

Time-dependent effects of maternal continuous propranolol on fetal lung development in rats

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Summary. Previous studies have demonstrated a role for the beta-adrenergic system in the maturation of the fetal alveolar epithelium. Chronic blockade of beta-adrenergic binding sites has been shown to adversely effect physiologic and biochemical indices of fetal lung maturation. In the present study timed-pregnant female Sprague-Dawley rats were treated with a continuous 0.5 mg/hr dose of propranolol HCl, or saline, via an osmotic pump. The treatment periods were days 18-21, or 20-23 of gestation. Fetal body weights were obtained, and the morphology of the fetal lungs studied by light and electron microscopy. Cytoplasmic volume densities of lamellar inclusion bodies and glycogen within developing type II alveolar epithelial cells were also determined. In addition, total phospholipids (as phosphorus) and glycogen content were determined biochemically. The fetuses from females treated from day 20-23 demonstrated no differences between saline-treated and propranolol-treated groups, in either fetal weight or the morphologic appearance of the developing lung. In contrast, the fetuses from mothers treated from day 18-21 with propranolol were significantly smaller, and their lungs appeared less mature than saline-treated counterparts. The glycogen content of developing type II alveolar epithelial cells was significantly more abundant (as judged by stereologic and biochemical analyses) in the propranolol-treated fetuses. In addition, total phospholipids were decreased in the propranolol-treated 21-day fetuses. The results of the present study suggest that the development of the alveolar epithelium is sensitive to continuous beta-adrenergic blockade by propranolol during a critical time late in gestation.

Key words: Lung development, Beta-blockade, Type II cells

Introduction

The development of the fetal lung is a complicated process which is regulated by a number of humoral and/or hormonal mediators. These may be endogenously active, or may exhibit substantial effects when administered exogenously (reviewed by Smith and Bagues, 1980; Gross, 1990). The maturation of the alveolar epithelium is particularly important, as it is this which determines the viability of the neonate. This occurs late in gestation, as cuboidal, undifferentiated, cells mature to thin type I cells, across which gas-exchange occurs, and secretory type II cells, the source of pulmonary surfactant. The maturation of the type II cells is characterized by the acquisition of lamellar inclusion bodies, the secretory granule, and the loss of glycogen (Williams, 1977). It is generally accepted that the glycogen is utilized as a substrate for the production of the principal lipids which form a large portion of the pulmonary surfactant of the lamellar inclusion bodies (Bourbon and Jost, 1982). The importance of glucose metabolism in lung cell proliferation, differentiation and maturation is summarized by Ito et al. (1999).

The possible role of the beta-adrenergic system in lung development and maturation has been a subject of some discussion. There is evidence that beta-adrenergic agonists stimulate the secretion of pulmonary surfactant, and, perhaps, synthesis as well (Mettler et al., 1981; Rooney 1985). In addition, fetal type II cells possess beta-adrenergic receptors (Sommers Smith and Giannopoulos, 1983; Ewing et al. 1992), and the number of these receptors at the alveolar level increases with gestational age (Lewis et al., 1990).

The regulatory role of endogenous catecholamines was investigated by Kudlacz et al. (1990), who treated pregnant rats with a continuous propranolol regimen beginning on day 7 of pregnancy. This treatment with propranolol, a potent beta-adrenergic blocker, resulted in a deleterious shift in basal lung compliance, a measure of surfactant effectiveness, as well as other changes. Petit and Nielson (1992) treated pregnant rats with 5-10 mg/kg/day propranolol from gestational day 10 onward and found decreased fetal growth and biochemical

evidence of impaired fetal lung development. Vilos et al. (1985) demonstrated that propranolol inhibits the maturational effect of adrenocorticotropins in fetal sheep lung. Previous studies in our laboratory have documented a fibrogenic response of adult rat lung following 1-3 weeks of continuous propranolol treatment (Smith and Sommers Smith, 1988; Sommers Smith and Smith, 1989). Considering the importance of epithelial/mesenchymal interactions in the development of the alveoli (Stiles et al., 1986), it was thought that derangement of the mesenchymal elements of the developing lung with propranolol could have a major effect on the morphologic, as well as biochemical, maturation of the fetal alveolar epithelium. The present study was undertaken in order to examine the effects of continuous propranolol treatment at specific times during late gestation. The results of this study reveal that propranolol treatment on days 20 through 23 has little noticeable effect, but similar treatment on days 18 through 21 has a profound effect on lung maturation, as well as overall fetal growth.

Materials and methods

A total of 40 timed-pregnant Sprague-Dawley female rats (Charles River, Wilmington, MA) were used. Day 1 of gestation was designated as the day following nocturnal mating. The animals were received by the Wellesley College vivarium at day 12-14 of gestation, were housed individually and maintained on a diet of Purina Formula Chow #5008 and water *ad libitum*. At day 18 or 20 animals (20 dams at each day) were weighed and anesthetized with 87 mg/kg ketamine and 3 mg/kg xylazine intramuscularly. The skin of the interscapular area was clipped and scrubbed with alcohol. A small (1.5-2.0 cm) skin incision was made and an osmotic pump (Alza model 2ML1) was implanted in a subcutaneous pocket, under aseptic conditions. This particular model of pump was utilized because the flow rate (approximately 10 μ l/hr.) was such that the propranolol was soluble at the appropriate concentration. The wound was closed with sterile wound clips and the animals allowed to recover. They were monitored closely for signs of infection and maintained for three days.

In half the animals the osmotic pump contained a solution of DL-Propranolol HCl (Sigma) in normal sterile saline, in a concentration such that the pump delivered 0.5 mg/hr propranolol. Control animals were treated in an identical fashion, except that the osmotic pump in these contained only normal sterile saline.

On day 21 or 23, respectively, of gestation the animals were again weighed and anesthetized, as above. The uterus was exposed by a mid-ventral incision and the most lateral and medial fetuses of the uterine horn which contained the most fetuses (or the right horn if the numbers were equal) were removed from their amniotic sacs. They were prevented from breathing by moderate pressure on the ventral neck, and their lungs were quickly obtained by thoracotomy. These were placed in

cold phosphate-buffered 2% glutaraldehyde and chopped, by hand, into pieces approximately 1 mm³. These tissues were processed for electron microscopy, as below. The remainder of the fetuses were removed, weighed, and their lungs were obtained and quickly frozen in liquid nitrogen for other uses. Fetal weights were statistically examined by obtaining the mean weight of the fetuses of each litter, and then the means and standard errors of all the control vs. propranolol-treated litters. Alternatively, all the propranolol and saline-treated fetal weights were pooled. Results were analyzed using Student's t-test.

The tissue for electron microscopy was fixed for one hour, washed several times in buffer, post-fixed in 1% osmium tetroxide for two hours, dehydrated in a graded ethanol series, infiltrated and embedded in epoxide (Polybed 812, Polysciences Inc.). Following polymerization two blocks from each fetus were selected at random, sectioned at 0.5 μ m, mounted on glass slides and stained with toluidine blue for light microscopic observation. Thin (approximately 60 nm by interference color) were obtained, mounted on 200 mesh copper grids and stained with uranyl acetate and lead citrate. These were examined in either a Zeiss EM-9 or a Phillips CM-10 electron microscope, for qualitative interpretation of ultrastructural features.

Ten randomly selected grids of the fetal lung from the groups killed at the 21st gestational day were then utilized for stereologic analysis of type II cell cytoplasmic volume densities. In this procedure each grid was scanned, beginning in the left upper corner, and the first 5 type II epithelial cells encountered were photographed at a standard magnification. In order to maintain as much uniformity as possible, only those cells in which the plane of section passed through the nucleus were photographed. This procedure resulted in photographs of at approximately 40 cells from both the control and propranolol-treated groups. These were enlarged to a final magnification of 15,550x and analyzed stereologically, employing the methods of Weibel (1973), as previously utilized for fetal rabbit type II cells (Smith et al., 1982) and adult rat type II cells (Smith and Griffin, 1987). Briefly, a transparent grid was placed over each micrograph and the intersections of the grid lines used as test points. The test system consisted of approximately 900 points per micrograph, and the cell structures which were analyzed included lamellar inclusion bodies, rough endoplasmic reticulum, mitochondria and glycogen granules. Results were expressed as cytoplasmic volume densities (hits per organelle over total test points; Weibel, 1963) and analyzed using Student's t-test.

The glycogen content of the rapidly frozen fetal lung tissue was analyzed using the methods of Murat and Serfaty (1974). Briefly, the glycogen is enzymatically degraded to glucose, which is then measured colorimetrically. In other samples, the lipids were extracted with chloroform, using the methods of Folch et al. (1957) and total phospholipids were analyzed colorimetrically as phosphorus, using a modification of

the methods of Bartlett (1959).

Results

There were no apparent differences in the gross appearance or behavior between propranolol-treated and control dams at any corresponding gestational day. There were no significant differences between the weights of the dams at any gestational day studied. The mean litter size for all groups was 12.2, and there were no differences between the litter sizes in any of the groups. All of the fetuses appeared normal by gross examination, and there was no sign of fetal loss in the propranolol-treated groups. However, the fetuses of the dams treated with propranolol from days 18-21, and then obtained on day 21, appeared smaller, on average, than those from saline-treated dams. This observation was confirmed by weighing all the fetuses. The results of this analysis are listed in Table 1. As may be seen, the mean fetal weight of the group treated with propranolol from days 18-21 was significantly less than controls (3.13 g vs. 4.10 g). There was no difference in the weights of fetuses treated from days 20-23. It should also be noted that there were no appreciable differences between members of the same

litter, thus the standard use of the most lateral and medial fetuses in each litter was deemed appropriate.

Light microscopic observation of fetal lung tissue from the various groups revealed that the lungs of the fetuses treated with propranolol from days 18-21 appeared less mature than those from saline-treated dams. These lungs appeared to have fewer well-defined developing alveolar spaces, there appeared to be less pulmonary surfactant in the potential alveolar spaces that did exist, and the type II alveolar epithelial cells appeared less well developed (Figs. 1-3). No differences

Table 1. Effect of propranolol on fetal weights values are mean grams \pm SEM and were calculated using the mean of the individual means from each litter.

GROUP	FETAL WEIGHTS
Day 21 Propranolol-treated	3.13 \pm 0.08*
Day 21 Saline-treated	4.10 \pm 0.14
Day 23 Propranolol-treated	5.25 \pm 0.15
Day 23 Saline-treated	5.45 \pm 0.2

*: $p < 0.001$

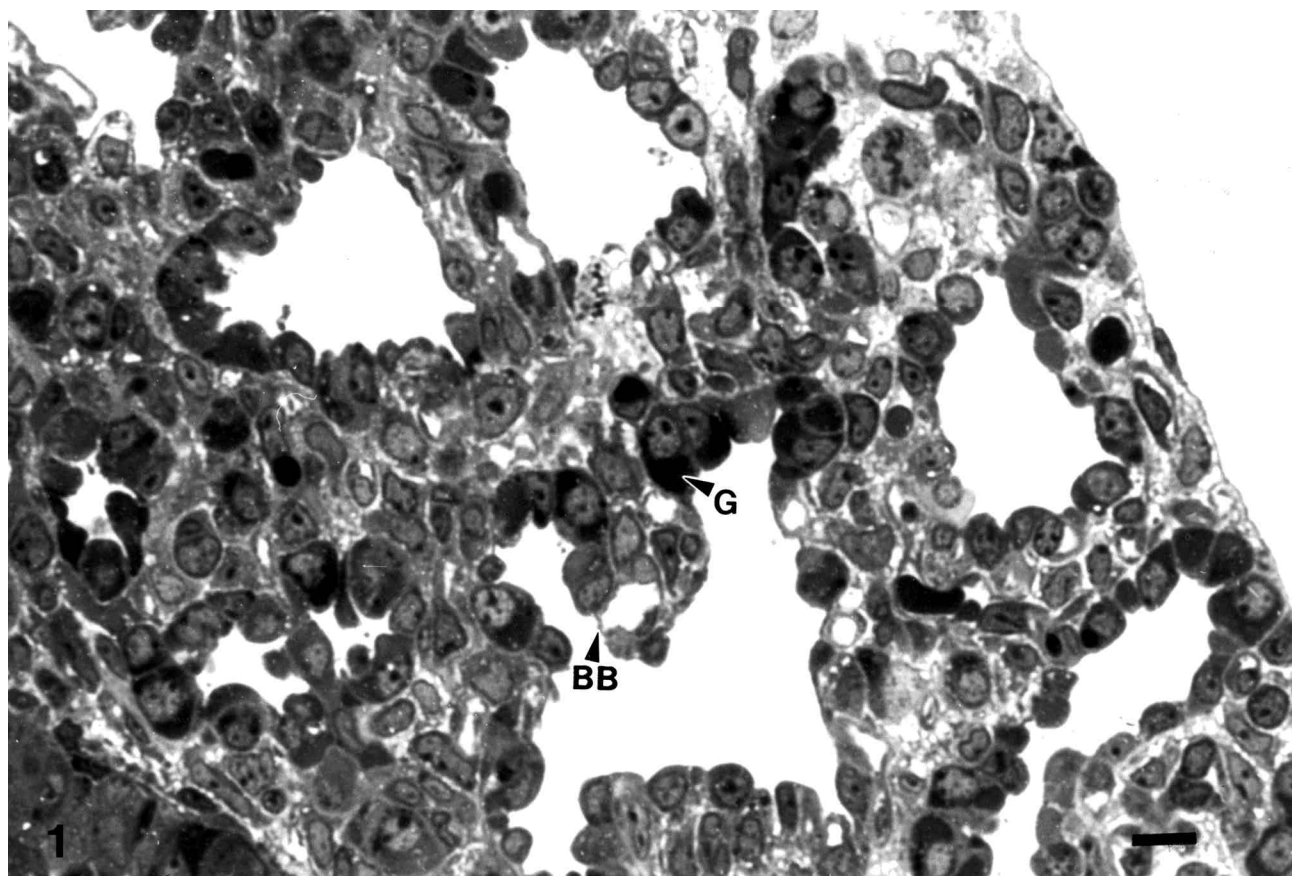


Fig. 1. Light micrograph of fetal lung tissue from group treated with saline on days 18-21. Note alveolar spaces lined with somewhat more mature epithelial cells, showing some blood-air barriers (BB). Note that some type II cells have substantial glycogen (G), but many have very little. x 800

could be observed between the lungs of animals treated with propranolol or saline from days 20-23.

Electron microscopic observation of the fetal lung tissue confirmed the impression of delayed maturation in the group treated from days 18-21, as may be seen in figures four through seven. Although, as always, there was some variation between individual cells and areas within any group, or animal, the overall impression was of thicker developing alveolar septa, fewer developing blood-air-barriers, and less mature type II cells in these propranolol-treated fetuses. The maturity of the type II cells was based on the number and size of lamellar bodies, which were counted and physically measured in the final micrographs, and by the amount of glycogen

within the cytoplasm.

The stereologic analysis of type II cell cytoplasmic contents revealed significantly more glycogen within the type II cells of the fetuses treated with propranolol from days 18-21 (volume density 27.09 vs 11.09; Table 2). This was confirmed by the biochemical assay of glycogen content, as presented in Table 3, which demonstrated significantly more glycogen in the lung tissue of the propranolol-treated 21-day fetuses (1.452 mg/g vs 0.514).

The morphologic observation of less surfactant in the developing air spaces of the propranolol-treated 21 day fetuses was confirmed by the biochemical analysis of total phospholipid content, which revealed more total phospholipids in the control group (4.10 µg/mg vs. 1.67;

Table 2. Stereologic analysis of fetal II cytoplasmic contents. Values are mean volume density measurements \pm SEM

CYTOPLASMIC STRUCTURES	PROPANOLOL-TREATED	SALINE-TREATED
Lamellar Bodies	4.44 \pm 0.59	4.64 \pm 0.69
Rough Endoplasmic Reticulum	4.38 \pm 0.72	3.10 \pm 0.33
Glycogen	27.09 \pm 2.09*	11.09 \pm 2.54

*: p<0.001

Table 3. Glycogen content of 21 day fetal lung. Values are mean mg glycogen/gram tissue \pm SEM

GROUP	GLYCOGEN CONTENT
Saline-Treated	0.514 \pm 0.14
Propranolol-Treated	1.452 \pm 0.38

Values differ at p<0.05

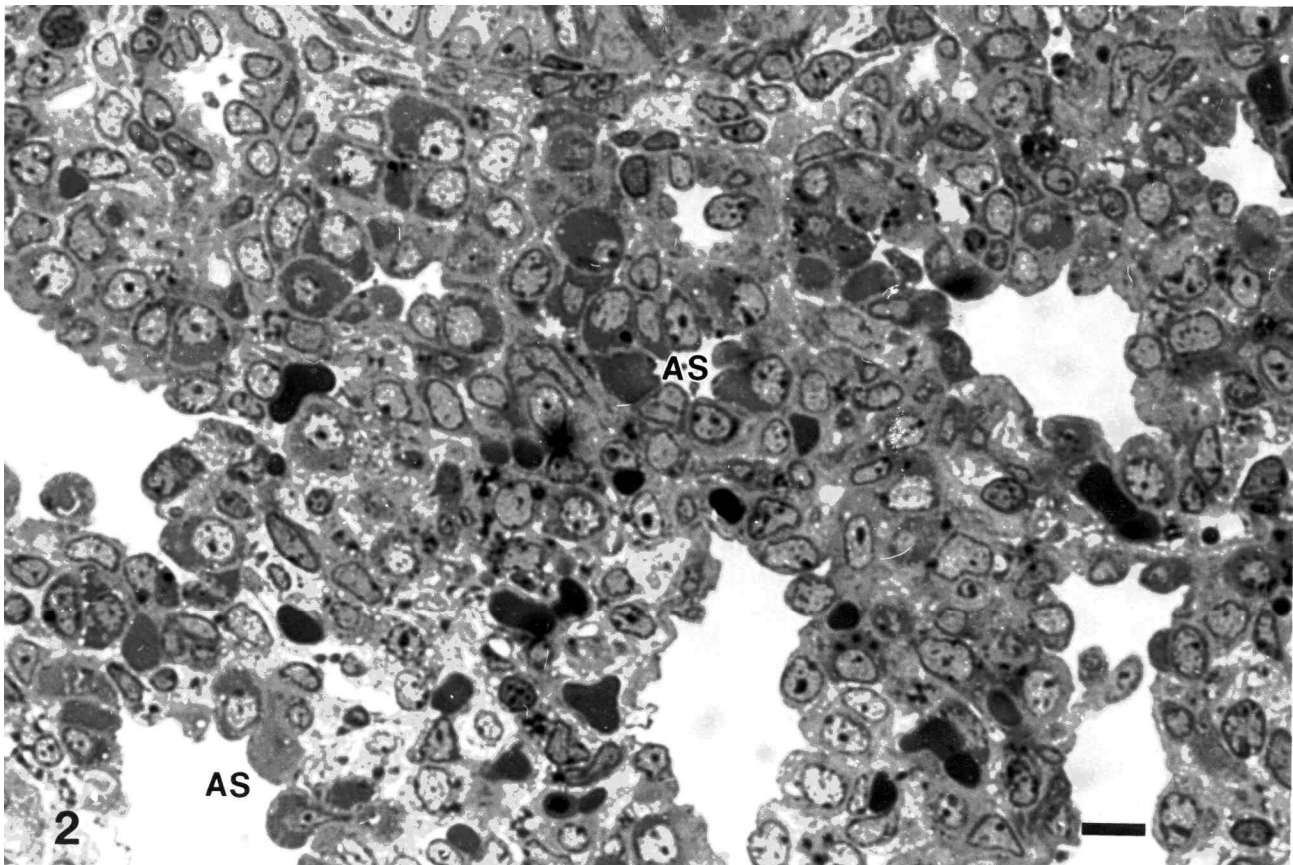


Fig. 2. Light micrograph of fetal lung tissue from a group treated with continuous propranolol on days 18-21. Many of the alveolar spaces are quite small and lined by cuboidal, poorly differentiated, cells. Many of the interstitial cells contain lipid droplets. Note large amounts of glycogen in cuboidal cells lining alveolar space (AS). x 800

Table 4), while the intracellular surfactant, as determined morphometrically, was similar in the two groups.

Discussion

The results of the present study demonstrate that continuous treatment of rat fetuses with propranolol, through a maternally-implanted osmotic pump, on days 18-21 of gestation, results in a generalized growth retardation. In addition, this treatment results in a specific retardation of alveolar epithelial cell maturation, which is particularly evident in the retention of vast glycogen stores in type II cells. This treatment did not seem to affect the presence or number of lipofibroblasts, which were present as previously described by others (Tordet et al., 1981; Marin, 1982) in all groups. Most

strikingly, similar treatment on days 20-23 of gestation had no visible effect on fetal body weight or lung development in the treated group, when compared to controls. These treatment regimens were chosen because the alveolar epithelium normally matures rapidly at the end of gestation.

The results of the present study seem to differ somewhat from those of Kudlacz et al. (1990), who treated fetal rats in a similar manner from day 7 through term. They did not report fetal growth retardation, but did demonstrate compromised lung function. The differences between the present study and that of Kudlacz et al. may be due to the dose of propranolol utilized. Kudlacz et al. administered 10 mg/kg/day, or approximately 0.135 mg/hr. The osmotic pumps in the present study delivered 0.5 mg/hr, in rats of approximately equal weight, and one of the goals of the present study was to use the maximum dose delivered continuously by the osmotic pump. However, the differences may also be due to the timing of the treatment, which appeared to be critical in the present study.

The results of the present study show a dramatic effect on fetal growth when propranolol is administered on days 18-21 of gestation. The exact mechanism of action in this case is unknown. However, intra-uterine

Table 4. Total phospholipid content of fetal lung. Values are mean μg phosphorus/mg tissue

Day 21 Propranolol-treated	1.67 \pm 0.23*
Day 21 Saline-treated	4.10 \pm 0.14
Day 23 Propranolol-treated	5.25 \pm 0.15
Day 23 Saline-treated	5.45 \pm 0.2

*: values differ at $p < 0.05$

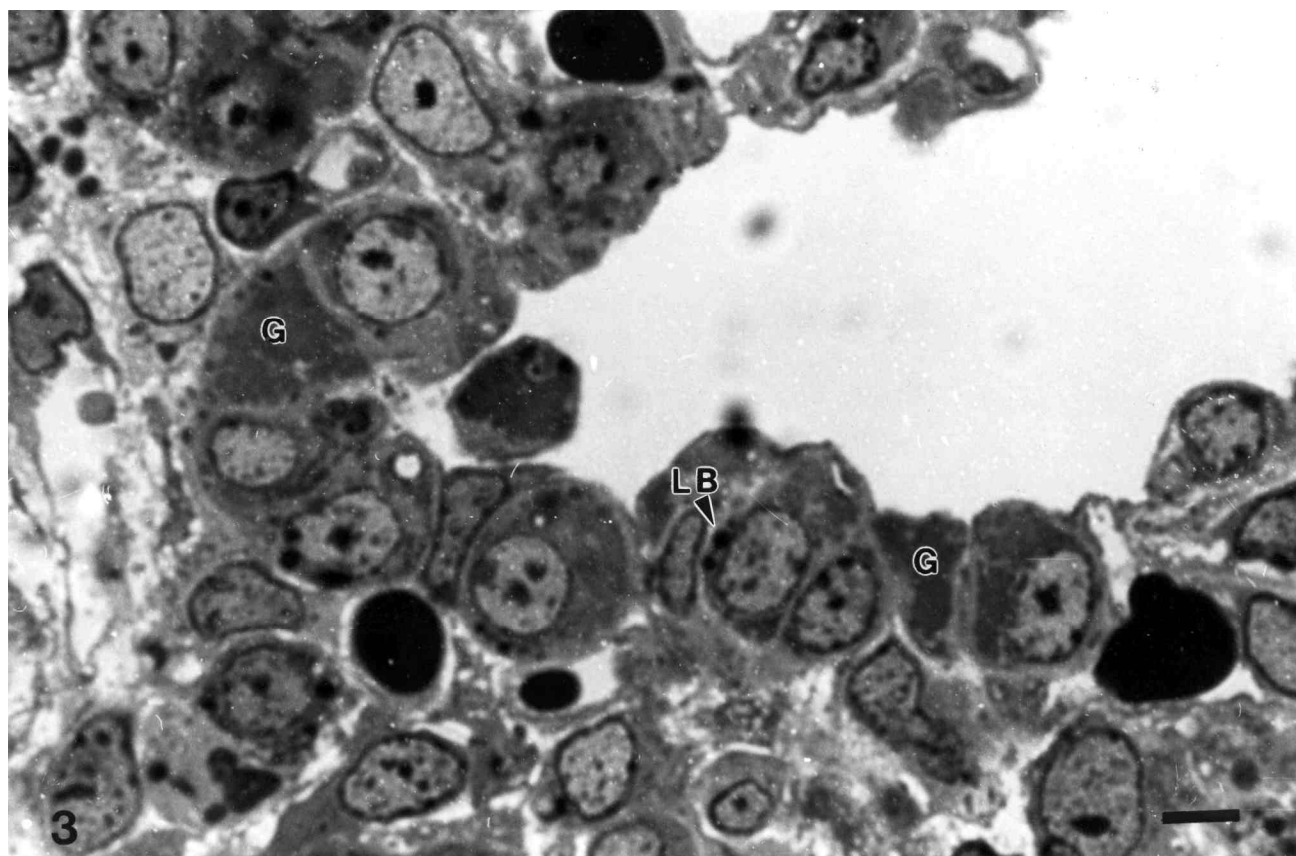


Fig. 3. Light micrograph of epithelial cells lining an alveolar space of fetal lung tissue from a group treated with continuous propranolol on days 18-21. Note few lamellar bodies (LB) and large amounts of glycogen (G). x 2,000

growth retardation as a consequence of propranolol treatment in humans has been described, but not universally. For example Pruyn et al. (1979) found a

strong correlation between long-term propranolol therapy in pregnancy and fetal growth retardation, but others dismiss the conclusion of an association between

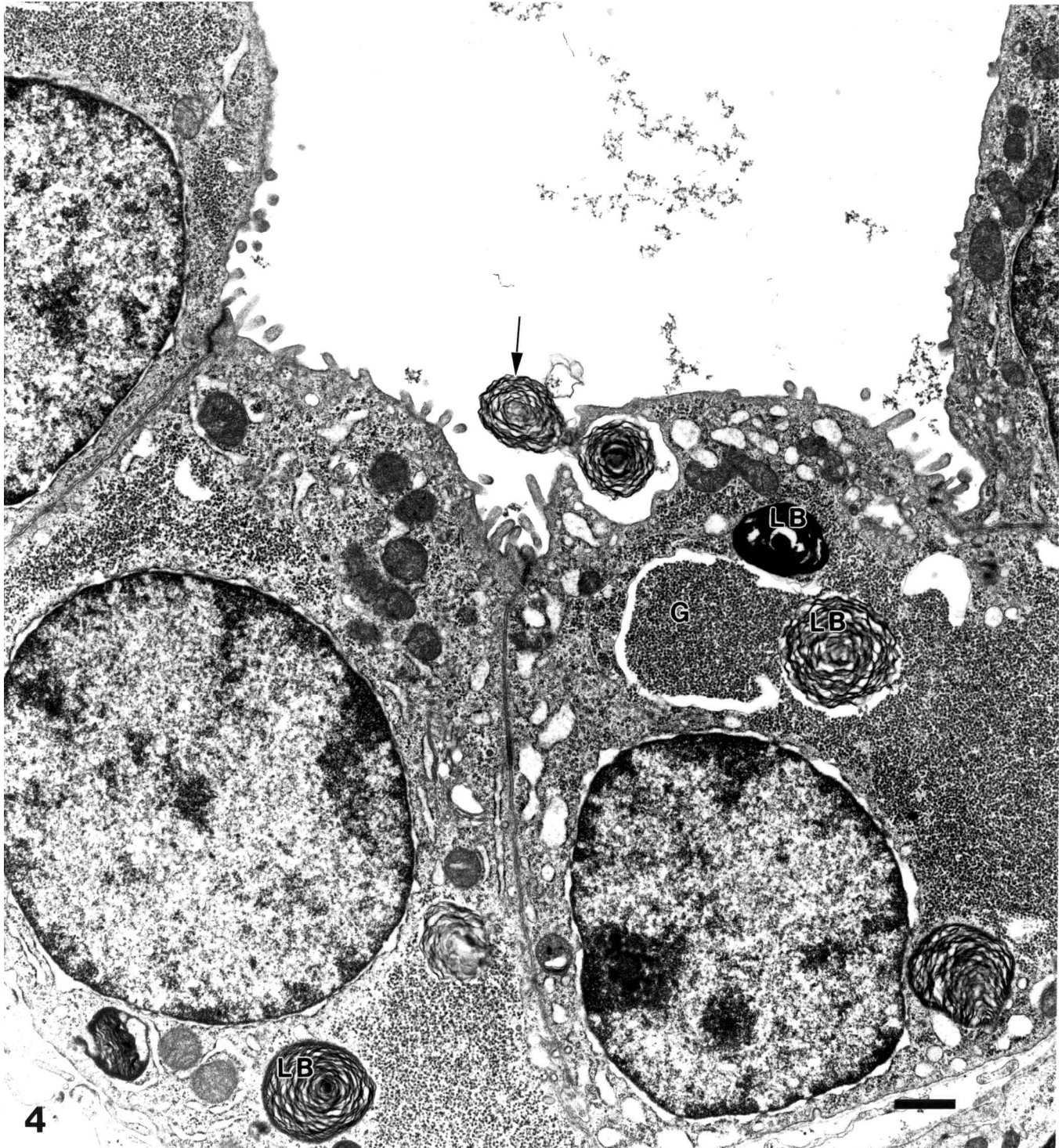


Fig. 4. Electron micrograph of epithelial cells lining alveolar space of fetal lung tissue from group treated with saline on days 18-21. While there is considerable glycogen (G) present, there are also well-developed lamellar inclusion bodies (LB), one of which is on the process of being secreted into the alveolar space (arrow). The cell at the upper right appears to be flattening, presumably to become a type I epithelial cell. x 9,800

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propranolol therapy and intrauterine growth retardation (O'Connor, et al., 1981; Rubin, 1981). It is appropriate to speculate as to whether the intra-uterine growth retardation demonstrated in the present study is responsible for the delayed maturation of the type II alveolar epithelial cells. While this is a possibility, small for gestational age infants typically have more mature lungs (Gluck and Kulovich, 1974; Sher et al., 1981).

There are other possibilities for the time-specific effect of propranolol on alveolar epithelial maturation. As originally anticipated, the blocking of beta-receptors could have a direct effect on alveolar epithelial cells. The

type II cells possess beta-receptors which increase in number towards term (Lewis et al., 1990). Rasmusson et al. (1988) have demonstrated a direct beta-adrenergic effect on surfactant synthesis, secretion and reutilization in fetal rabbit lung. In a related study, Warburton et al. (1987) have demonstrated that a beta agonist depletes glycogen in fetal lamb lung, which correlates to the finding of decreased glycogen use in the beta-blockade of the present study. In addition, McDonald et al. (1986) found that beta-blockade interferes with surfactant release in the perinatal lung, which agrees well with the present study, where less surfactant was observed in the

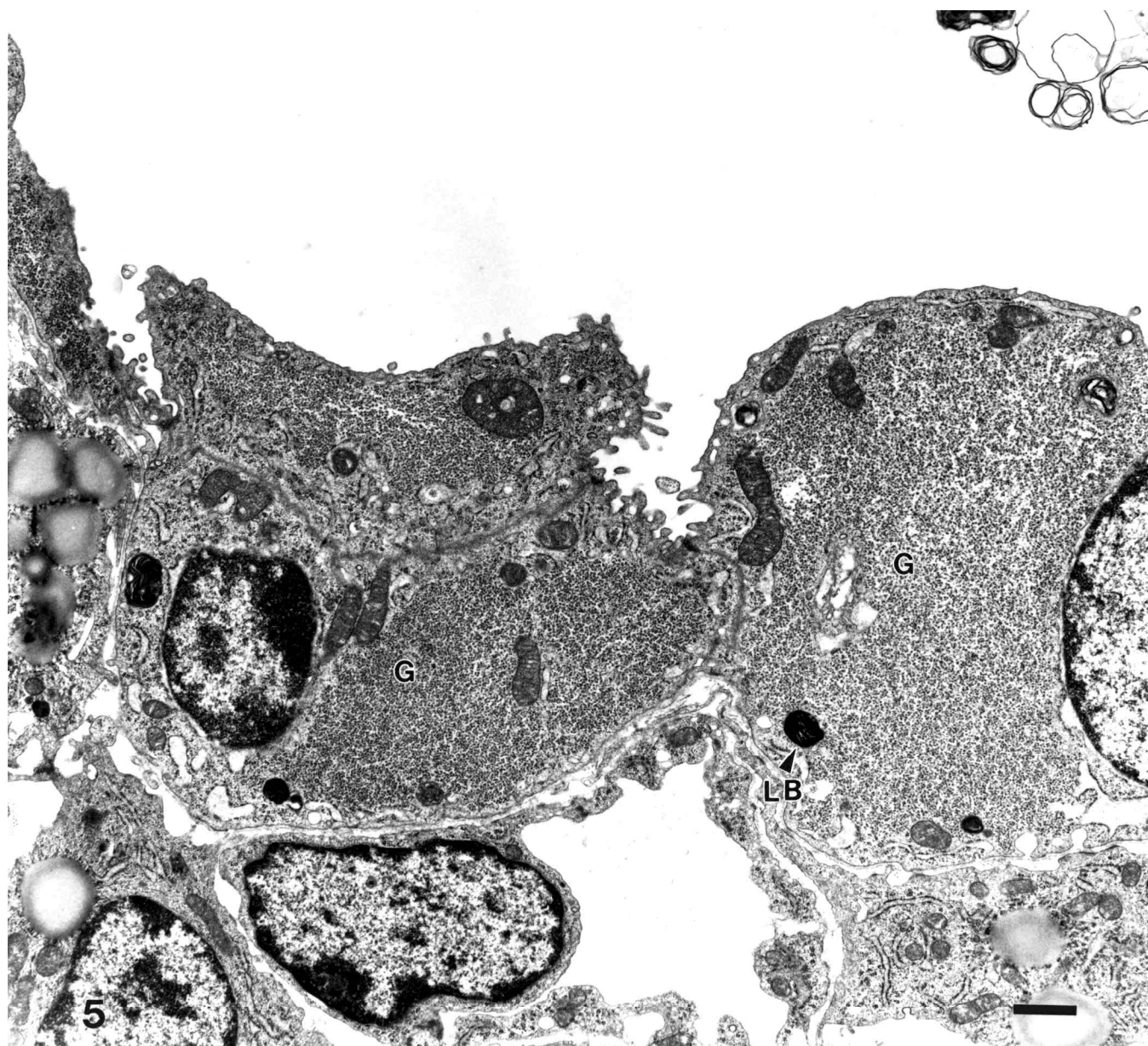


Fig. 5. Electron micrograph of epithelial cells lining an alveolar space of fetal lung tissue from group treated with continuous propranolol on days 18-21. Much of the cytoplasm of these cells is taken up with glycogen particles (G). There are a few, small, lamellar bodies in the basal area of the cells (LB). x 9,500

developing alveolar spaces.

Alternatively, the effect may be indirect and mediated through the fibroblasts underlying the epithelium. Epithelial/mesenchymal interaction is extremely important in the maturation of the epithelium (Stiles et al., 1986), and fetal rat lung fibroblasts apparently produce a factor which can block type II cell maturation (Torday and Kourembanas, 1990). Disruption of the collagen matrix around the type II cells may also interfere with their maturation (King and Adamson, 1987). Previous studies in our laboratory have demonstrated that continuous propranolol treatment of adults results in alterations in the connective tissue components of the alveolar walls which we characterize as fibrogenesis (Smith and Sommers Smith, 1988;

Sommers Smith and Smith, 1989). Such obvious changes in the connective tissue of the fetal lungs were not observed in the present study, but more subtle effects could alter fibroblast function, and thus its role in alveolar maturation. The parallels between tissue repair in pulmonary fibrosis and lung development have recently been further solidified by the work of Terasaki et al. (2000).

It should be stressed, however, that the effect observed in the present study is very time-dependent, which argues for a precise mechanism of action which is inherently time-dependent. Substrate utilization in the perinatal lung is a controlled, and fundamentally important function (Patterson and Rhoades, 1989; Hart et al., 1998). Kudlacz et al. (1990) have reported that

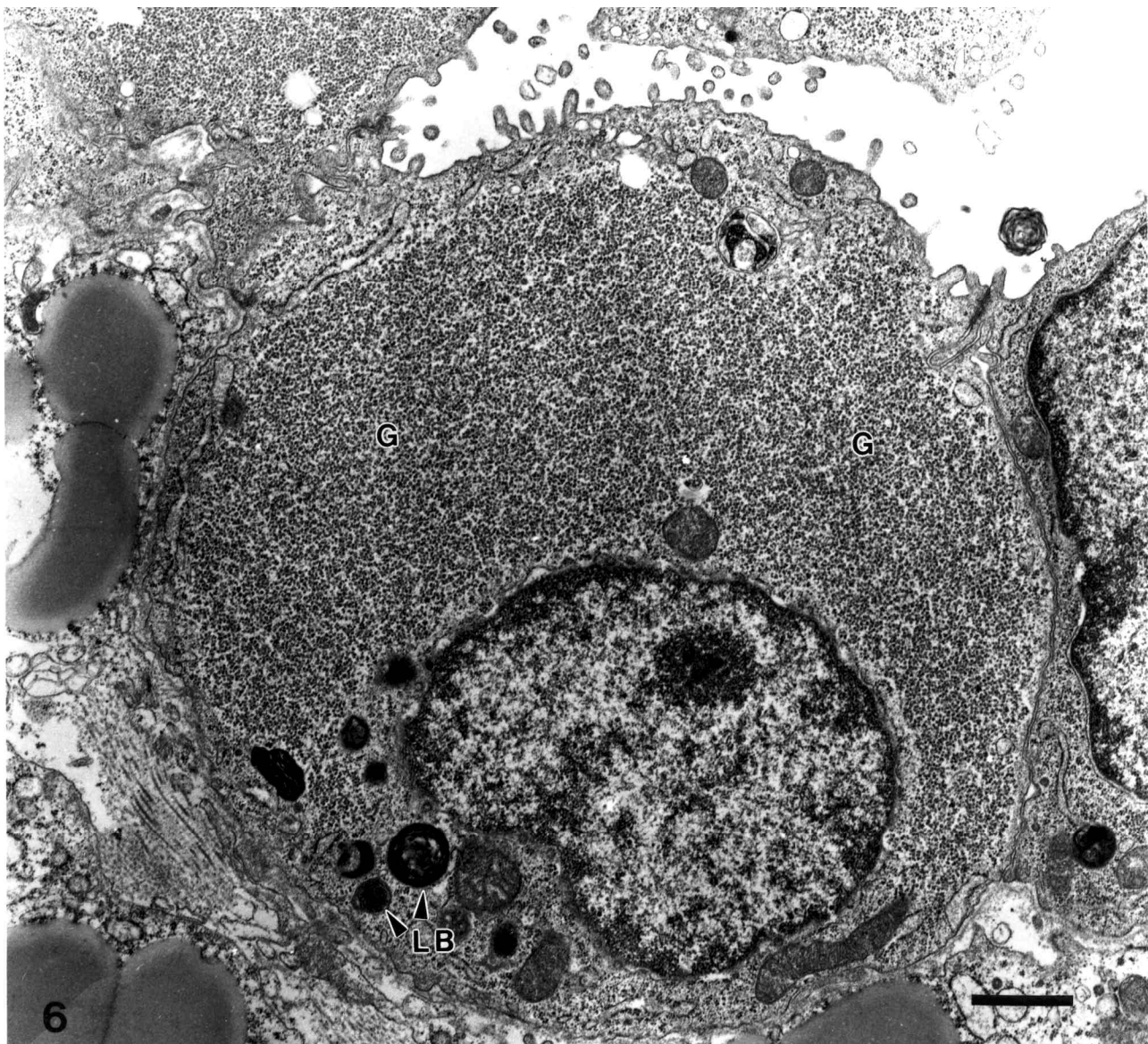


Fig. 6. Higher magnification electron micrograph of an immature type II cell of fetal lung from group treated with continuous propranolol on days 18-21. Again, note that most of the cytoplasm contains glycogen (G), and there are only a few, small, lamellar bodies (LB) in the base of the cell. x 14,700

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continuous propranolol treatment interfered with normal basal activity of the enzyme ornithine decarboxylase. Their results indicate that endogenous catecholamines mediate the sequential coupling mechanisms which are required for establishing normal trophic responses. Kudlacz et al. (1989) have also reported that beta-adrenergic stimulation may have a profound effect on normal patterns of tissue development and responsiveness in the peripheral lung. The results of the

present study suggest that periodic blockade of endogenous beta-adrenergic input at critical periods has a profound effect on glycogen utilization, overall body growth, and maturation of the alveolar epithelium. This effect may be due to interference with an enzyme system, such as ornithine decarboxylase.

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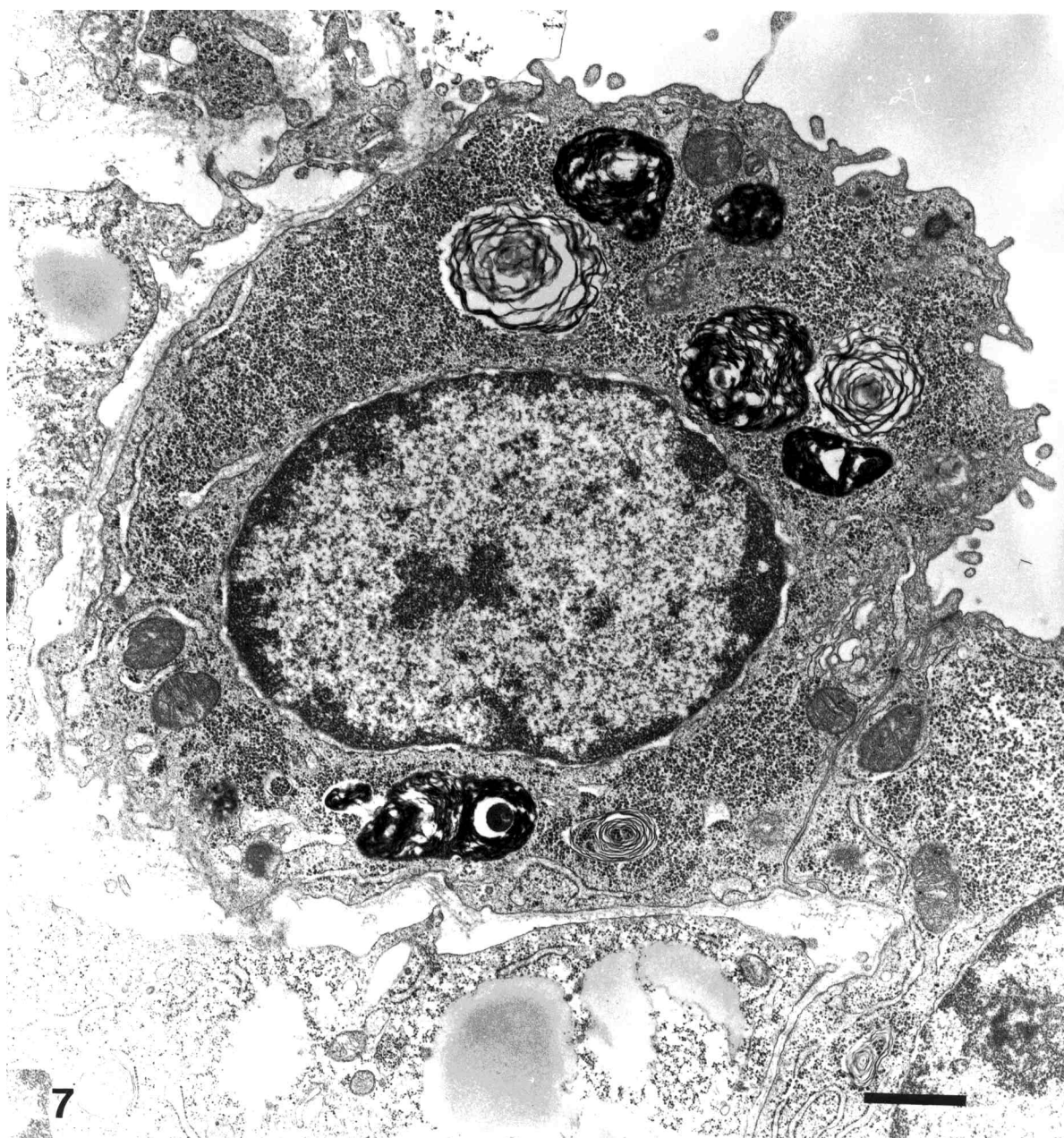


Fig. 7. Electron micrograph of a type II alveolar epithelial cell of fetal lung from group treated on days 20-23 with continuous propranolol. Although there is still considerable glycogen in the cell, there are now a number of well-developed lamellar inclusion bodies, many of which are located in the apex of the cell. These cells were somewhat variable, but overall did not differ from controls in any predictable manner. x 14,700

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