicular period), bronchiolar epithelium contained both ciliated and nonciliated or primitive Clara cells (Figs. 5A-D, 6A-C and 7A-C). Primitive Clara cells were identified before the ciliated cells. Primitive Clara cells contained abundance of glycogen and the nuclei were deeply notched (Figs. 5A-D, 6A-C and 7A-C). The ciliated cells had numerous mitochondria with developed cristae in the cytoplasm (Fig. 7A,C).

DISCUSSION

During late pseudoglandular and part of the canalicular stages of lung development the bronchiolar cells are similar and cuboidal in shape. During late canalicular period and subsequent periods two types of cells are recognized. One cell type is ciliated and the other is the nonciliated or primitive Clara cell. The primitive Clara cells have dome-shaped protrusions projecting into the bronchiolar lumen high above the luminal surface of the ciliated cells. Primitive Clara cells were first identified before the appearance of ciliated cells.

Jeffery and Reid[7] studied the bronchilar cells of 16-week human fetus and found Clara cells to have abundance of intracellular glycogen with occasional profiles of rough endoplasmic reticulum and polysomes. At 23 weeks, Jeffery and Reid[7] found that some smooth endoplasmic reticulum had ribosomes associated with it. In the fetal rabbit, Clara cells lack secretory granules but have

large amounts of glycogen and few organelles.[4] In the rat, the primitive Clara cell has supranuclear glycogen and some rough endoplasmic reticulum by 16 to 17 days gestation.[1] By 21 days gestation, small electron-dense granules are seen at the Clara cell apex but the cell is immature and lacks the abundance of smooth endoplasmic reticulum seen in the mature Clara cell.[1] In bovine fetal lung, Clara cells lack secretory granules but have large amounts of glycogen and few organelles. The nuclei of the Clara cells are deeply notched which is characteristic of bovine Clara cell. This result is similar to the observation of Clara cells in the rabbit.[4] It appears that the structure of Clara cells during fetal period varies from species to species.

The function of Clara cells is not known, but morphological and cytochemical features are compatible with a secretory function.[8] Some experimental studies with tracer particles show the Clara cells in neonatal rabbit to be capable of phagocytizing foreign particles.[9] Clara cells have also been proposed as the cells of origin for bronchioloalveolar carcinoma of the lungs.[10] Evans and co-workers[11] concluded that Clara cells were progenitor cells for the bronchiolar epithelium. In the bovine, the nonciliated or primitive Clara cells were seen to have abundant glycogen and this may remain into neonatal life when Clara cells differentiate. In contrast, the ciliated cell loses its glycogen during fetal life and matures earlier than the Clara cell. It seems, therefore, that adult Clara cells develop from an early glycogen laden nonciliated cells by gradual diminution of their glycogen. Later small electron-dense granules appear, and finally a smooth endoplasmic

reticulum, which, in the adult, fills the cell apex. The fact that primitive Clara cells were seen before the ciliated cells may suggest that the ciliated and future bronchiolar cells are derived from the Clara cell. Mature Clara cells have been reported in calf.[12]

Neurosecretory cells observed in human and rat, and intermediary cells in rat bronchioles were not seen in the bovine.

In conclusion, two cell types, ciliated and primitive Clara cells were seen during fetal periods in the bovine. The nonciliated cells were present before the ciliated cells were detectable which may suggest that ciliated cells develop from the primitive Clara cells. Primitive Clara cells have abundant glycogen but small electron-dense granules were not observed.

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