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FREEZE-FRACTURE STUDY OF THE RAT LUNG

III. Ripple marks on the lamellae—a possible artefactual change of lamellar inclusion bodies in freshly processed lung tissue.

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Abstract

The lamellar inclusion body of the rat alveolar type II cell was studied using freeze-fracture replication technique without chemical fixation or glycerinization. Ripple marks with 18-20 nm spacing were observed in occasion. Such marks were considered to be a product of artefact, resulting from rearrangement of phospholipid molecules of the lamellae during rapid freezing.

INTRODUCTION

There is no established standard procedure in processing tissues for freeze-etching or freeze-fracture replication. Different preparatory methods may yield a different cleaving behavior and/or fracture face appearance.

Most commonly used method would be that with glutaraldehyde fixation and glycerinization[1]. Therefore, we used structural features obtained with this preparatory method as a standard of reference, and compared them with others[2,5]. Among other methods, processing without chemical fixation, but with 30 % glycerol at 38°C provided one unusual feature, which has been reported in the provious communication[3]. Here, we like to describe other changes present only in the specimen processed without chemical fixation or glycerinization.

MATERIALS AND METHODS

Wister strain rats (300 gm.) were anesthetized by intraperitoneal injection of Nembutal. The trachea was exposed and was tied off. The thorax was opened and the lung was removed *en bloc*. Lungs were gently and carefully diced into about one-mm^3 tissue blocks with two sharp razor blades sliding

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against each other. Then, tissue blocks were rapidly frozen in liquid Freon 22 and were stored in liquid nitrogen. Nothing was used to adhere the tissue to the specimen carrier. Neither chemical fixation nor glycerinization was used. Tissues were fractured in a Balzers' freeze-etching device (BAF-301) at -110°C (10^{-6} Torr). Using an electron-beam gun, platinum-carbon was casted at a 45° angle, followed by carbon coating. Mostly, fracture was performed while platinum-carbon was kept on casting, but some of the specimen were shadowed immediately after the fracture. Replicas were cleaned with sodium hypochlorite, rinsed in distilled water, and mounted on 300 mesh uncoated copper grids. They were examined in a Hitachi H-500 electron microscope.

RESULTS

Except for the small area of the lung tissue edge, alveoli appeared well-expanded. The general appearance of the rat lung, especially of alveolar type II cells in freshly processed lung tissue without chemical fixation or glycerinization was almost identical to that seen in the glutaraldehyde-fixed and glycerinized lung tissue except for two points. The alveolar lining layer was well-preserved only with this preparatory method, and it was never observed in glutaraldhyde-fixed specimens. Partial preservation could have been obtained in the specimens immersed in glycerol for a short period (unpublished observation). The appearance and formation of the surface film in the alveolar lining layer is described elsewhere[4]. Another finding distinct from those revealed with other procedures, is the one we are describing in this communication. The lamellar surface of occasional lamellar inclusion bodies showed ridges of low height, running parallel with a periodicity of 18-20 nm. Some of them branched away to show "arrow-wing"-like image. This structure is referred to as "ripple marks" on the lamellae, here. Ripple marks were not associated with so-called membrane associated particles (15 nm) in any forms, although they were somehow granular and in close apposition to the granules of lamella itself (5-8 nm). They were seen only on the lamellar face, but strangely enough cross-edges of the same lamellae were straight without ridge formation corresponding to ripple marks. Not all lamellar bodies showed such ripple marks, and the incidence of their occurrence was rather low (one out of fifty lamellar bodies). There was a slight tendency for them to appear near the area where ice-crystals were formed in large numbers.

DISCUSSION

Currently, we are under investigation of the effect of freeze-fracture preparatory methods on the appearance of lamellar inclusion bodies. As have

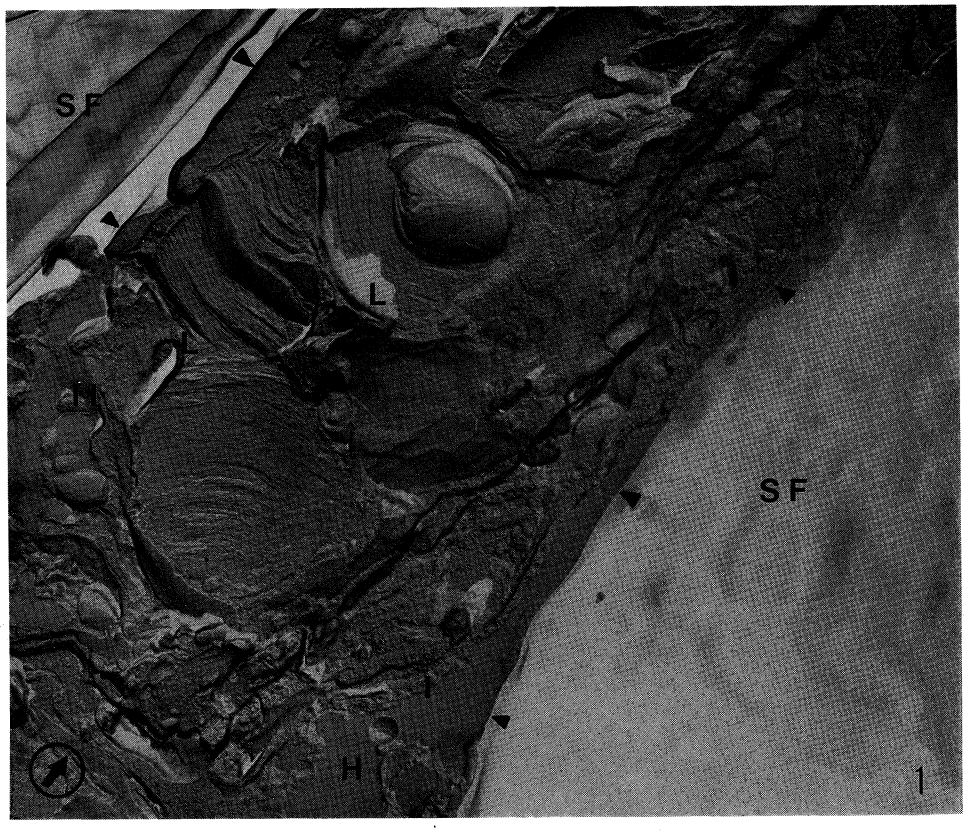

Fig. 1. Type II alveolar epithelial cell (II) on the alveolar wall. The surface film (SF) is well-preserved only in this procedure. Arrowheads indicate the edge of the surface film.
H: Hypophase I: Type I cell L: Lamellar body
The encircled arrow of this and succeeding photographs indicates the direction of shadowing.
Magnification. ×21,000

been described[2,3], seven procedures were compared. Technically, tissue processing without chemical fixation or glycerinization troubled us in several points. First of all, ice-crystals were readily formed. A rapid and careful freezing with liquid Freon 22 and then liquid nitrogen was essential. Small ice-crystals were frequently seen particularly when there was a brief pause between fracture and replication. By performing fracture and replication almost simultaneously, this could be eliminated to some extent. Being strictly a freeze-fracture replication, simultaneous processing was critical. This eliminates a chance of water sublimation. After final trimming, a knife was put over the tissue and

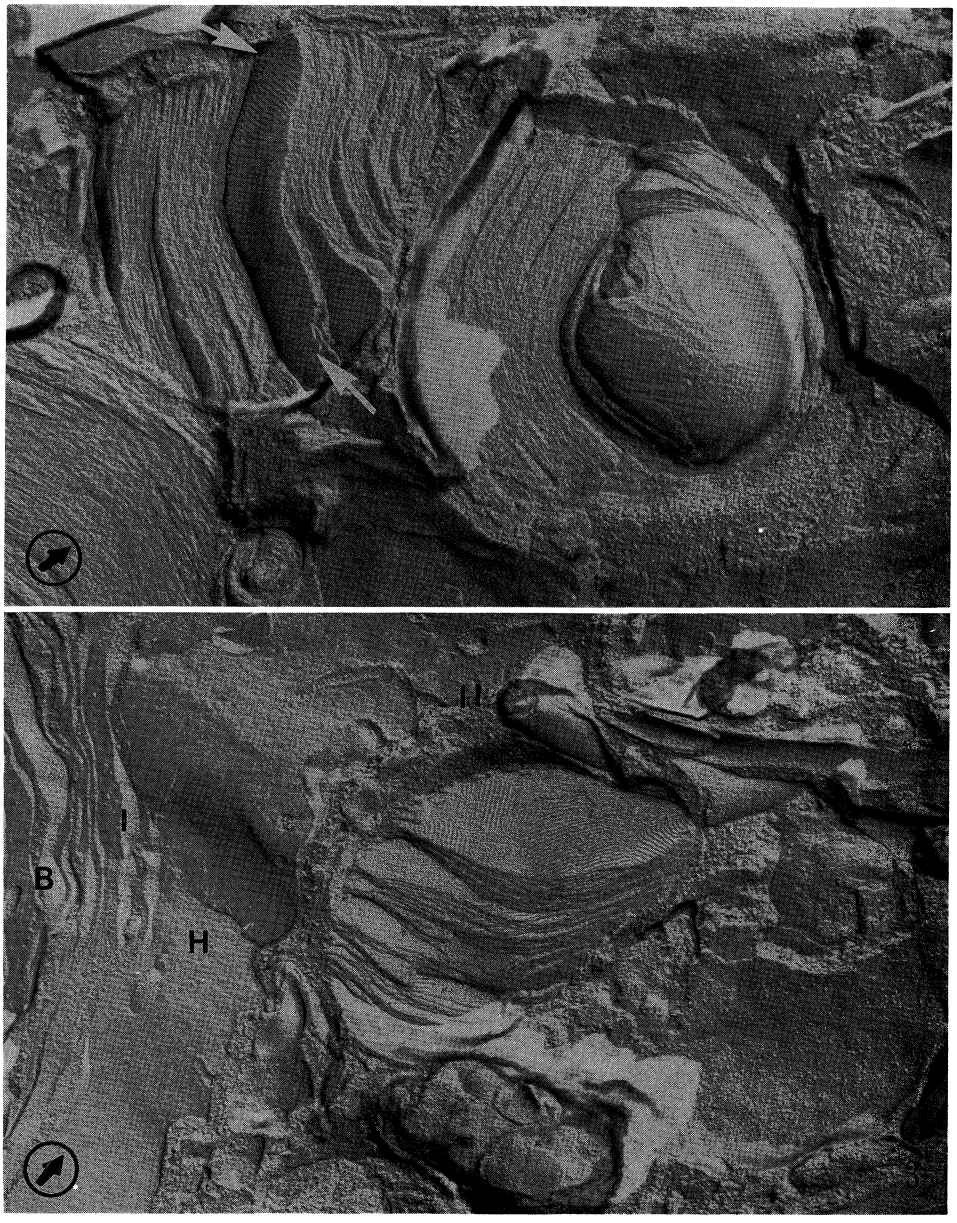

Fig. 2. As seen at higher magnification, it is evident that the lamellar face has linear sculpturing described as ripple marks with 18-20 nm periodicity (white arrows).
Magnification. ×46,000

Fig. 3. Ripple marks. Linear ridges of ripple marks are somehow granular and in close apposition to the granules of the lamella. Also note that the limiting membrane is studded with membrane-associated particles.
H: Hypophase I: Type I alveolar epithelial cell
II: Type II alveolar epithelial cell B: Blood vessel
Magnification. ×35,000

then lowered properly. Platinum-carbon was started to cast at this moment. Then, a final cut was done with platinum-carbon kept on shadowing. With this procedure, ice-crystal formation was reduced. About 50 % of recovered replicas were entirely free from ice-crystals. The rest of them contained mostly large ice-crystals which could not be eliminated by the above-mentioned procedure. These were considered to have been produced during the process of freezing.

Secondly, the digestion of freshly processed tissue with sodium hypochlorite was frequently prolonged or impossible. The solution of sodium hypochlorite kept in air-free environment is recommended to be used. Thirdly, replicas were frequently torn apart when the tissue was dissolved in sodium hypochlorite solution. This was probably because some alveolar walls facing large air-filled spaces were neither shadowed nor coated with platinum-carbon and carbon respectively so that the continuous replica film could not be formed. Lastly, the preservation of the dome-like structure of alveoli was difficult. In order to preserve this structure intact, the supporting film such as formvar was not used and grids with large number were used. In addition, electron beam-gun did not always work perfectly. After all, about 70 % of the trials provided usable replicas for the observation of the lung ultrastructure, either partially or completely, and replicas of probably 35 % of the attempted were perfect, while replieas from glutaraldehyde-fixed and glycerinized specimen were excellent in 80 %.

General appearance of the alveolar type II cell and lamellar inclusion bodies is similar to that seen in the glutaraldehyde-fixed and glycerinized lung tissue. The alveolar lining layer, that is composed of surface film and hypophase, however, could be clearly demonstrated only in the specimen processed in this way. In addition, occasional lamellar faces had linear sculpturings (ripple marks) with 18-20 nm spacing. Such ripple marks were never seen in the lungs processed other way, and since many replicas were taken and compared, this structure is considered distinctive in this procedure even if its infrequent occurrence is taken into consideration. Smith *et al.*[5,6] demonstrated regular linear sculpturing with about 45 nm spacing on some lamellae of the inclusion of type II cells of adult rat lung. In contrast to ours, they proces-

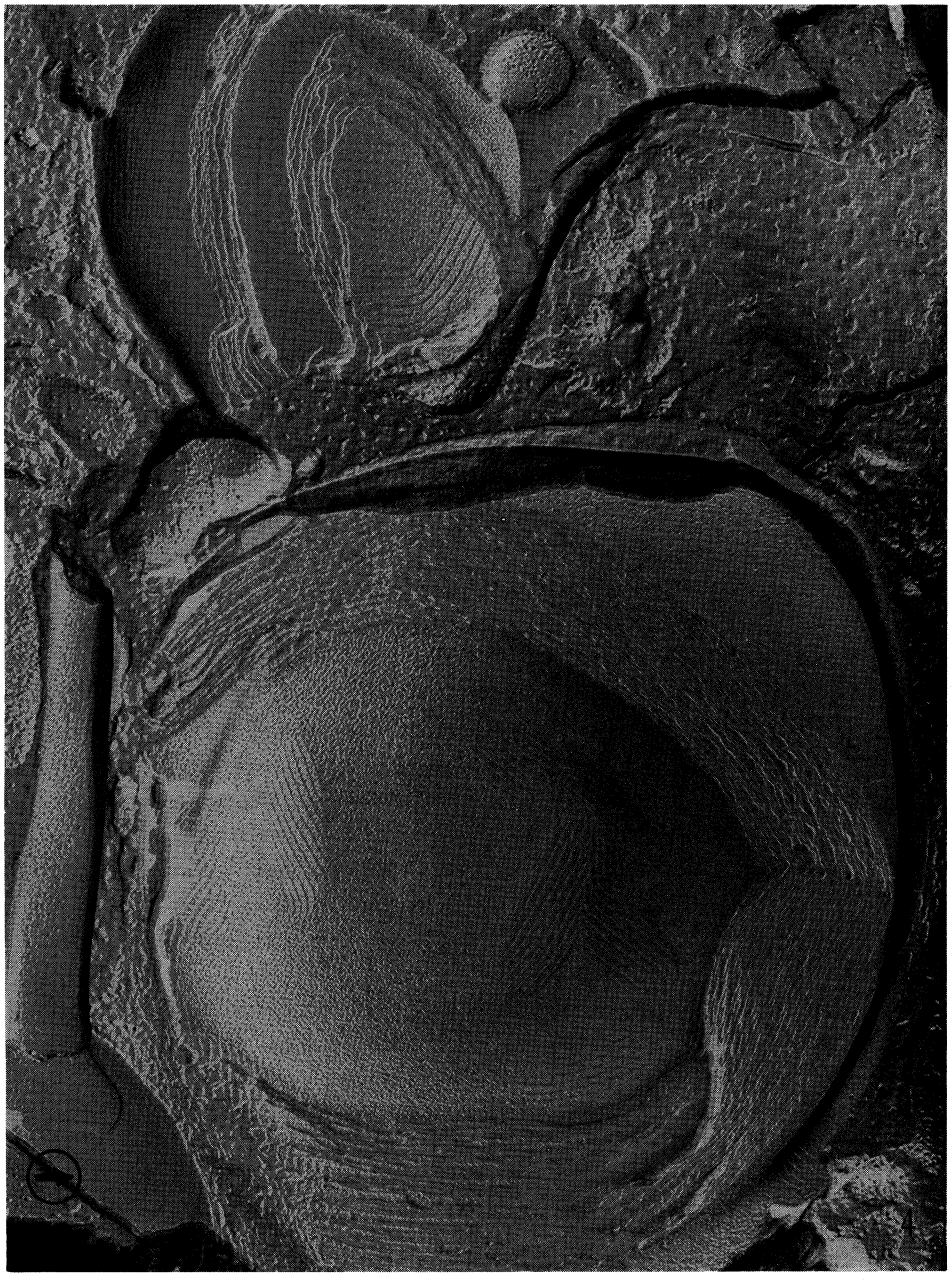

Fig. 4. This electron micrograph clearly shows that linear ridges curve and branch to form arrow-wing image. In this sample, replication was performed after fracturing.
Magnification. ×69,000

sed tissues with glutaraldehyde fixation and glycerinization before freezing. On structural basis, ripple marks described here differ from theirs; the periodicity is narrow and interposing rows of 10 nm particles like described by Smith *et al.* are lacking.

It remains to be elucidated whether ripple marks on the lamellae were a product of artefact or a real structure demonstrable only by this prodecure. Its infrequent occurrence, preponderance of its appearance near ice-crystal-formed areas, and a lack of such a structure in specimens processed with other methods, however, suggest its possible artefactual occurrence. In this respect, further investigation should be continued.

Structurally, on the other hand, it seems to be a disarrangement of phospholipid molecules forming lamellae, since linear ridges were somehow granular and were in close apposition to the granules on the lamellae nearby. The preparatory method without chemical fixation or glycerinization for freeze-etching or freeze-fracture replication study is generally believed to provide more true-to-life features of organells, cells or tissues. It is indeed so, to greater extent, when the conventionally processed thin section materials are compared.

The presence of ripple marks on the lamellae in the freshly processed tissue, however, indicates that it is not entirely artefact-free, and that the artefactual change of the molecular rearrangement may be induced even in freshly processed freeze-fracture specimens.

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