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Morphological effects of chronic tracheal ligation and drainage in the fetal lamb lung

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INTRODUCTION

The potential air spaces of the fetal lung are well expanded with liquid (Addison & How, 1913). If the flow of this liquid through the fetal trachea is wholly or partially impeded, overdistension of the lung results, indicating that fetal lung liquid is actually formed within the lungs. This is observed in infants with congenital atresia of the bronchi (Potter & Bohlender, 1941; Griscom *et al.* 1969) and in animals following experimental tracheal ligation (Jost & Policard, 1948; Carmel, Friedman & Adams, 1965; Lanman *et al.* 1971). However, no detailed ultrastructural descriptions of such overdistended lungs have been presented.

Several other physical and biochemical alterations in the intra-uterine environment of the developing fetus have been shown to alter the morphological development of the lung (see Knelson & Hill, 1971); in particular the administration of glucocorticoids (Kikkawa *et al.* 1971; Platzker, Kitterman, Clements & Tooley, 1972) and alterations to the pituitary–adrenal endocrine axis of the fetus as a result of experimental decapitation *in utero* (Blackburn, Travers & Potter, 1972; Chiswick Ahmed, Jack & Milner, 1973; Meyrick *et al.* 1975). In these last-named experiments decapitation was achieved by ligating the fetal neck. This, however, may well occlude or sever the trachea, causing distension or drainage of the lung.

It was to clarify the effects at a cellular level of tracheal ligation and drainage that we examined the lungs of fetal lambs 21–28 days following either ligation or continuous drainage *in utero*. We have used high resolution light microscopy of plastic sections, transmission electron microscopy, scanning electron microscopy and morphometry to determine the effects of these basic manipulations on pulmonary alveolar development.

MATERIALS AND METHODS

Experimental preparation

The chronic *in utero* fetal sheep preparation, previously described in detail (Adamson *et al.* 1975; Maloney *et al.* 1975), was somewhat modified in this series of experiments. Six ewes pregnant for 105–110 days were anaesthetized with halothane, and, through uterine wall incisions, the fetal lambs were partially delivered.

The tracheas of three lambs were ligated approximately 2 cm distal to the larynx. In a further three lambs the tracheas were exteriorized by inserting 4 cm of a cannula (50 cm total length) into the trachea distal to the larynx and pointing towards the lung. The cannulae were brought out through the sutured uterine wall and the abdomen of the ewe and connected to a collecting bag placed on the ewe's flanks. Fetal lung liquid was collected and measured daily.

In all preparations electrocardiogram electrodes were placed subcutaneously over the left shoulder of the fetus, with the reference electrode on the skin surface. All six lambs were returned to the uterus, the uterine and abdominal incisions were sutured and the animals allowed to recover. Twenty one to twenty eight days later, at a second Caesarian section, the lambs were again partially delivered. Cannulae were inserted into the tracheas which had been ligated. Tracheal pressures were monitored prior to lung liquid removal using a Statham transducer (P 23BB or P 23D6) connected to a Gilson M 8PM polygraph. All lungs were fixed *in utero* by removing fetal lung liquid and replacing it with an equal volume of fixative.

For comparison, five further animals were used as normal controls, one each at gestational ages 108 and 110 days and three at gestational ages 133–134 days. After fixation all lungs were handled in identical fashion. The weight of each lung was determined and lung volume was measured by displacement, care being taken to submerge the tracheal stub (approx. 10 cm in length) in each case. Thin slices were removed from five pulmonary lobes in each animal and processed for electron microscopy. Measurements of fetal weight and crown–rump length were taken post-mortem in all cases.

Histological techniques

Transmission electron microscopy. The fixative contained 2·5 % glutaraldehyde and 2 % paraformaldehyde in 0·1 M sodium cacodylate buffer at pH 7·4. The lung slices were chopped into blocks, washed in 0·1 M sodium cacodylate buffer and post-fixed in 1·5 % osmium tetroxide for 1 hour. Following a second buffer wash the blocks were stained in 5 % magnesium uranyl acetate for 1 hour. After dehydration in graded acetone solutions the blocks were embedded overnight in Spurr's resin and cured. Silver sections were cut on a Huxley ultramicrotome, stained with lead citrate, and viewed in a Siemen's Elmiskop 1 at 80 kV.

Thick plastic sections (0·5–1·0 μm) were cut on the Huxley ultramicrotome and stained with 1 % toluidine blue at 95 °C.

Scanning electron microscopy. After 24 hours in fixative 2–4 mm slices of lung tissues were washed in 0·1 M sodium cacodylate buffer for $\frac{1}{2}$ hour and post-fixed in 1 % osmium tetroxide for 1 hour. Following dehydration in graded solutions of ethane diol, and finally cellosolve, the slices were dried in the critical point dryer using liquid carbon dioxide as infiltrating medium. The slices were mounted on stubs, shadow coated with carbon and gold and viewed in a Siemen's Autoscan at 20 kV.

Morphometry. Toluidine blue stained, 1 μm plastic sections were viewed $\times 400$ through a light microscope with a 10×10 line graticule in one eyepiece. One hundred points of the graticule were used in the point analysis of the tissue section, making it possible to determine the percentage volume occupied by tissue and future air spaces, according to the methods of Weibel (1963). Areas for analysis were randomly

Table 1. *Gross parameters of fetal lamb preparations*

Preparation ...	Normal Early gestation			Normal Late gestation			Tracheal ligation			Tracheal drainage		
Gestational age (days)	108		110	134	133	133	131	132	131	130	132	130
Average	109			133			131			131		
Total body weight (kg)	1·6		1·4	3·0	4·2	3·7	3·0	4·2	4·3	3·4	3·2	2·5
Average	1·5			3·6			3·8			3·0		
Total lung weight (g)	97		110	183	187	161	385	698	560	62	90	59
Average	104			177			548			70		
Lung liquid volume (ml)	38		24	70	30	35	187	331	307	*5	*7	*6
Average	31			45			275			6		
Lung 'tissue weight' (g)	59		86	113	157	128	198	367	253	57	83	53
Average	73			132			273			64		
Lung 'tissue weight'/ total body weight (g/kg)	36·9		62·3	37·6	37·4	34·1	66·0	87·4	58·8	16·8	25·9	21·2
Average	48·6			36·4			70·7			21·3		

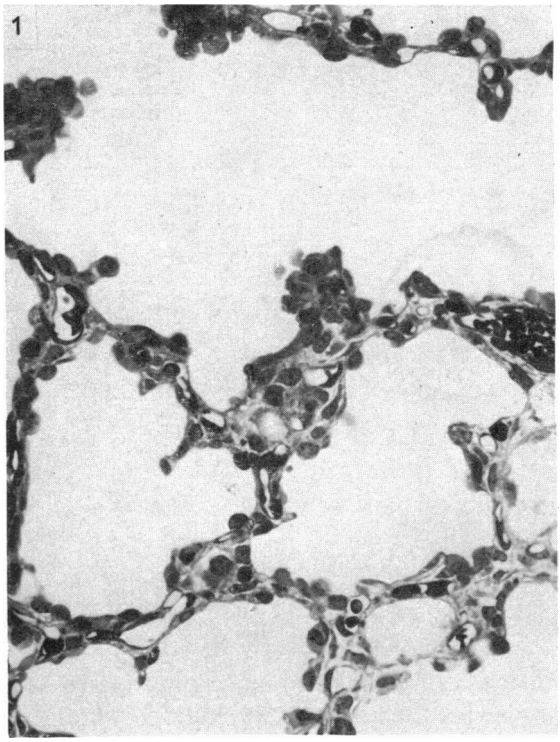
* The cumulative volumes drained from these lungs were respectively: 4·1 litres over 21 days; 8·2 litres over 24 days; 4·3 litres over 25 days.

displayed at low power, centred without focusing, and turned into high power ready for analysis. Pre-selection of areas was avoided. Approximately 25 sections were used from each preparation, giving 49 sections of early gestation normal lungs, 72 sections of late gestation normal lungs, 67 sections of tracheally ligated lungs and 74 sections of tracheally drained lungs. Sections in which staining was not adequate, or in which very large airway or vascular structures predominated, were not counted. This number of sections minimized random counting errors. Statistical differences from the late gestation normal were confirmed using an unpaired *t* test.

RESULTS

Physical parameters

Fetal heart rates monitored during the 21–28 days of continuous ligation or drainage were normal. The average flows of lung liquid from the three tracheal drainage lambs were 8·3 ml/hour (4·1 litres over 21 days); 14·3 ml/hour (8·2 litres over 24 days) and 7·3 ml/hour (4·3 litres over 25 days). At the time of fixation tracheal pressures were +7·5 and +7·5 cm H_2O for the two early gestation normals; +2·0, −0·7 and −10·0 cm H_2O for the three late gestation normals; +9·3, +8·2 and +8·2 cm H_2O for the three tracheal ligated animals and −1·1, 0 and −8·8 cm H_2O for the three tracheal drainage animals. The body weights and crown–rump lengths of the fetuses used in this series of experiments were within the normal range of those used in earlier series of experiments in this laboratory (Adamson *et al.* 1975) so that neither continuous ligation nor drainage had apparently affected general fetal growth. The body weights and crown–rump lengths of the early and late gestation fetuses were also within the expected range.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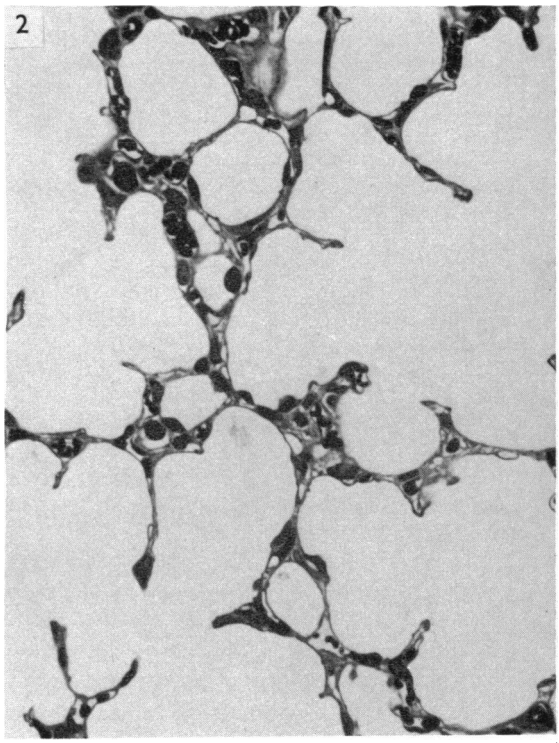
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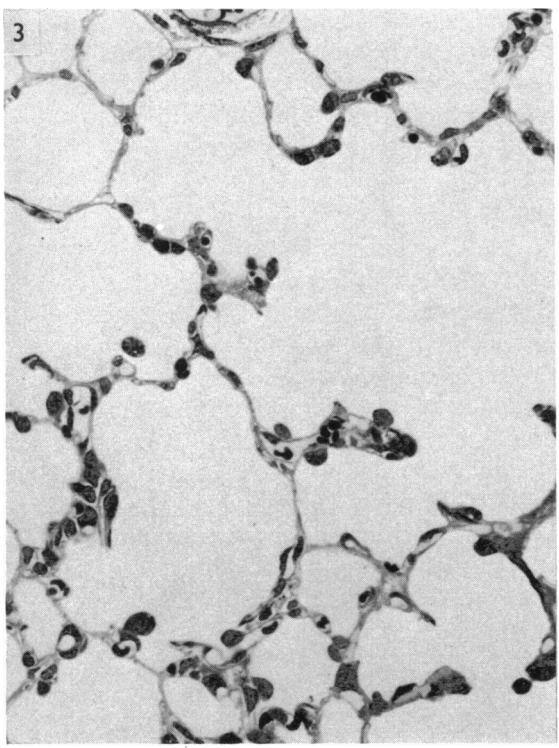
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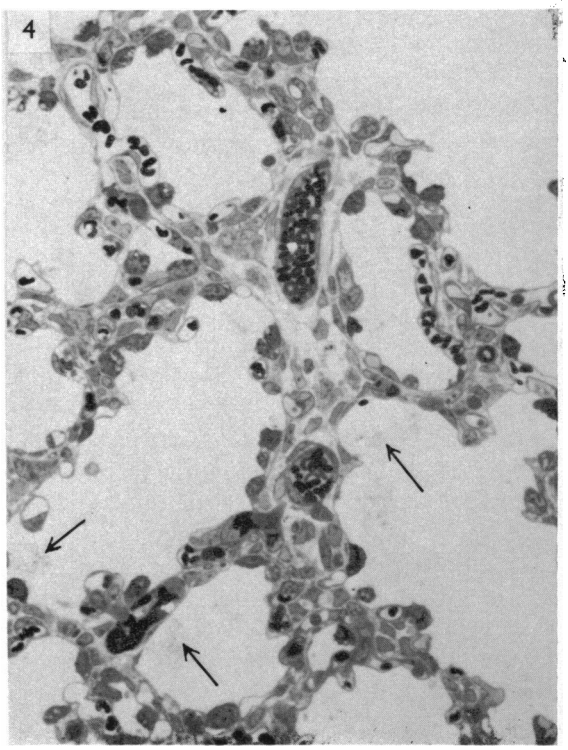
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Table 2. *Morphometry results*

Preparation	Normal Early gestation	Normal Late gestation	Tracheal ligation	Tracheal drainage
Gestation (days)	109	133	131	131
No. of sections counted	49	72	67	74
Percentage total air space	59·2	68·4	72·8	50·3
Mean ± S.E.	±0·77	±0·78	±0·75	±1·3
t test (significance levels)	$P < 0{\cdot}001$	—	$P < 0{\cdot}001$	$P < 0{\cdot}001$

Lung size

The gross measurements of the lungs, both of those used for comparison and of those following tracheal ligation and drainage, are given in Table 1, as well as the gestational ages at the time the lungs were prepared. The most dramatic effect of ligation or drainage was on lung size. Ligated lungs were larger than normal lungs of a similar gestational age, whereas drained lungs were smaller. Because the fetal lung consists of both fluid and tissue, we decided to obtain estimates of tissue weight to determine whether the changes in lung size could be attributed solely to changes in fluid retention or whether changes in lung tissue weight were also involved.

Two methods of estimating tissue weight were used. In the first, the weight of the volume of lung liquid removed at the time of fixation was subtracted from the fetal lung weight and the remaining 'tissue weight' was then expressed as a ratio of fetal body weight (Table 1). This is not a precise indication of actual lung tissue weight (see Discussion), nevertheless it does provide a means of comparing the effects of ligation and drainage. An alternative estimate of 'tissue weight' is given below (see Morphometry).

The 'tissue weight' of ligated lungs was, on the average, increased by approximately 95% compared with the normal at the same gestational age. In contrast the 'tissue weight' of drained lungs was, on the average, decreased by approximately 40% as compared with the normal lung at the same gestational age. In fact, when the 'tissue weight' of drained lungs is compared with the 'tissue weight' of normal lung at the start of the experimental period, it can be seen that there had been no effective tissue growth over the 21–28 days' drainage period.

Fig. 1. Normal fetal lamb lung at 110 days of gestation. Large liquid filled channels are lined with a flattening epithelium; many cuboidal cells are present. Thick walls are composed of interstitial tissue and many capillaries. Toluidine blue stained, plastic section. × 400.

Fig. 2. Normal fetal lamb lung at 133 days of gestation. In comparison with Fig. 1 future air spaces are lined by a thinner epithelium and wall projections subdivide the earlier larger channels. Walls have thinned as a result of a decrease in the amount of interstitial tissue and a singling out of capillaries. Toluidine blue stained, plastic section. × 400.

Fig. 3. Tracheally ligated lungs at 132 days of gestation. Future alveolar walls are thinner than in Fig. 2. They are lined by a thin squamous epithelium sandwiching capillaries and very little interstitial tissue. Toluidine blue stained, plastic section. × 400.

Fig. 4. Tracheally drained lungs at 132 days of gestation. Future alveolar walls are thick. A pulmonary epithelium consisting of cuboidal and squamous cells sandwiches increased amounts of interstitial tissue as well as many capillaries. Much lamellar material (arrows) is found in the future air spaces. Toluidine blue stained, plastic section. × 400.

Morphometry

Table 2 indicates the percentages of the lung that is occupied by future air spaces in the early and late normals, the ligated and the drained lungs.

Early gestation normal lungs had a greater percentage of tissue, and therefore a lesser percentage of future air space, than late gestational normal lungs. Percentage potential air space incorporation is marginally, but significantly, enhanced in the ligated lungs, and retarded in the drained lungs. In fact the drained lungs had less percentage potential air space than did early gestation normal lungs.

The second method for estimation of lung weight employed the results of Table 2. Given the mean volume percentage of future air space (lung liquid volume), the approximate weight of tissue can be estimated using the mean values of lung weight for each group. These 'tissue weights' (using the average lung weight values from Table 1) are calculated as 42 g for early gestation normals, 56 g for late gestation normals, 149 g for ligated lungs and 35 g for drained lungs. Using these values, ligated lungs increased in tissue weight by 166% and drained lungs decreased in tissue weight by 38%, when compared with late gestation normals.

Morphology

(*a*) *Normal lungs.* At the early stage in gestation (Fig. 1) (110 days) the lung was composed of large future air spaces partitioned by thick walls. Many of the cells lining the walls were reminiscent of the early cuboidal epithelial cells of the primitive mammalian lung. They contained abundant cytoplasmic glycogen, few organized intracellular organelles and appeared in scanning electron micrographs (Fig. 5) as cobblestone bumps lining the larger airways, frequently protruding from the walls of the canal. Some lining cells had begun to flatten (Fig. 9), sending out thinning cytoplasmic extensions over the surface of the future alveolar region. Alveolar type II epithelial cells were not apparent at this stage.

By 133 days of gestational age (Figs. 2 and 6) the development of future alveolus-like structures was progressing. This development was characterized by a thinner walled appearance of the lung parenchyma, with projections subdividing the earlier

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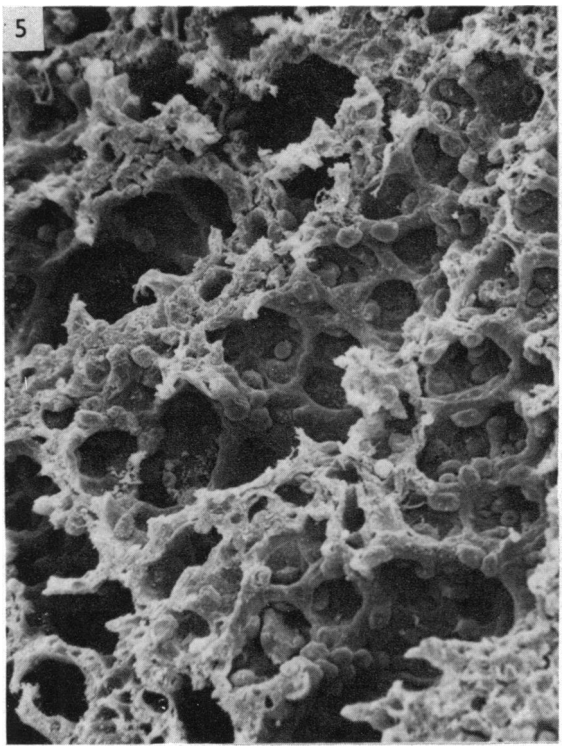
Fig. 5. Scanning electron micrograph of normal fetal lamb lung at 110 days of gestation. Many cuboidal cells line the larger channels. Ridges subdivide these larger structures, suggesting future alveolar formation. Approx. × 600.

Fig. 6. Scanning electron micrograph of normal fetal lamb lung at 133 days of gestation. Future alveolar walls have thinned and are lined by a squamous and cuboidal epithelium, many of the bumps being nuclei of epithelial type I cells. Red blood cells have fallen into some of these spaces during preparation. Approx. × 600.

Fig. 7. Scanning electron micrograph of tracheally ligated fetal lamb lung at 132 days of gestation. Future alveoli are stretched out and have the saucer-shaped appearance of inflated adult lung. The lining epithelium consists largely of squamous cells. Cuboidal cells are also evident. Approx. × 600.

Fig. 8. Scanning electron micrograph of fetal lamb lung at 132 days of gestation following continuous *in utero* tracheal drainage of lung liquid. Walls are lined by a cuboidal and squamous epithelium and, due to the collapsed nature of the lung, pulmonary capillaries are often outlined in the walls of the future alveoli (arrows). Approx. × 600.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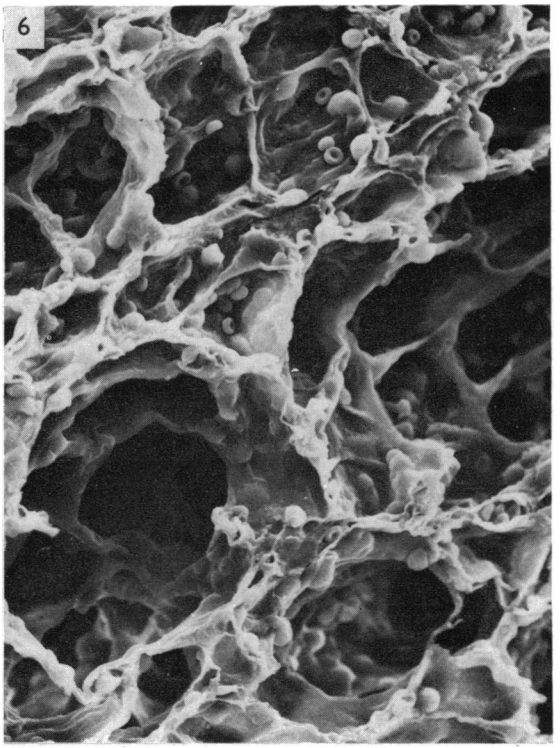
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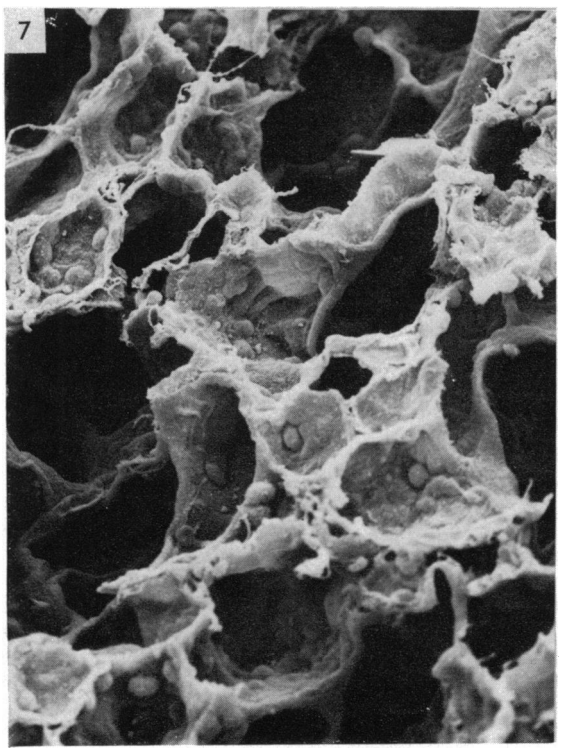
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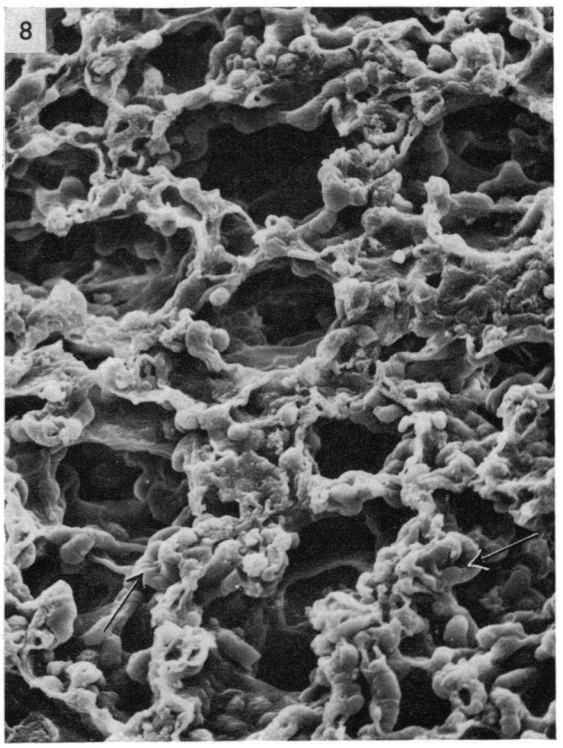
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larger spaces. Wall thickness had decreased, accomplished by a thinning of the interstitial tissue, a singling out of pulmonary capillaries within these walls, and a thinning of the newly developed pulmonary epithelium giving the alveolar epithelial type I cell. Such cells had little cytoplasmic glycogen, a few organelles positioned around the nucleus, and well formed cytoplasmic extensions (arrow, Fig. 10). The alveolar epithelial type II cell (Fig. 10) was frequently observed and possessed the normal characteristics of lamellar inclusions, well developed Golgi apparatus, many elongated mitochondria, and enhanced endoplasmic reticulum. The surface of the cell gave rise to conspicuous microvilli.

(*b*) *Tracheally ligated lungs.* In lungs from tracheally ligated animals (Fig. 3) thinner future alveolar walls had developed than in normal lungs at the same age. In scanning electron micrographs (Fig. 7) the ligated lungs had the smooth walled saucer shaped alveolus-like structures characteristic of inflated adult mammalian lungs. Alveolar epithelial type II cells (Fig. 11) were infrequently observed, and little lamellar material was seen in the future alveolus. The future alveolar capillary membrane had thinned.

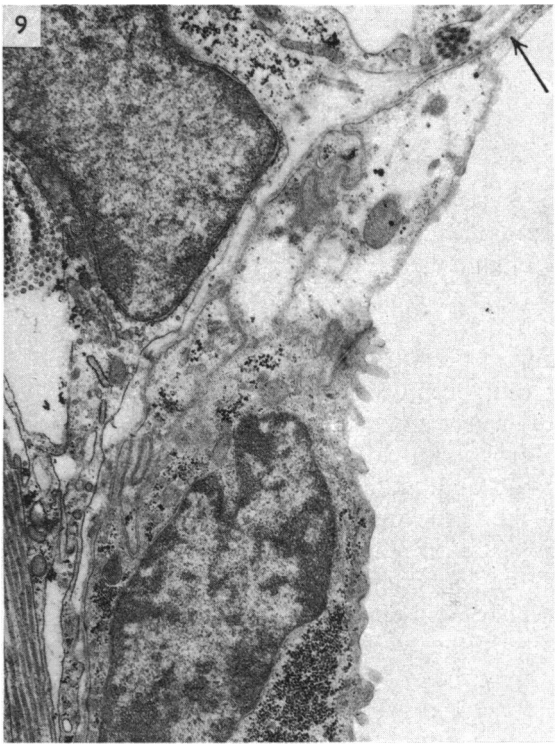
(*c*) *Tracheally drained lungs.* In lungs from tracheally drained animals (Fig. 4) the walls between the primitive air spaces were still thick and had altered little from those of early gestation normals (Fig. 1). Projections subdividing primitive air spaces were not apparent. Alveolar epithelial type I cells were not as prevalent as in normal lungs at the same gestational age. Many of the cuboidal cells lining the large spaces were reminiscent of the undifferentiated primitive pulmonary lining cells, whilst others were well differentiated alveolar epithelial type II cells (Fig. 12). These latter cells were frequently observed: they contained many lamellar inclusions and were associated with excesses of lamellar material within the future alveolar space (Figs. 4

Fig. 9. Normal fetal lamb lung at 110 days of gestation. The future alveolar walls are lined by glycogen-filled cuboidal cells as well as by cells with tapering processes (arrow). Underneath this epithelium interstitial cells and fibrous material constitute walls of the channels. Stain: lead citrate, magnesium uranyl acetate. Approx. × 12000.

Fig. 10. At 133 days of gestation the future alveolar portion of the normal fetal lamb lung consists of two major cell types. The prominent alveolar type II cell contains lamellar inclusions, elongated mitochondria, conspicuous Golgi apparatus and much smooth endoplasmic reticulum. This particular cell has been apically sectioned and would join the epithelial lining where there are prominent junctional complexes. The surface of the type II cell is thrown into well developed microvilli. Lamellar material is obvious within the future air space. The majority of the future alveolar surface is covered by the second epithelial type I squamous cell. This cell's tapering extensions (arrows) cover the underlying pulmonary capillaries and small amount of interstitial tissue. Stain: lead citrate, magnesium uranyl acetate. Approx. × 11250.

Fig. 11. Following tracheal ligation the future alveolar portion of the fetal lamb lung at 132 days of gestation shows a similar ultrastructure to the normal except that the alveolar type II cell is not as frequently observed. The future alveolar wall is mainly composed of thinned squamous epithelial type I cells (arrows) overlying pulmonary capillaries. Lamellar material is not as apparent within the future air space, and none is visible in this plate. Stain: lead citrate, magnesium uranyl acetate. Approx. × 10500.

Fig. 12. Following *in utero* tracheal drainage the future alveolar portion of the fetal lamb lung at 132 days of gestation is composed mainly of thick walls lined by well developed alveolar type II cells. Squamous epithelial type I cells were present, but are not obvious in this micrograph. The epithelium overlies thick walls composed of pulmonary capillaries and increased amounts of interstitial tissue. Excessive amounts of lamellar material are found within the future air space (see also Fig. 4). Stain: lead citrate, magnesium uranyl acetate. Approx. × 10500.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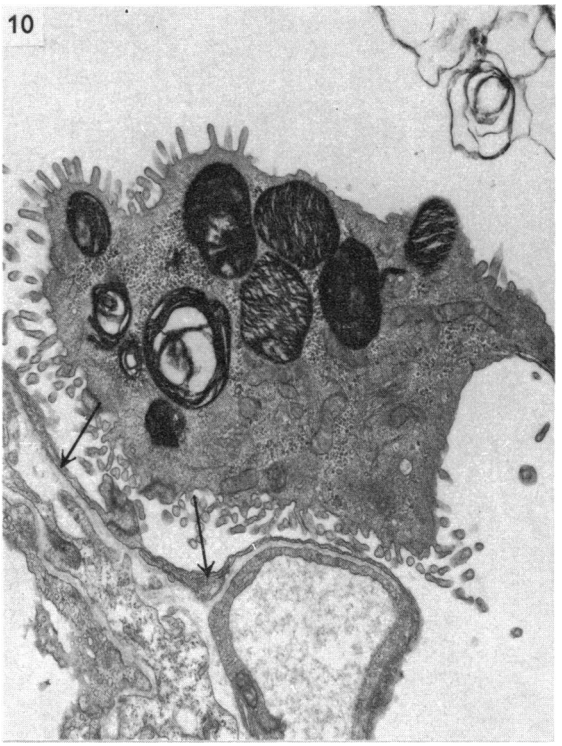
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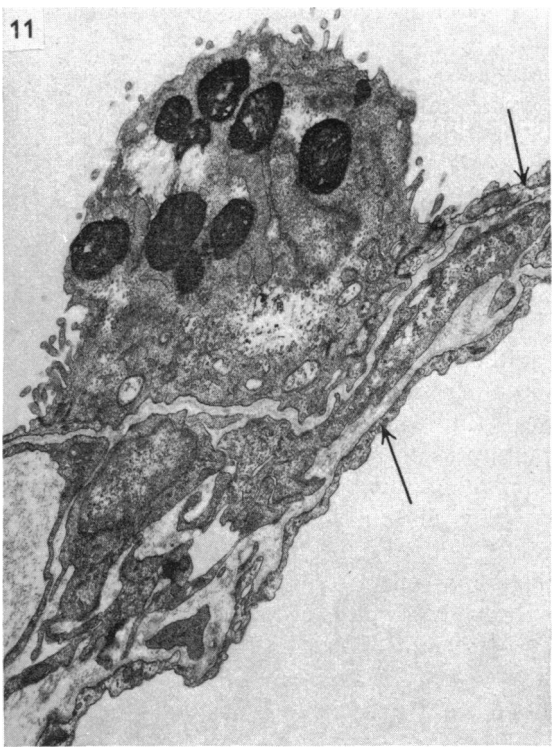
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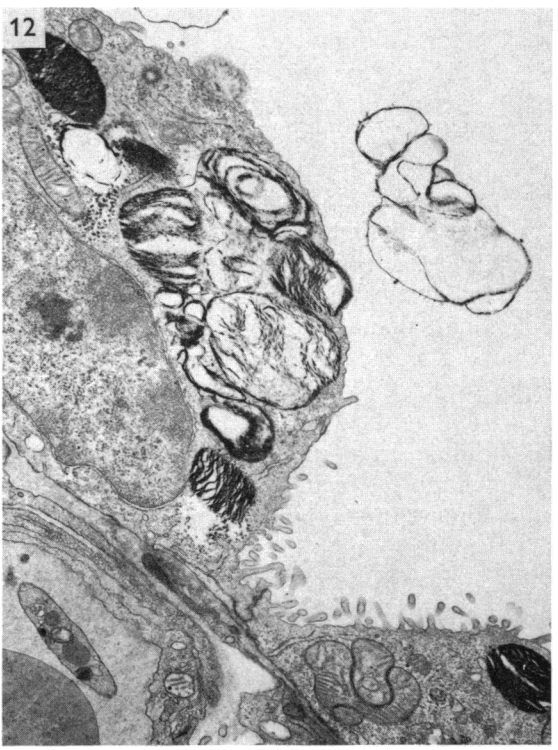
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and 12). In scanning electron micrographs (Fig. 8) the greater tissue density of this drained lung is obvious. Many pulmonary capillaries (arrow, Fig. 8) were outlined beneath the future air space epithelium.

DISCUSSION

This present study has confirmed the hypothesis that over a period of time gross alterations in lung liquid dynamics can affect fetal lung growth. In lungs in which the trachea was ligated, not only was the lung enlarged, as reported by earlier workers (Jost & Policard, 1948; Carmel *et al.* 1965; Lanman *et al.* 1971), but tissue growth and the thinning of the future alveolar wall had been accelerated as compared with normals of the same gestational age. Thinning of the future interalveolar walls, and better defined alveolar formation, have generally been accepted as postnatal events (see Thurlbeck, 1975). Furthermore, the greater percentage increase in tissue weight of ligated lungs, as shown by the second morphometrical technique, suggests that the future gas exchanging areas of the lung had undergone the most tissue growth. In lungs chronically drained of lung liquid the tissue weights were approximately half those of the normal lungs at the same gestational age (133 days) (mean 64 g compared with 132 g estimated by method 1 and 35 g compared with 56 g estimated by method 2). This suggests that tissue growth had been retarded during the drainage period. Furthermore 'tissue weights' of drained lungs were on the average actually less than 'tissue weights' of normal lungs at the time of operation (109 days) (mean 64 g compared with 73 g estimated by method 1 and 35 g compared with 42 g estimated by method 2). These results suggest that lung tissue growth had ceased during the drainage period and that lack of lung liquid alone would not account for the small lung size.

It is clear that neither method of estimating 'tissue weight' gives an accurate measure of the actual tissue weight of the whole lung. In the first method 'tissue weight' is over-estimated, as it is well established that only approximately 85% of the total lung liquid can be withdrawn from the fetal lung (Humphreys, Normand, Reynolds & Strang, 1967; Normand *et al.* 1971; Scarpelli, Condorelli & Cosmi, 1975). In our second estimation of 'tissue weight' the fields used for morphometry were selected from the future gas exchanging area of the lung, omitting larger airway and vascular structures. However, both provide valid comparisons, and each indicates that 'tissue weight' alters dramatically.

The unexpected enhancement of alveolar epithelial type II cells, and the increased presence of lamellar material within the potential air space of drained lungs, indicate that not only lung mass and the formation of future alveoli are affected by lung liquid drainage, but alveolar epithelial cell maturation is also influenced. This effect cannot be related to the general operational procedures as both ligated and drained preparations involved comparable fetal surgery, and in ligated lungs alveolar epithelial type II cells were less apparent than in normal lungs of the same age.

These findings highlight the importance of the controlled release of lung liquid for the normal maturation of the lung. Excessive lung liquid release through the trachea results in small lungs, deficient in alveoli but with well differentiated alveolar epithelial type II cells. In contrast, inhibition of lung liquid release results in large lungs.

with well defined future alveoli, but lacking the abundant numbers of alveolar epithelial type II cells. The relationship between the lamellar inclusions of these cells and the presence of pulmonary surfactant is well established (McDougall & Smith, 1975). The mechanism by which lung liquid release controls lung growth is not yet understood. Our findings suggest a pressure or volume regulation of lung growth, with the liquid acting as an internal template or splint. Because of the high compliance of the developing fetal lung, a volume difference of approximately 800% between ligated and drained lungs is accompanied by an average intratracheal pressure difference of only 12 cm H_2O.

The ability of glucocorticoids to produce changes in the developing lung has been widely reported. Kotas & Avery (1971) demonstrated that glucocorticoids inhibited lung growth in favour of differentiation, whilst Smith, Torday & Giroud (1974) demonstrated a biphasic action of cortisol. Using trypsin dispersed lung cells from rabbit fetuses they demonstrated that cortisol may decrease fetal pulmonary cellular growth in early gestation whilst enhancing maturation and slowing growth as term approaches.

Meyrick *et al.* (1975) and Blackburn *et al.* (1972) have looked at fetal lung growth in decapitated fetuses, using this as a model where adrenal function, and hence glucocorticoid secretion, is impaired. In the former study the fetal lungs were collapsed and in the latter study the lungs were distended. In neither case was the patency of the trachea known, and the changes found may well have been related to an upset in fetal lung liquid dynamics in addition to impaired glucocorticoid secretion.

SUMMARY

The relationship between lung liquid flow and fetal lung development has been studied at the cellular level using ultrastructural techniques. Continuous *in utero* tracheal ligation and drainage (over a period of 21–28 days) both result in malformations of the developing fetal lamb lung. Ligated lungs are larger, and drained lungs are smaller, than normal lungs at a similar gestational age. These changes are not merely due to altered lung liquid volume, but actual tissue growth has been affected. Future alveolar wall thinning is enhanced in ligated lungs and inhibited in drained lungs, whilst the presence of differentiated alveolar type II cells (probably related to surfactant production) is decreased in ligated lungs and markedly enhanced in drained lungs. These results indicate the importance of fetal lung liquid in the regulation of pulmonary development in the fetus.

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