Virchows Arch [Pathol Anat] (1981) 390:313–324

*Virchows Archiv A*


# Studies of the Lung in Diabetes Mellitus

## I. Ultrastructural Studies of the Lungs in Alloxan-Induced Diabetic Rats

Kazuhiro Sugahara[1], Kazuo Ushijima[1], Tohru Morioka[1], and Gentaro Usuku[2]

[1] Department of Anesthesiology, Kumamoto University Medical School, Honjo 1-1-1, Kumamoto City 860, Japan

[2] Department of Pathology, Institute of Constitutional Medicine, Kumamoto University

**Summary.** To evaluate the effects of chemically induced diabetes on lung tissue, we examined the ultrastructure of the lung of alloxan-induced diabetic rats. Fifty male Wistar rats were made diabetic by a single intraperitoneal injection of alloxan (200 mg/kg of body weight): they were sacrificed from one to four weeks later. The alloxan-induced diabetes produced significant morphological alterations in the lung. These include marked dilatation of the cisterna of the granular endoplasmic reticulum, dilation of the Golgi saccules and the appearance of glycogen granules as a cluster in the cytoplasm of the granular pneumocytes and the interstitium. These findings were well correlated with the severity of diabetes mellitus. The altered granular pneumocytes were observed in about 50% of animals and in most (87.5%) of the observed pneumocytes 2 weeks and 4 weeks after alloxan treatment respectively. The average number of lamellar inclusion bodies per granular pneumocyte decreased to about half of that of the control in diabetic rats 4 weeks after alloxan treatment, and minimum thickness of the capillary basement membrane was approximately 35% thicker than that of the control (diabetics; $879 \pm 189$ Å, controls; $649 \pm 100$ Å).

The ultrastructural alterations of the lung in diabetic rats indicate disorders in the pulmonary capillaries and in the metabolism of pulmonary surfactant, which may cause pulmonary dysfunction in diabetic patients.

**Key words:** Diabetes mellitus, experimental – Pulmonary alveoli – Pulmonary surfactant – Basement membrane – Endoplasmic reticulum.

### Introduction

It is well known that diabetes mellitus induces metabolic abnormality, not only of carbohydrate, but also of lipids and protein. These abnormalities are associated with generalized angiopathy (Stauffacher et al. 1971). In addition to atheromatous changes in the large vessels, diabetics are prone to develop specific lesions of small vessels (arterioles, capillaries and venules) in such diverse

*Offprint requests to:* Kazuhiro Sugahara, Department of Anesthesiology, Kumamoto University Medical School, Honjo 1-1-1, Kumamoto City 860, Japan

0340-1227/81/0390/0313/$02.40

tissues as the retina, renal glomerulus, muscle and skin (Bloodworth 1963). One well-documented morphological manifestation of small vessel disease is the thickening of the capillary basement membrane associated with diabetic retinopathy and nephropathy (Williamson et al. 1976). Maternal diabetes predisposes newborn infants to respiratory distress syndrome (Robert et al. 1976). Furthermore, we have demonstrated that arterial oxygen tensions and pulmonary carbon monoxide diffusing capacities in diabetic patients are often lower than those in a normal corresponding age group (Sugahara et al. 1978; Sugahara et al. 1979). These previous observations suggest that diabetics may also develop functional and histological disorders in the pulmonary capillaries and in the synthesis of pulmonary surfactant in the lungs.

At this moment, however, little information is available concerning the morphological alterations in the lung in diabetes mellitus. The present experiments were therefore undertaken in an attempt to examine the ultrastructure of the lungs, and to assess the effects of chemically induced diabetes on the lung tissue.

## Materials and Methods

*Experimental Animals.* Fifty male rats of Wistar derived strain, weighing 100 to 200 g, were used. Diabetes was induced by an intraperitoneal injection of a single dose of alloxan monohydrate (200 mg/kg) after a 24 h-fasting period. In each experiment, half of the animals subjected to alloxan treatment were selected at random from groups of uniform age, and the remainder were used as normal controls. The control rats received an equal volume of isotonic saline without alloxan. The animals were fed ad libitum and were kept from one to four weeks before sacrifice.

Development of induced diabetes was confirmed by observing animal behavior and examining the glucose level in the blood taken from the tail vein and in the urine. This was verified by postmortem histological examination of the pancreas.

*Samples.* The rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and the trachea was cannulated after tracheotomy. Through the cannula, 2% glutaraldehyde in 0.2 mol phosphate buffer (pH 7.4) was infused under a constant pressure of 14 cm $H_2O$ to fix the lungs in situ. Portions of the left lobe of each fixed lung were cut into 1 mm cubes. The cubes were immersed in phosphate buffer overnight, and further fixed in 1% osmium tetroxide at 4° C for 2 h. They were dehydrated in a series of ethanol and then infiltrated with propylene oxide. Finally the specimens were embedded in Epon 812. Using glass knives and an LKB Ultrotom III, 1 μm sections were cut for light microscopy and pale gold-thin sections for electron microscopy. The ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a Hitachi HU-12A electron microscope.

*The Number of Lamellar Inclusion Bodies.* More than four specimens of the lung tissue taken from each animal were examined in order to observe ten granular pneumocytes in a mid section plane, having a large nucleus surrounded by well-preserved cytoplasm and lamellar inclusion bodies. The number of lamellar bodies and mitochondria per cell in the control group and the 4 week diabetic group were counted in each granular pneumocyte which had a large nucleus visible in the ultrathin sections. The means were compared using Student *T*-test.

*Measurement of Capillary Basement Membrane Thickness of the Lung.* All tissue samples of the control and 4 week diabetic animals were examined under a Hitachi HU-12A electron microscope, and photographed at an initial magnification of approximately × 8,000. The instrument was calibrated at monthly intervals with a carbon grid and suitable corrections were made when the thickness of the basement membrane was measured for statistical analysis. All electron micrographs were obtained in a magnification ratio of approximately × 8,000 to eliminate possible errors in changing the ratio. In the present observations, capillaries of 40–80 $\mu$ in diameter were examined, since

there is a possibility that the thickness of the basement membrane might vary, depending upon the size of the capillary. The thickness of the basement membrane was measured by a technician who was not informed about the source of tissues. Estimation of capillary basement membrane thickness was carried out according to one of the methods described by Williamson et al. (1969); the capillary basement membrane was measured at two points where the membrane was the thinest, but at least 1 cm apart from each other on micrographs enlarged 3.0 times to a final magnification of approximately $\times 24{,}000$. The means of the basement membrane thickness and standard deviation were derived from the averages of these two measurements taken on ten to fifteen capillaries of each specimen.

## Results

### *1. Effects of Alloxan Injection*

Rats treated with alloxan showed hyperglycaemia and glucosuria from the first day after the alloxan injection. Blood glucose levels in the control and alloxan-treated animals are shown in Fig. 1. Ketonuria started to occur on the second day and persisted thereafter in all animals which showed a blood glucose level above 300 mg/dl. Marked polyuria was quite common; the daily amount of urine often exceeded 10% of the body weight. Most of the alloxan-treated animals looked quite sick, especially after the initial 2 days, but ate well. Body weight usually remained at or near the initial value, while the control rats at the same stage of development gained approximately 30–40 g weekly.

Histological examination of the pancreas in the alloxan-treated animals showed that 24 h after the injection, the majority of the beta cells were greatly shrunken, and in some cases the cell coalesced into an almost homogeneous debris in which individual cells could not be recognized. The number of islets was counted in ten fields under a light microscope at a magnification of $\times 40$. From the first week on, the number of the islets decreased to about half of the control (diabetics; $4.00 \pm 1.26$, control; $10.57 \pm 1.27$, $P < 0.001$). The islets showed a practically normal appearance with the routine haematoxylin-eosin stain. However, special stains (Aldehyde-Fuchsin) revealed that they consisted of agranular and alpha cells only; beta cells were completely absent. The alpha cells and the surrounding pancreatic tissue remained intact without showing any evidence of damage.

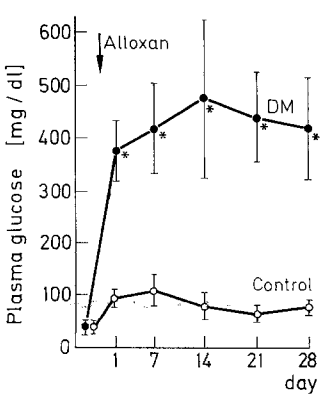


**Fig. 1.** Plasma glucose values after alloxan injection in rats. $*P < 0.001$ compared with the control. Alloxan treated animals (*DM*) showed significant increase in plasma glucose levels, compared with the control by the first day; these levels were maintained during the 4 weeks

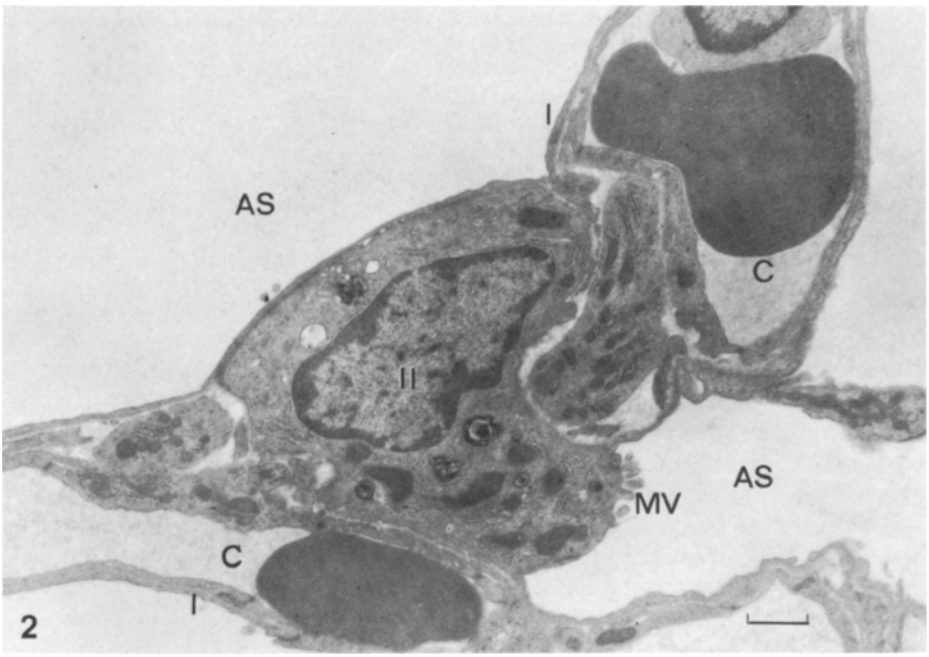


**Fig. 2.** Electron micrograph of the lung taken from an animal of the control group. *I*, membranous pneumocyte, *II*, granular pneumocyte, *AS*, alveolar space, *C*, capillary, *MV*, microvilli. ×10,000; calibration;1 μm

## 2. *Morphological Observations*

Light microscopic examination of the lung showed no particular differences between the pulmonary structures of the control and alloxan-treated animals; alveolar spaces were well inflated, pulmonary capillaries were dilated and there was no evidence of congestion of the pulmonary vasculature. However, electron microscopic examination revealed lesions which were widespread in the lungs of the diabetic rats.

*Control Rats.* The lungs of the control group showed a normal lung structure which has type I alveolar cells (membranous pneumocytes), type II alveolar cells (granular pneumocytes), capillary endothelial cells and interstitial cells (Kuhn III 1977; Fig. 2). The membranous pneumocytes were simple squamous cells which formed a thin layer over most of the alveolar walls. Usually the nucleus was centrally placed in the cell and surrounded by a thin cytoplasm in which ribosomes, numerous pinocytic vesicles, a few microtubules and a few mitochondria were found. The alveolar surface of the cell was smooth. The granular pneumocytes were large and cuboidal with a single central nucleus. Microvilli were found on the exposed surface of the cell. The cytoplasm of the granular pneumocytes contained more organelles than that of the membranous pneumocytes, and moderate numbers of elongated mitochondria, narrow

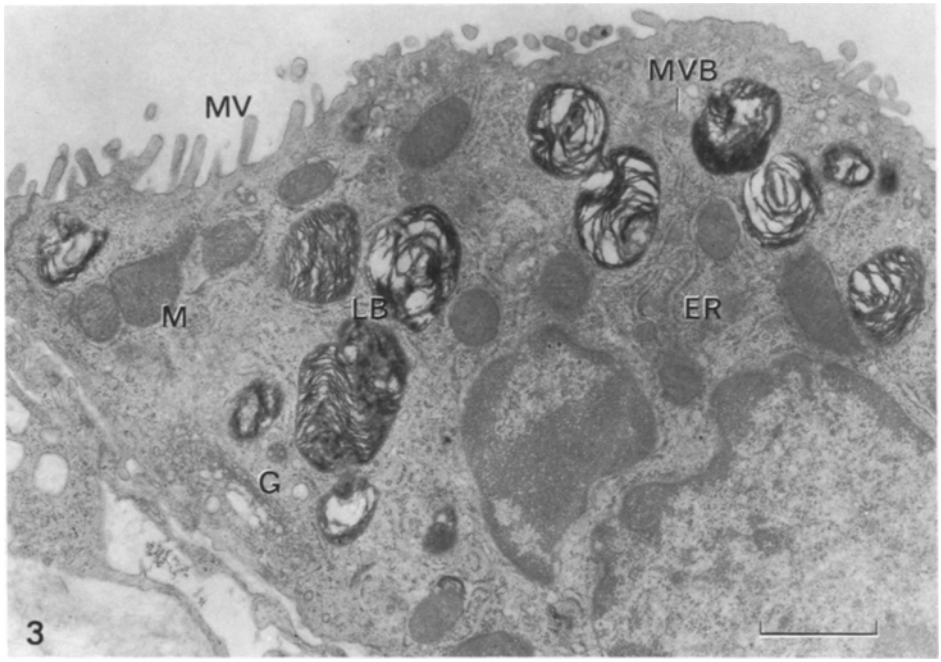


**Fig. 3.** General appearance of a granular pneumocyte taken from an animal of the control group. The cytoplasm is abundant with mitochondria (*M*), narrow profiles of granular endoplasmic reticulum (*ER*), lamellar inclusion bodies (*LB*) of varying sizes, multivesicular bodies (*MVB*), Golgi apparatus (*G*) and microvilli (*MV*) on the cell surface facing the alveolar space. ×20,000; calibration = 1 μm

strands of endoplasmic reticulum, free ribosomes, a small Golgi apparatus and multivesicular bodies. The granular pneumocytes were characterized by moderate numbers of osmiophilic lamellated bodies (Fig. 3).

*Diabetic Rats.* At 3, 6, 9 and 12 h after the alloxan injection, the lungs showed no significant changes compared to the control group.

One week after the alloxan injection, slight alterations of the granular pneumocytes were observed in the diabetic rats; the endoplasmic reticulum and Golgi apparatus were slightly dilated (Fig. 4). There were no significant alterations observed in other cells of the lungs.

Two weeks after alloxan treatment, the dilation of the endoplasmic reticulum of the granular pneumocytes became more marked (Fig. 5). The degree of the dilation varied considerably, depending upon individual cells and endoplasmic reticulum. Some of them showed localized dilatation, while others were massively expanded. The inside of the dilated endoplasmic reticulum appeared more electron-lucent than the surrounding cytoplasm. The Golgi apparatus was also more dilated than those of the rats examined one week after the alloxan injection. These changes were observed in about 50% ($49.1 \pm 5.3\%$) of the granular pneumocytes (264 cells examined in 6 diabetic rats). The membranous pneumocytes, the interstitial cells and the capillary endothelium appeared almost normal.


**Fig. 4.** Electron micrograph of a granular pneumocyte taken from an animal one week after alloxan-treatment. ×20,000; calibration = 1 μm

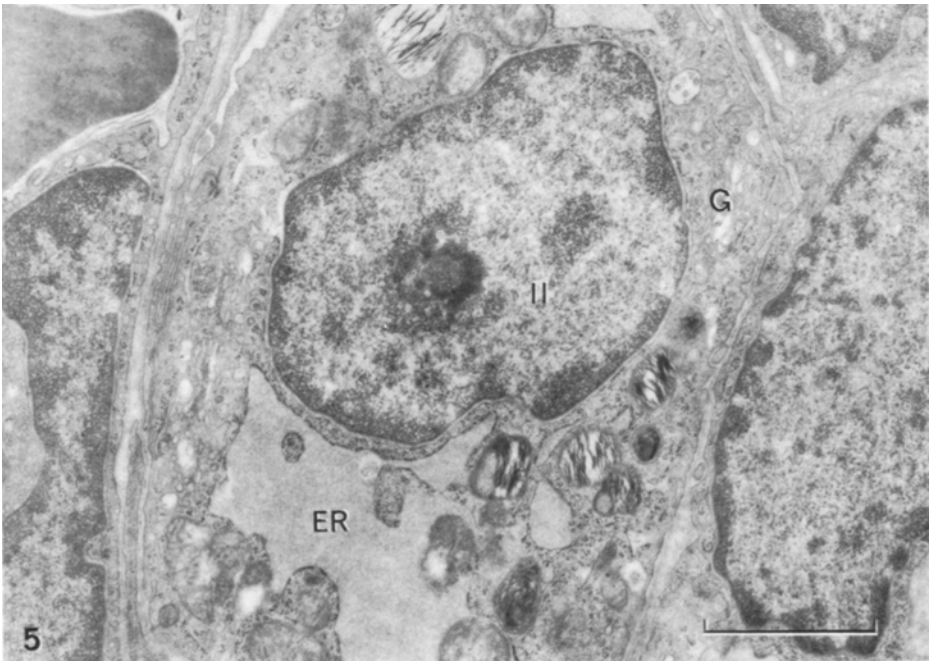


**Fig. 5.** Electron micrograph of a granular pneumocyte taken from an animal two weeks after alloxan-treatment. ×30,000; calibration = 1 μm

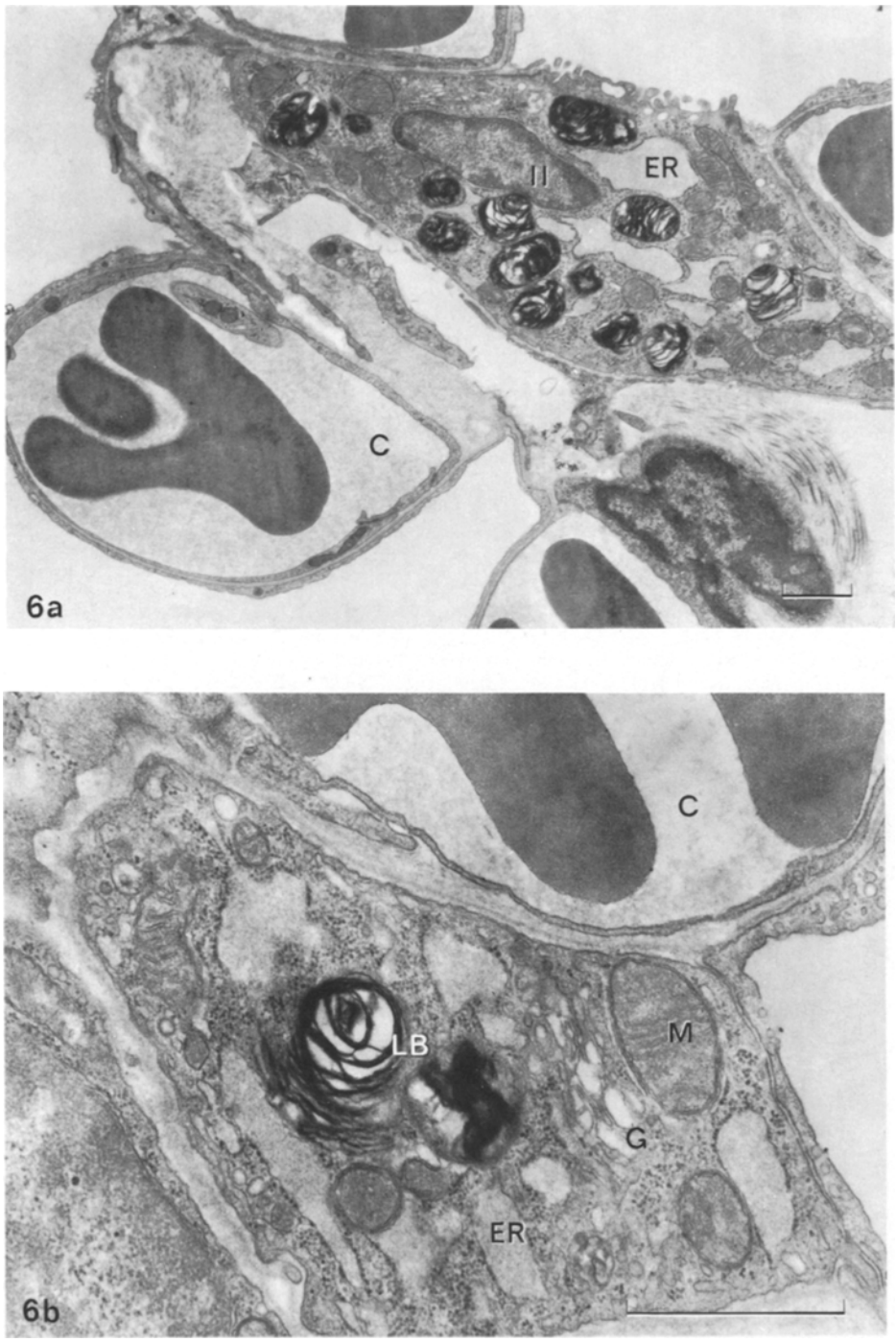


**Fig. 6. a** Electron micrograph of a lung taken from an animal 4 weeks after alloxan-treatment. ×13,000; calibration = 1 μm. **b** Electron micrograph of a lung taken from an animal 4 weeks after alloxan-treatment. ×40,000; calibration = 1 μm

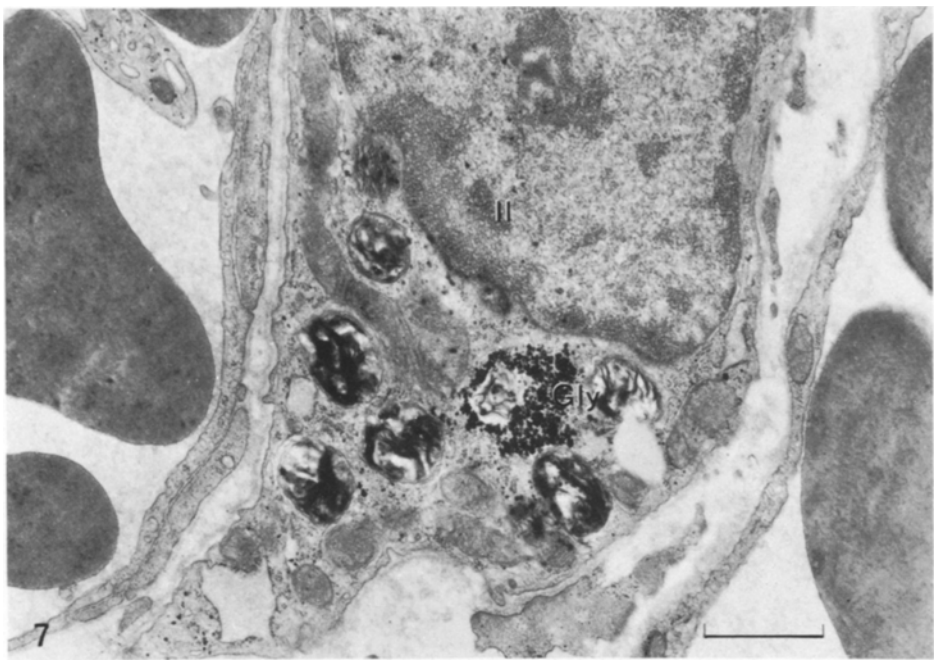


**Fig. 7.** The glycogen granules appeared as a cluster in the cytoplasm of a granular pneumocyte taken from an animal 4 weeks after alloxan-treatment. ×21,000; calibration = 1 μm

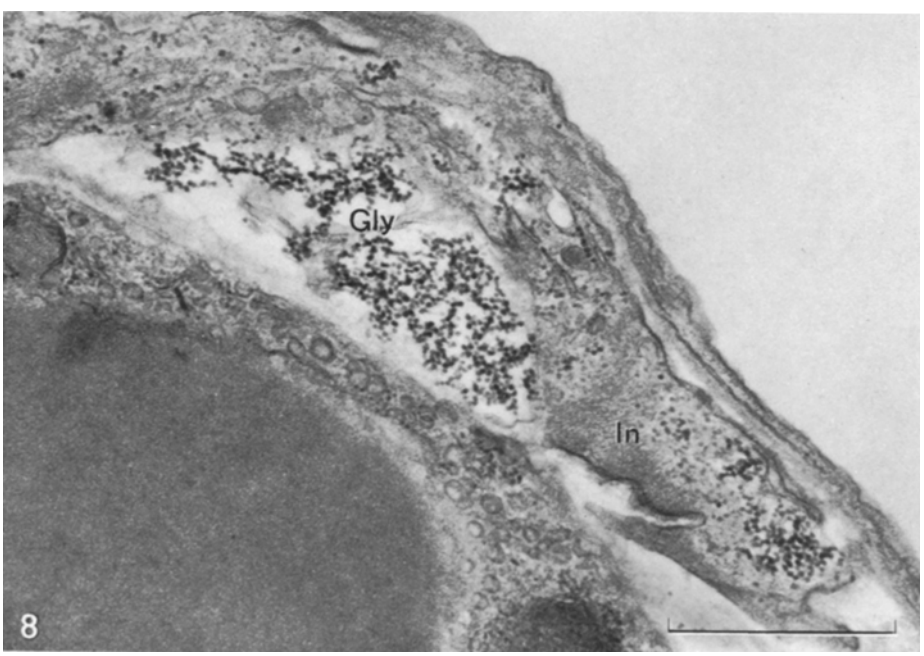


**Fig. 8.** The glycogen granules in the interstitium (*In*) taken from an animal 4 weeks after alloxan-treatment. ×39,000; calibration = 1 μm

Four weeks after alloxan, the dilated endoplasmic reticulum of the granular pneumocytes increased in number (Fig. 6a, b). The Golgi apparatus was also dilated. The altered granular pneumocytes were observed in most of the diabetic rats (87.5±4.0% in 238 cells examined in 6 rats). Dense small granules of 100–200 Å in diameter frequently appeared and occasionally aggregated as a cluster in the cytoplasm of the granular pneumocytes (Fig. 7). They were also found in the interstitium (Fig. 8). These granules were confirmed as glycogen granules by an α-amylase digestion test. The degree of aggregation of the glycogen granules varied. The capillary basement membrane of the lungs was slightly thickened (Fig. 6b). The other components of the lungs showed no significant changes.

### *3. The Number of the Lamellar Inclusion Bodies per Granular Pneumocyte*

The number of the lamellar bodies and mitochondria per granular pneumocyte was counted 4 weeks after the alloxan injection when morphological changes were most pronounced in the granular pneumocytes, and then compared with the controls. Differences of the number of mitochondria per cell were not statistically significant, but the number of the lamellar bodies per cell in the diabetic rats reduced to about half of the control (Table 1). However, the lamellar bodies of the diabetic rats showed practically no difference in shape and size from those of the control rats.

### *4. Thickness of the Capillary Basement Membrane of the Lungs*

Preliminary studies were undertaken to determine the validity and reproducibility of the technique for measuring basement membrane thickness. Different persons performed independent measurements of the basement membrane thickness on 10 capillaries from 5 randomly selected control or diabetic rats. *T*-tests revealed no significant differences between the first and second sets of the measurements ($P > 0.5$, 0.5, 0.4, 0.5 and 0.4 respectively). Therefore, the method employed to measure the basement membrane thickness was considered to be reliable. Typical examples of capillaries of the control and diabetic rats are shown in Fig. 9. The density of the diabetic basement membrane did not differ significantly from that of the control, nor could any difference in the internal structure of the membrane be noticed. Results of measurements of basement membrane thickness are summarized in Table 2. The minimum thickness of the capillary

**Table 1.** Lamellar inclusion bodies and mitochondria per granular pneumocyte in lung tissue of alloxan-diabetic rats

| | No. of animals | Lamellar bodies/cell | Mitochondria/cell | No. of cells observed |
|---|---|---|---|---|
| Control | 4 | 9.83±4.69 | 10.75±4.53 | 56 |
| Diabetics (4 week) | 4 | 5.33±3.62* | 11.31±5.87 | 51 |

Values are mean±standard deviation. *$P < 0.05$ compared with control

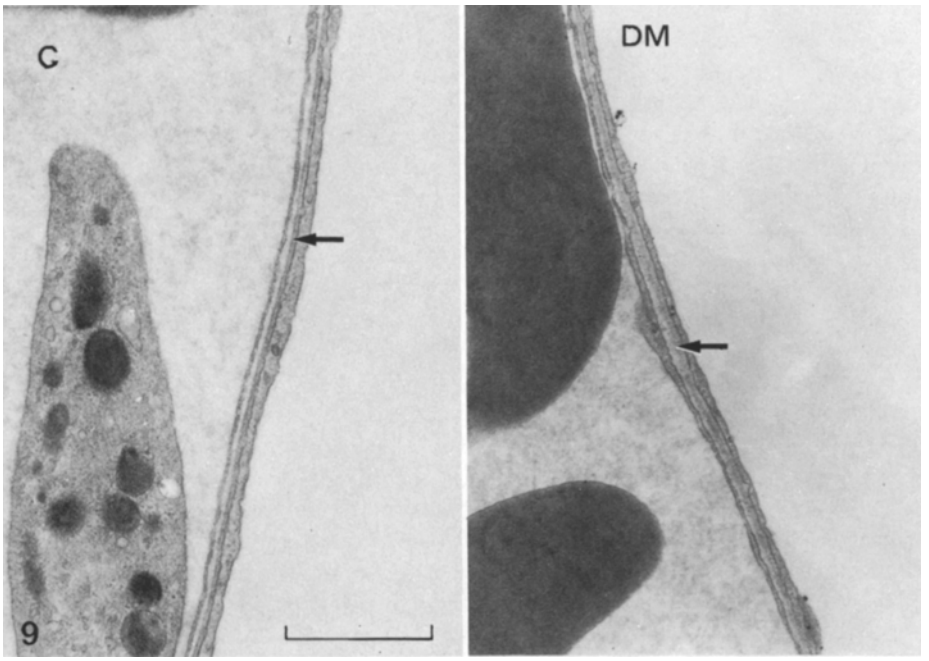


**Fig. 9.** Electron micrograph of a pulmonary capillary taken from both control (*C*) and diabetic (*DM*) animals. The basement membranes (*arrow*) of the control is narrow and thin, but that of the diabetic is thicker. ×24,000 Both magnifications are the same. Calibration = 1 μm

**Table 2.** Comparison of estimates of pulmonary capillary basement membrane thickness in control and diabetic rats

| | No. of measurements | Minimum |
|---|---|---|
| Controls | 89 | 649 ± 100 Å |
| Diabetics | 103 | 879 ± 189 Å |
| | | $P < 0.001$ |

basement membrane in the diabetics (879 ± 189 Å) was approximately 35% thicker than that of the control (649 ± 100 Å).

## Discussion

The present experiments have demonstrated that the alloxan induced diabetes mellitus produces significant morphological alterations in the lung of the rat. They include marked dilatation of the cisterna of the granular endoplasmic reticulum, dilation of the Golgi saccules and the appearance of glycogen granules as a cluster in the cytoplasm of the granular pneumocytes and the interstitium. These findings were well correlated with the severity of diabetes mellitus. There is the possibility that these morphological alterations observed in the lung after the alloxan treatment might be due to the direct action of alloxan per se, since it has been reported that a large dose of alloxan (by an intravenous injection) produced pulmonary oedema (Houssay 1947; Aufdermaur 1948) and

changes in both the capillary endothelium and the alveolar epithelium of the lungs (Cottrell et al. 1967). However, the morphological changes observed in the present experiments were clearly different from those obtained by previous investigators. Therefore, the alterations in the lung structures of the rats treated with alloxan are most likely due primarily to diabetes mellitus induced by alloxan. Furthermore, the dilated endoplasmic reticulum and Golgi saccules were also observed by Plopper et al. (1978) in the fine structure of the granular pneumocytes in streptozotocin-diabetic rats, and they attributed those findings to a depression of the pulmonary metabolic functions associated with diabetes mellitus.

In diabetes mellitus, glycogen deposits were observed in the epithelial cells of the distal segment of the proximal convoluted renal tubules, the descending loop of Henle, hepatocytes, beta cells of pancreatic islets and cardiac muscle (Scotti 1977). In the present study, it was shown that the intracellular accumulation of glycogen also occured in the granular pneumocytes and the interstitium of the lungs in diabetic rats. Abnormal accumulation of glycogen is found in glycogen storage diseases, and is attributable to a change in the intracellular metabolism of glucose, which shifts the metabolism of glucose to the deposition of glycogen (Scarpelli et al. 1977). The metabolic fate and the role of glucose as a metabolic substrate in the lungs have not been adequately defined. Nevertheless, most interest has been focused on the role of glucose with respect to lipogenesis and surfactant metabolism (Tierney et al. 1977). Diabetic rat lungs show depressed glucose oxidation (Morishige et al. 1977) and a reduced rate of glucose incorporation into neutral lipids and phospholipids (Moxley et al. 1975). These observations suggest that the disorder of glucose metabolism in diabetes mellitus may lead to a disturbance of the synthesis of the pulmonary surfactant in the lungs. In this connection, the decrease in the number of lamellar inclusion bodies per granular pneumocyte in the diabetic rats observed in the present experiments is of particular importance, since the granular pneumocyte is the source of pulmonary surfactant (Kikkawa et al. 1975), and the lamellar inclusion bodies are the storage organelles (Page-Roberts et al. 1972; Gil et al. 1973). Therefore, the present finding may also indicate that the synthesis of the pulmonary surfactant in diabetes mellitus is decreased, since the number of these bodies was diminished as the surfactant decreased (Klaus 1962; Schaefer et al. 1964).

In recent years, numerous qualitative and quantitative electron microscopic studies have shown that, in general, capillary basement membranes are thicker in diabetics than in non-diabetics (see Kilo et al. 1972). Furthermore, these studies have demonstrated that the capillary bed of most, if not all tissues, are affected to a varying degree. To our knowledge, however, there are very few reports on the capillary basement membranes of the lung in diabetics. In the present study, we have shown that capillary basement membranes are thickened in the lungs, as well as in other tissues. Basement membrane is known to consist essentially of glycoprotein and an increased accumulation of basement membrane substance has been reported in the kidney of diabetic patients (Spiro 1971). On the basis of our present findings, it is possible to speculate that the abnormality in polysaccharide metabolism may also be responsible for the pulmonary lesion, as well as other lesions in diabetes mellitus.

The present findings indicate disorders in pulmonary capillaries and in the metabolism of pulmonary surfactant in diabetic rats, which may cause pulmonary dysfunction in diabetic patients.

*Acknowledgments.* We thank Prof. W. Doerr, Direktor des Pathologischen Institutes der Universität Heidelberg and Prof. K. Nishi, Dept. of Pharmacology, Kumamoto University Medical School for their kind and helpful advices in the preparation of this manuscript.

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Accepted October 20, 1980