THE ANATOMICAL RECORD 202:23-31 (1982)

Ultrastructural Stereologic Analysis of Fetal Rabbit Type II Alveolar Epithelial Cells Following Maternal Pilocarpine Treatment or Fasting

DENNIS M. SMITH, SUE A. SHELLEY, AND JOHN U. BALIS
Departments of Pathology and Anatomy, Loyola University Stritch School of Medicine, Maywood, Illinois 60153

ABSTRACT We have previously reported that subcutaneous administration of 5 mg/kg pilocarpine HCl, a muscarinic agonist, to pregnant white rabbits on days 24 through 27 of gestation results in an acceleration of fetal lung maturation. In the present study the ultrastructural differences observed within alveolar type II cells of 28-day fetuses from the pilocarpine and control groups were quantified by stereologic analysis. In addition, maturational changes occurring within the lungs of 28-day fetuses obtained from pregnant rabbits which were fasted for 7 days (days 21-28) were similarly analyzed.

Stereologic analysis revealed that the fetal type II cells in the pilocarpine-treated group possessed a significantly greater volume density of lamellar inclusion bodies and rough endoplasmic reticulum and a significantly smaller volume density of glycogen than did controls. The volume density values for fetal type II cells of the fasted group were, with the exception of the Golgi apparatus, found to be intermediate between those of the control and the pilocarpine-treated groups. It was also noted that a high percentage of the lamellar inclusion bodies within type II cells of the fasted group appeared morphologically abnormal, being characterized by a highly irregular outline and tightly packed lipid membranes.

The results of this study substantiate and quantify our previously reported observation of accelerated maturation in pilocarpine-treated fetuses. In addition, they suggest a similar, though not as pronounced, effect of maternal fasting prior to delivery. However, the abnormal morphology of the lamellar inclusion bodies in the fasted group may reflect a qualitative change in the pulmonary surfactant produced by these fetuses.

There is a great deal of interest in the identification and characterization of factors which may influence or regulate the development and maturation of the mammalian lung, particularly the type II alveolar epithelial cells. This interest stems from the established relationships between the type II cells, the production of pulmonary surfactant, and the etiology of the respiratory distress syndrome of the newborn (RDS).

The search for pharmacologic means of enhancing fetal lung maturation has led to a large body of evidence documenting the effects of adrenal glucocorticoids on lung development (for reviews see Avery, 1975; Olson, 1979). However, controversy remains as to the efficacy of prenatal corticosteroid administration in humans for the prevention of RDS (Frank and Roberts, 1979). Other agents which have been reported to hasten fetal lung maturation include thyroid hormones (Hitchcock, 1979) and β-adrenergic agonists such as terbutaline (Bergman et al., 1979).

We previously reported (Smith et al., 1979a, b) that, in rabbits, maternal administration of pilocarpine, a muscarinic agonist, results in an acceleration of fetal lung maturation. The evidence for this includes morphologic correlates of increased maturation, a lower glycogen content, as determined biochemically, and de-

Portions of this work were presented at the Ninety-Second Meeting of The American Association of Anatomists, Miami, Florida.

Dennis M. Smith's present address is Department of Biological Sciences, Wellesley College, Wellesley, Massachusetts 02181.

Sue A. Shelley and John U. Balis's present address is Department of Pathology, University of South Florida School of Medicine, Tampa, Florida 33612.

Received July 15, 1980; accepted July 20, 1981.

0003-276X/82/2021-0023$03.00

creased cell proliferation in the lungs of pilocarpine-treated fetuses. In the present study, in order to further characterize and quantify these findings, ultrastructural stereologic analysis of the morphologic changes occuring within the type II cells of the fetal lungs was performed.

In addition to pharmacologic manipulation, there is evidence that the nutritional status of the pregnant female may play a role in the maturative process occurring in the fetal lung. Naeye et al. (1974) reported significantly higher Lecithin/Sphingomyelin (L/S) ratios and shake-test values for amniotic fluid lecithin in human infants born to undernourished mothers. In addition, the lungs of infants born to the most poorly nourished mothers revealed a more mature histologic pattern when compared to neonates of better-nourished mothers. Finally, the incidence of death due to hyaline membrane disease was lower in premature offspring of mothers who were undernourished or on low-calorie diets before delivery. These findings were substantiated by Fairbrother and coworkers (1975) who, based on the results of foam tests, concluded that malnourished fetuses had a higher concentration of surfactant in the amniotic fluid than well-nourished counterparts.

The second portion of the present study was designed to determine the effects of maternal fasting on the maturation of the rabbit fetal lung. In this report we compare results obtained through pharmacologic manipulation of fetal lung maturation (pilocarpine) with those obtained following nutritional deprivation. Portions of this work have previously been presented in abstract form.

MATERIALS AND METHODS

Young white New Zealand female rabbits (Langshaw Farms, Augusta, Michigan) ranging in weight from 3.2 to 4.0 kg were mated under direct observation and the time of mounting was considered to be time zero of gestation. Control and pilocarpine-treated does were at all times maintained on a standard diet of approximately 90 gm of lab chow per day. Does of the fasted group were fed similarly prior to the beginning of the fast. All animals were allowed water ad libitum.

Some of the pregnant does received the identical pilocarpine regimen as utilized in our previous studies (Smith et al., 1979a, b)—5 mg/kg administered subcutaneously on days 24 through 27 of gestation—and are designated as the pilocarpine-treated group. The fasted group consisted of pregnant does subjected to complete withdrawal of food on days 21 through 28 of gestation. Pregnant does injected with saline served as controls for both groups, since pilot experiments utilizing non-injected controls demonstrated no significant differences from the saline-injected rabbits in any parameter studied.

In all cases the does were killed using an air embolism and the fetuses were obtained by an immediate hysterotomy at the 28th day of gestation. The fetuses were not allowed to breathe and therefore the lungs were maintained in the fetal condition when removed and fixed for electron microscopy. The fixative utilized was 2% glutaraldehyde in 0.2 M Sorensen's phosphate buffer (pH 7.2). In order to maintain uniformity, the most lateral fetus of the right uterine horn was always utilized for electron microscopic studies. In the fasted group, the lungs and the livers of the remaining fetuses were removed and frozen in liquid nitrogen. These were then analyzed for glycogen content using the method of Murat and Serfaty (1974). This was done in order to compare the results from this group with those of pilocarpine-treated and control fetuses which we have previously reported (Smith et al., 1979a).

The fetal lung fixed in glutaraldehyde was processed and sectioned for electron microscopy utilizing procedures detailed in our previous study (Smith et al., 1979a). It should be stresed that, in order to obtain optimal preservation of glycogen within the fetal type II cells, Sorensen's phosphate-buffered solutions and a somewhat abbreviated ethanol dehydration were employed. Thin (60 nm) sections were obtained from randomly selected blocks from the right upper and right lower lobes of six fetuses from different litters in each group. These were mounted on 200-mesh copper grids and examined in a RCA EMU-3H electron microscope. Beginning in the left upper corner, each grid was scanned and the first five alveolar type II cells which were encountered were photographed at a standard magnification of 5,800. The scope was calibrated periodically in order to maintain uniformity. Identification of type II cells was based on cuboidal shape, presence of microvilli, and, most importantly, the presence of at least one lamellar inclusion body. In addition, in order to maintain as much uniformity as possible, only cells in which the plane of section passed through the nucleus were photographed. This process resulted in 60 photographs from each

group (30 from each of the upper and lower lobes) which were enlarged to a final print size of 8 × 10 and a magnification of 20,000. These photographs were then analyzed stereologically employing the methods of Weibel (1973), in order to determine the cytoplasmic volume densities of components involved in pulmonary surfactant production in the fetus.

The test system for the stereologic analysis consisted of a transparent acetate sheet imprinted with a 0.25-inch grid which was randomly applied to the micrographs. By using the intersections of the grid lines as reference points, the test system employed approximately 1,200 test points per micrograph, with 800–1,000 falling over the area occupied by the entire type II cell.

The cell components which were analyzed in the present study included glycogen, lamellar inclusion bodies, rough endoplasmic reticulum, and Golgi apparatus. The number of point "hits" falling over each of the structures of interest were tallied for each cell, as well as the number of total point "hits" falling over the entire cytoplasmic compartment of the cell. Point "hits" falling over the nucleus were not included. Using the equation Point Intercept / Point Total (P_i / P_t) = Point Density (P_p) = Profile Density on a Test Area (A_a) = Volume Density (V_v) (Weibel, 1963, 1972), cytoplasmic volume densities were obtained for each of the structures of interest.

The use of the test system detailed above resulted in a maximum relative error of approximately 10% in measuring the organelles of least volume density (i.e., Golgi). While it would have been acceptable to utilize far fewer test points for the volumetry of other structures, the test system used yielded results with minimal relative errors. All data were compared using Student's t-test.

It is appropriate to mention that the stereologic methods employed in this study may have introduced two levels of bias. The first is due to the sampling method which selected only profiles of cells which included the nucleus. This may have overestimated the volume density of organelles occurring primarily in the central core of the cell, and underestimated those occurring more in the periphery. However, since the goal of this study was the comparison of type II cells from the various experimental groups, this bias is of negligible concern because identical sampling techniques were utilized for all groups.

A second level of bias may have been introduced by measuring the volume densities of the organelles of interest relative to total cytoplasmic volume. It may be theorized that changes in the absolute volume of total cytoplasm following maturational stimulation could skew the volume densities of the organelles in question since they are determined relative to cytoplasmic volume. However, the point totals were not significantly different in the various groups of this study and therefore this does not appear to be a problem.

RESULTS

Mean maternal weight gains or losses during the appropriate experimental period were: control, +2.4% (days 24–27); pilocarpine, −3.8% (days 24–27); fasted, −11.1% (days 21–28). The average weight of fetuses from seven litters in the fasted group was 25.57 gm (± 0.81) as compared to our previously reported values of 26.95 gm (± 1.97) for the pilocarpine-treated group and 35.42 gm (± 1.42) for control fetuses (Smith et al., 1979a). There were no apparent abnormalities in fetuses of any group and their lungs appeared comparable by gross inspection.

In fetuses of the fasted group light microscopy revealed evidence of increased maturation in the lungs including thinner alveolar septa and a marked decrease in periodic acid-Schiff (PAS)-positive material within lung parenchymal cells. This PAS-positive material disappeared after diastase pre-treatment and thus was interpreted as having been glycogen. Similar light microscopic findings were previously reported in the lungs of the pilocarpine-treated fetuses. Indeed, no marked differences were noted, by light microscopy, between lungs of pilocarpine-treated fetuses and those of the fasted group, both appearing more mature than controls.

The biochemical determination of glycogen content (Table 1) indicated that the glycogen content of the lungs of fasted fetuses was intermediate between our previously reported values for control and pilocarpine-treated fetuses, which are included here for completeness. There was also a decrease in liver glycogen content in the fasted group which was significant when compared to either the control or pilocarpine-treated group. It should be noted that there was no significant difference in liver glycogen content between the control and pilocarpine-treated group.

Stereologic analysis of type II alveolar epithelial cells from control and pilocarpine-treated fetuses confirmed the morphologic differences previously noted by electron mi-

TABLE 1. Glycogen content (mg/gm tissue)

	Number of litters	Number of fetuses	Lung	Liver
Control[1]	5	17	4.11 ± 0.33	16.98 ± 2.03
Fasted	5	13	3.26 ± 0.35[2]	8.29 ± 1.17[3]
Pilocarpine[1]	7	25	2.65 ± 0.13	14.98 ± 1.30

[1]Smith, et al. (1979a).
[2]No significant difference between fasted values and values for control and pilocarpine-treated groups.
[3]Significantly lower ($P < 0.001$) than both control and pilocarpine-treated groups.

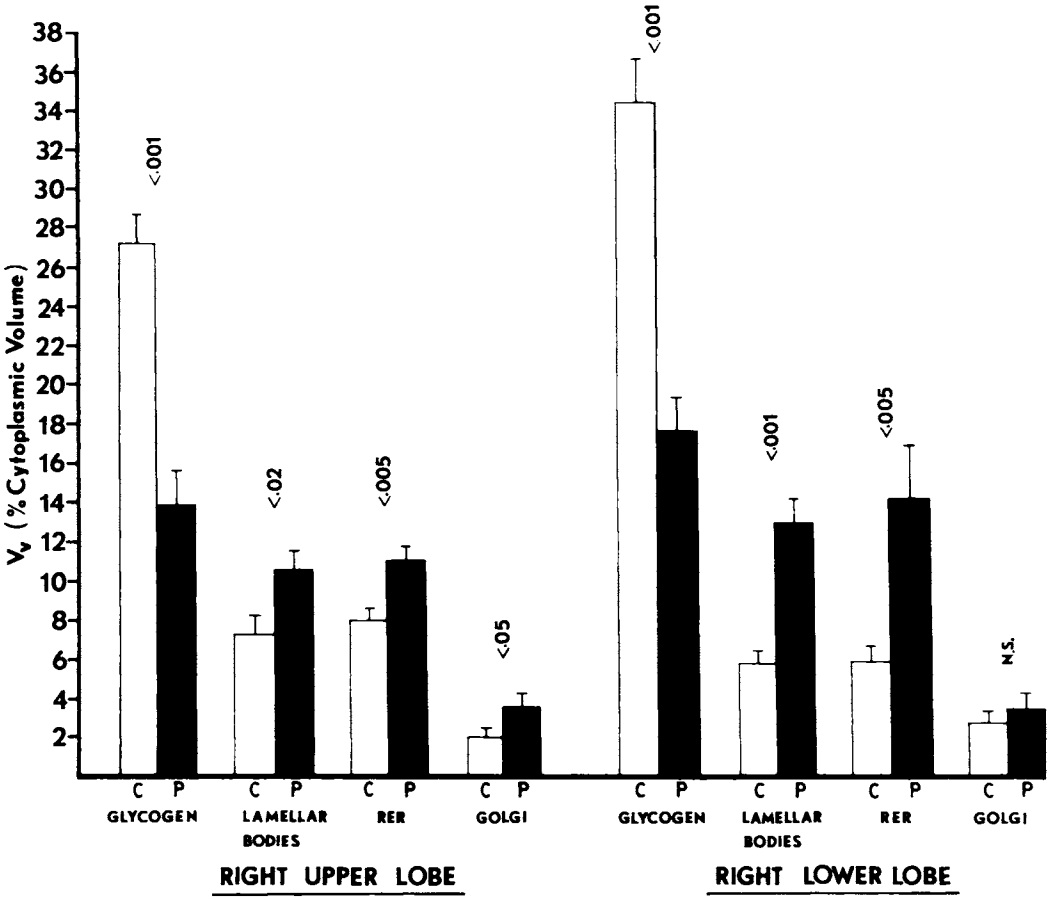

Fig. 1. Histogram illustrating the results of the ultrastructural stereologic analysis of cytoplasmic components within type II alveolar epithelial cells of control (C) and pilocarpine-treated (P) fetuses. RER, rough endoplasmic reticulum.

croscopy (Smith et al., 1979a). As illustrated in Figure 1, when compared to controls, the cytoplasmic volume density of lamellar inclusion bodies and rough endoplasmic reticulum were increased significantly in the type II cells of the pilocarpine-treated group, in both the upper and lower lobes. The Golgi apparatus was increased significantly in the cytoplasm of the type II cells of the upper lobe, but not the lower. In addition, there was a significant decrease in the volume density of glycogen present within these cells, again in both the upper and lower lobes.

Electron microscopic observation of fetal lungs of the fasted group confirmed the impression of increased maturation in these

lungs gained through light microscopy. The type II cells of the fasted fetuses appeared to contain more numerous lamellar inclusion bodies when compared to controls of the same gestational age (compare Figs. 3 and 4). In addition, there appeared to be a decrease in the glycogen content of these cells. It was observed, however, that many of the lamellar inclusion bodies within these cells appeared morphologically abnormal. These were characterized by a highly irregular outline and intense osmiophylia of tightly packed membranous profiles (Figs. 4, 5). It was noted, at higher magnification, that the periodicity of the lamellae within the "abnormal" inclusion bodies was identical to that of the "normal" inclusion bodies present within the type II cells. The periodicity of both types was subsequently determined to be 4 nm. This finding would lend support to the view that these "abnormal" inclusion bodies were morphologic variants of lamellar inclusion bodies and not inclusions of some other origin. Although this type of inclusion body was extremely rare in the type II cells of control or pilocarpine-treated fetuses, they accounted for 41% of all the inclusion bodies in analyzed type II cells of the right upper lobe in fasted fetuses, and 31% in the right lower lobe.

Stereologic analyses of type II cells from fasted fetuses were consistent with the impressions gained by routine electron microscopic observation. When these results are compared to those obtained from type II cells of control and pilocarpine-treated fetuses (Fig. 2) it becomes apparent that, with the exception of the Golgi apparatus, values for the analyzed structures in the fasted group fall between the values for control and pilocarpine-treated fetuses. Summarizing maturational changes, the type II cells of the fasted fetuses contained significantly less glycogen than did controls, in both the upper and lower lobes. In addition, those of the lower lobe contained a significantly greater volume density of lamellar inclusion bodies when compared to controls.

DISCUSSION

Our previous observations suggested that maternal pilocarpine administration results in accelerated maturation of the fetal lung. These

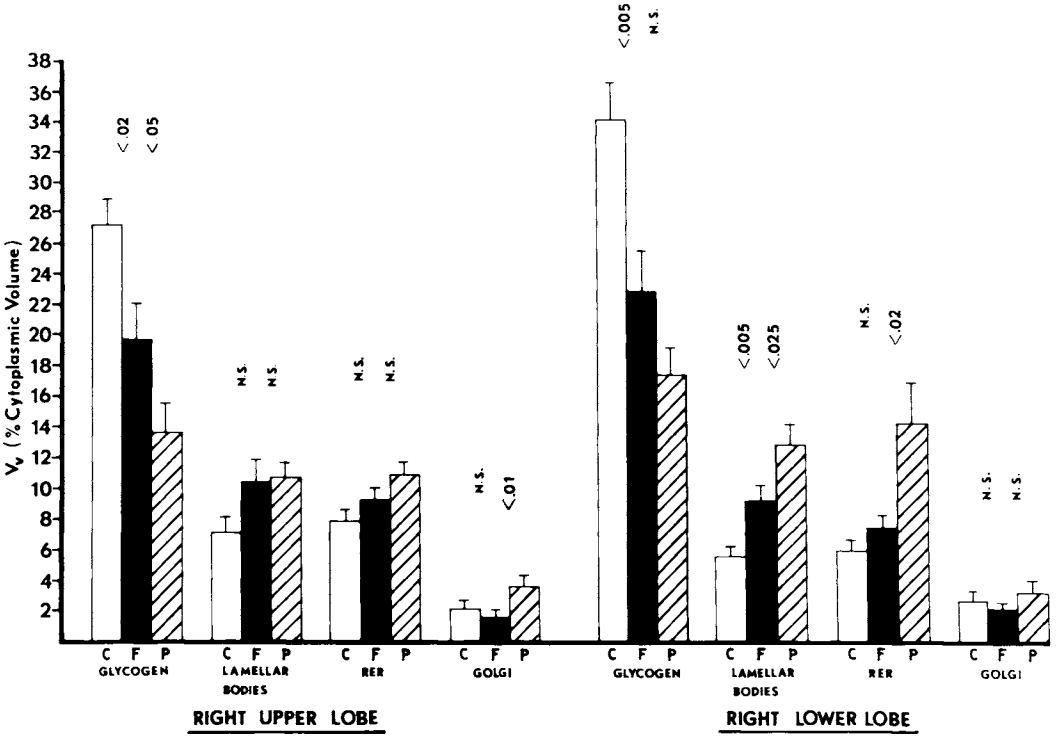

Fig. 2. Histogram comparing the results of the ultrastructural stereologic analysis of cytoplasmic components within type II alveolar epithelial cells of fetuses in the fasted group (F) with the results of control (C) and pilocarpine-treated fetuses (P) as presented in Figure 1.

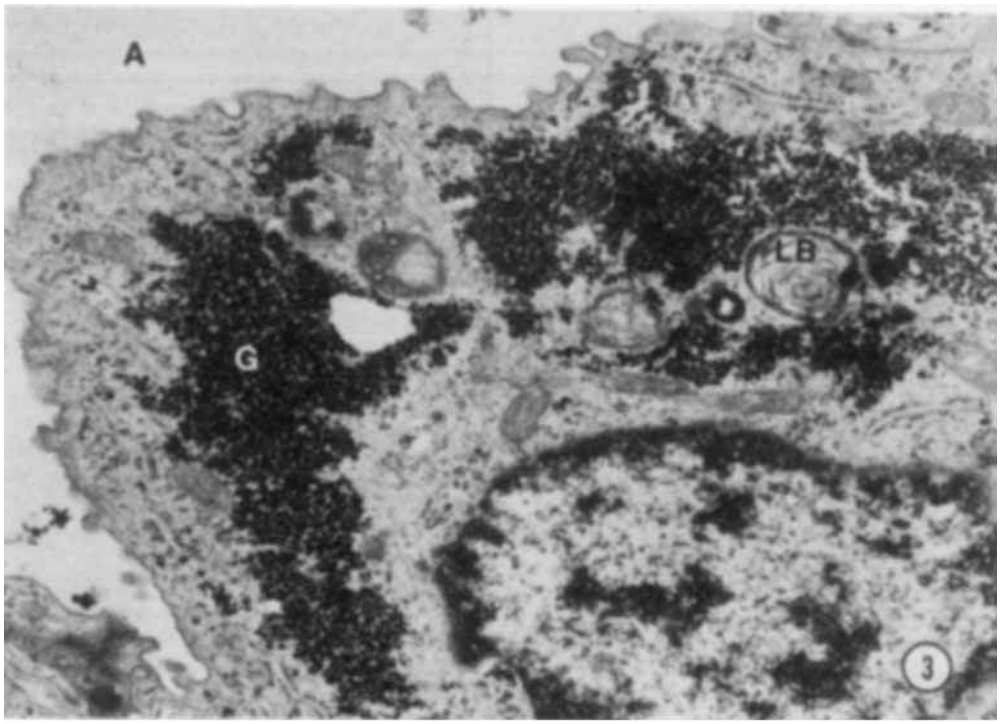

Fig. 3. Electron micrograph of a type II cell from a control fetus. These cells typically contained abundant glycogen (G) and relatively few lamellar inclusion bodies (LB), usually containing sparse lamellae. A, Alveolar space. × 21,000.

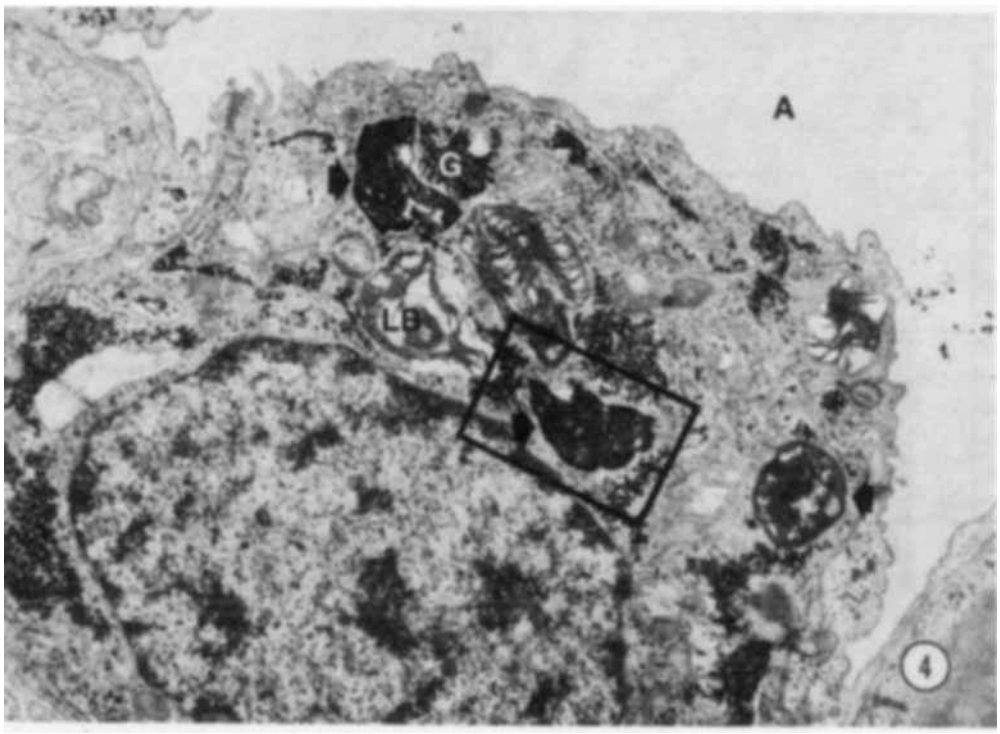

Fig. 4. Electron micrograph of a type II cell from a fetus of the fasted group. Glycogen (G) was less abundant in these cells and lamellar inclusion bodies (LB) were more frequent. A large percentage of the lamellar inclusion bodies in these cells were morphologically abnormal (arrows). A portion of this cell (box) is shown at higher magnification in Figure 5. A, Alveolar space. × 14,300.

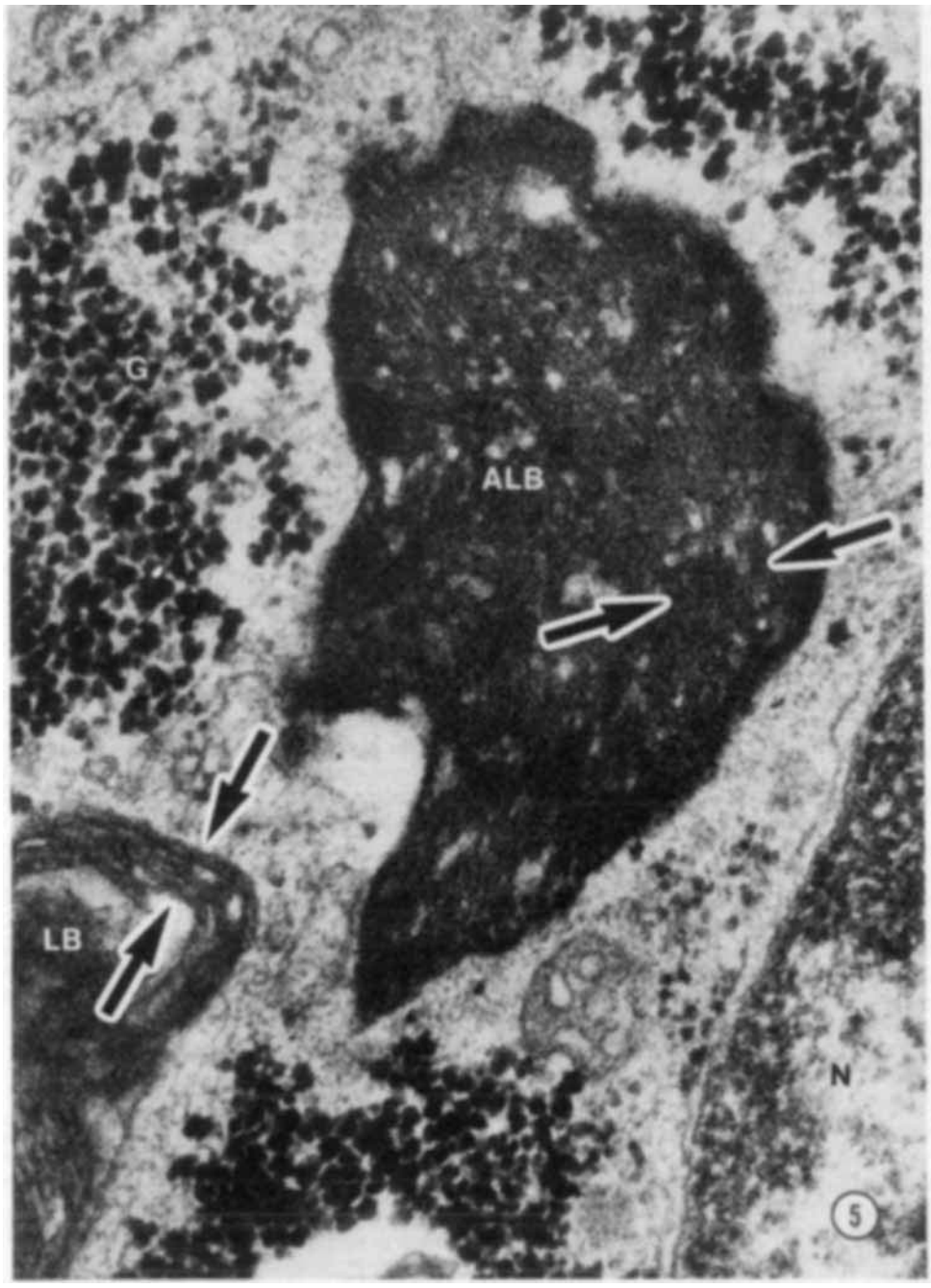

Fig. 5. Higher magnification of a portion of the cell seen in Figure 4. The periodicity of the lamellae in the abnormal lamellar inclusion body (ALB) may be seen to be identical with that in the normal lamellar inclusion body (LB). The periodicity of the lamellae is particularly evident in the areas demarcated by the arrows. G, glycogen; N, nucleus. × 92,200.

findings included thinning of the type I cells to form the blood-air barriers, significant increases in the number of mature type II cells which contained more and larger inclusion bodies, and substantial reductions in the glycogen content of alveolar epithelial cells (Smith et al., 1979a), as well as a reduced rate of cellular proliferation (Smith et al., 1979b). The conclusion that pilocarpine enhances fetal lung maturation is substantiated by stereologic analysis made in the present study. Muscarinic agents, including pilocarpine and oxotremorine, have previously been reported to stimulate surfactant synthesis and secretion in the adult, neonatal and fetal lung; however, the mechanism by which pilocarpine produces this effect remains unclear. The literature in this field has been recently reviewed (Smith et al., 1979a, Smith and Bogues, 1980).

The present study also provides quantitative data indicating that maternal fasting results in an acceleration of maturation in the fetal lung. The 7-day duration of the fast may appear severe. However, the adult rabbit stores a great deal of food in its stomach and blood glucose levels do not begin to fall until 72 hours after the onset of fasting (Kozma et al., 1974).

There is relatively little information pertaining to the effects of malnutrition or fasting on the lung surfactant system. In apparent contrast to the findings of the present study, Curle and Adamson (1978) described delayed maturation, including retention of glycogen within type II cells, in the lungs of rat fetuses born to mothers fasted in late gestation. In addition to possible interspecies variation, however, the pregnant rats in the study of Curle and Adamson were allowed free access to water supplemented with 2% glucose. Since glucose is a primary energy substrate supplied to the fetus by the mother (Simmons et al., 1974), and fetal glucose levels mimic those of the maternal system (Hill and Longo, 1980), this would result in a major difference between these experiments and those of the present study.

Faridy (1975) has reported a reduction in lecithin content and increased minimal surface tension in the lungs of fetal rats obtained from mothers deprived of food for 72 hours late in gestation; however, these puppies did not experience respiratory problems after birth. This is interesting in light of the clinical findings of Naeye et al. (1974) and Fairbrother and coworkers (1975), which would indicate increased maturation of the lung and a decreased incidence of RDS in neonates born to undernourished mothers. In addition, data recently presented by Roepke et al. (1980) indicated that infants born to mothers experiencing small weight gains during pregnancy had significantly better apgar scores than those born following large maternal weight gains. It is also of interest that populations known to have a high incidence of malnutrition have a significantly lower incidence of postnatal death due to hyaline membrane disease (i.e., India, Hadley et al., 1958).

In the present study the effect of maternal fasting was similar in many respects to that of pilocarpine treatment, though not as marked. However, a high percentage of the lamellar inclusion bodies within the type II cells of the lungs of these fasted fetuses appeared morphologically abnormal with an irregular outline and intense osmiophylia. This morphologic appearance suggests that the biochemical nature of the pulmonary surfactant contained within these abnormal lamellar inclusion bodies may be different from that which is normally present.

In conclusion, the results of this study substantiate and quantify our previously reported observation of accelerated maturation in pilocarpine-treated fetuses. In addition, they suggest a similar, though not as pronounced, effect of maternal fasting prior to delivery. Furthermore, the abnormal morphology of many of the lamellar inclusion bodies in the fasted group suggests a qualitative change in the pulmonary surfactant produced by the type II cells of these fetuses.

ACKNOWLEDGMENTS

This study was supported by NIH grant HD-04434.

The authors wish to thank Doctor Gissur Brynjolfsson, Emeritus Professor, Department of Pathology, Loyola University Stritch School of Medicine, for his strong support and encouragement of this study.

LITERATURE CITED

Avery, M.E. (1975) Pharmacologic approaches to the acceleration of fetal lung maturation. Br. Med. Bull., *31*:13-17.

Bergman, B., T. Hedner, and P. Lundborg (1979) Effect of terbutaline on lecithin content in alveolar lung wash in fetal rabbits. Acta Physiol. Scand., *105*:378-380.

Curle, D.C., and I.Y.R. Adamson (1978) Retarded development of neonatal rat lung by maternal malnutrition. J. Histochem. Cytochem., *26*: 401-408.

Fairbrother, P.F., I.L. DuToit, and R.L. Cheifitz (1975) The amniotic fluid foam test and fat cell count in malnourished and well-nourished fetuses. Br. J. Obstet. Gynecol., *82*:182-186.

Faridy, E.E. (1975) Effect of maternal malnutrition on surface activity of fetal lungs in rats. J. Appl. Physiol., *39*:

535-540.
Frank, L., and R.J. Roberts (1979) Effects of low-dose prenatal corticosteroid administration on the premature rat. Biol. Neonate, *36*: 1-9.
Hadley, G.G., E.W. Gault, and M.D. Graham (1958) A study of pathology of still-births and neonatal deaths in south India. J. Pediatr., *52*: 139-148.
Hill, E.P., and L.O. Longo (1980) Dynamics of maternal-fetal nurtrient transfer. Fed. Proc., *39*: 239-244.
Hitchcock, K.R. (1979) Hormones and the lung. I. Thyroid hormones and glucocorticoids in lung development. Anat. Rec., *194*: 15-40.
Kozma, C., W. Macklin, L.M. Cummins, and R. Mauer (1974) Anatomy, physiology, and biochemistry of the rabbit. In: The Biology of the Laboratory Rabbit. S. H. Weisbroth, ed., Academic Press, New York, pp. 49-72.
Murat, J.C., and A. Serfaty (1974) Simple enzymatic determination of polysaccharide (glycogen) content of animal tissues. Clin. Chem., *20*: 1576-1577.
Naeye, R.L., R.K. Freeman, and W.A. Blanc (1974) Nutrition, sex and fetal lung maturation. Pediatr. Res., *8*: 200-204.
Olson, E.B. (1979) Role of glucocorticoids in lung maturation. J. Anim. Sci., *49*: 225-238.
Roepke, J.L., A. Kirksey, and H.E. Clark (1980) Influence of prepregnant weight and weight gain during pregnancy on the size and apgar scores of newborn infants. Fed. Proc., *39*: 563.
Simmons, M.A., G. Meschia, E.L. Makowski, and F.C. Battaglia (1974) Fetal metabolic response to maternal starvation. Pediatr. Res., *8*: 830-836.
Smith, B.T., and W.G. Bogues (1980) Effects of drugs and hormones on lung maturation in experimental animal and man. Pharmacol. Ther., *9*: 51-74.
Smith, D.M., S.A. Shelley., and J.U. Balis (1979a) The maturation of the rabbit fetal lung following maternal administration of pilocarpine. Am. J. Anat., *154*: 163-178.
Smith, D.M., S.A. Shelley, and J.U. Balis (1979b) Reduced cell proliferation in fetal lung after maternal administration of pilocarpine. A scintillation autoradiographic study. Am. J. Anat., *155*: 131-137.
Weibel, E.R. (1963) Morphometry of the Human Lung. Academic Press, New York.
Weibel, E.R. (1972) A stereologic method for estimating volume and surface of sarcoplasmic reticulum. J. Microscosc. *95*: 229-242.
Weibel, E.R. (1973) Stereologic techniques for electron microscopy. In: Principles and Techniques of Electron Microscopy. M. Hayat, ed. Von Nostrand Reinhold, New York pp. 237-296.