ELECTRON-MICROSCOPIC DETECTION OF LUNG SURFACTANT PROTEINS WITH PROCION BRILLIANT BLUE H5GS

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Mammalian lung surfactant *in situ* is a lipid—protein complex. The presence of albumin, IgG-γ-globulin, and of two specific apoproteins with molecular weights of 38,000 and 32,000 daltons in the composition of its surface-active function has been conclusively demonstrated by immunochemical methods [5, 6]. It has been shown autoradiographically [3] that ^{3}H-leucine is incorporated into the secretory granules (osmiophilic lamellar bodies) of the type II alveolocytes — cells responsible for synthesis of the surfactant material. Meanwhile structural histochemical protein analysis of the alveolar extracellular lining (the surfactant complex) has so far been undertaken only by one or two workers in rat and rabbit lungs [2, 8]. This is partly due to the absence of a sufficiently simple histochemical method capable of revealing these substances under the transmission electron microscope. The only electron-immunohistochemical method which has been used for this purpose involves the combination of laborious and time-consuming operations [8], the need for which is due to the necessity of obtaining specific antibodies against the surfactant proteins and their subsequent processing.

The writers recently developed a sufficiently simple method by means of which protein structures located on the plasmalemma of plant and animal cells can be selectively stained with the copper-containing phthalocyanin dye Procion Brilliant Blue H5GS [1]. In the investigation described below an attempt was made to use this dye for the electron-microscopic detection of proteins in the alveolar extracellular lining in order to study their localization and structural-histochemical features in rats and guinea pigs.

EXPERIMENTAL METHOD

Noninbred rats weighing 250 g and guinea pigs weighing 330-380 g were used. The animals were anesthetized with hexobarbital, the chest opened, and the lungs were washed to remove blood by perfusion with physiological saline through the pulmonary artery for 2-3 min under a pressure of 30 cm water. The supply of physiological saline was then stopped and a dye-fixing mixture containing equal volumes of 3.6% glutaraldehyde, 0.2 M cacodylate buffer, pH 5.6-6.0, and 0.3% aqueous solution of procion Brilliant Blue H5GS (from ICI, West Germany) was passed through the pulmonary artery for 8-10 min under the same conditions. Segments of parenchymatous tissue of the lung measuring 5-6 cm^3 were cut out and placed in the same mixture, initially for 1 h and, after cutting up into smaller pieces each with a volume of 1-2 cm^3, for a further 30 min. The material was washed for 10 min with 0.1 M cacodylate buffer, pH 7.3-7.4, and placed for 30 min in a saturated aqueous solution of thiosemicarbazide, used as ligand — the connecting link between the metal of the dye and osmium [4]. After washing for 15 min in buffer the pieces were postfixed in 1% OsO_4 in 0.1 M cacodylate buffer, pH 7.3-7.4. All stages of treatment of the material were carried out at room temperature. The material was quickly dehydrated in acetones, soaked for 1.5 h in a mixture of propylene oxide + Epon-Araldite (1:1), and embedded in the pure resin by the usual method. The total time required to prepare the material for investigation was seven to eight h. Ultrathin sections were examined in the IEM-100B electron microscope without additional staining.

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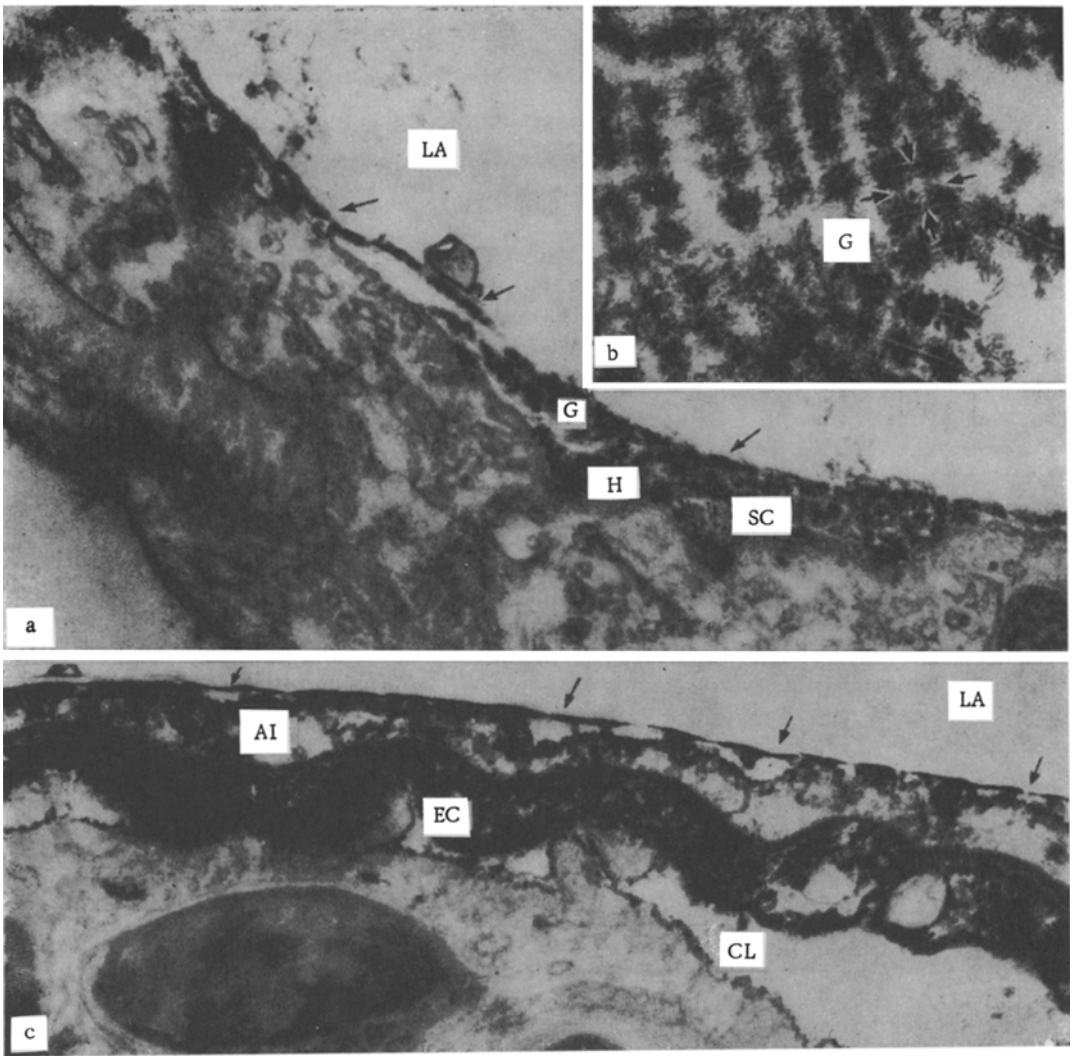

Fig. 1. Localization of reaction product in structural components of rat and guinea pig surfactant complex: a) in outer membrane (arrow), membrane of "grids" and matrix of hypophase in rat. Magnification 57,000 ×; b) fragment of "grid" of electron-dense material in corners and center (arrow) of its square compartments. Magnification 115,000 ×; c) in outer membrane (arrow) and matrix of hypophase of guinea pig. Magnification 49,000 ×. LA) Lumen of alveolus; CL) capillary lumen; SC) surfactant complex; G) "grids"; H) hypophase; AI) type I alveolocyte; EC) endothelial cell.

EXPERIMENTAL RESULTS

The product of the reaction with Procion Brilliant Blue H5GS was revealed in the alveoli of the rats and guinea pigs in the form of a fine globular material with high electron-optical density. It was located in all structural components of the surfactant complex (Fig. 1a): in the outer film-membrane lying directly on the air—liquid phase boundary, in the compactly arranged membranous structures of the surfactant ("grids"), lying in the hypophase, in the substance of the hypophase proper, filling the hollows in the epithelial lining of the alveolus. The dye bound directly with the proteins composing the membranes of the surfactant, as shown by the appearance of granules in them and the increase in electron-optical density. The reaction product in the "grids" of the surfactant also was located on the surface of the membranes, forming concentrations of osmiophilic material in the corners of their square compartments (Fig. 1b). The central part of each square was filled with material against the background of which the osmiophilic globules 15-20 mm in diameter stood out; they were symmetrically arranged, one opposite each corner.

No significant differences were found in the structure and character of distribution of the reaction product in the alveolar extracellular lining of rats and guinea pigs. Since the surfactant "grids" were found much less frequently in the alveoli of guinea pigs than

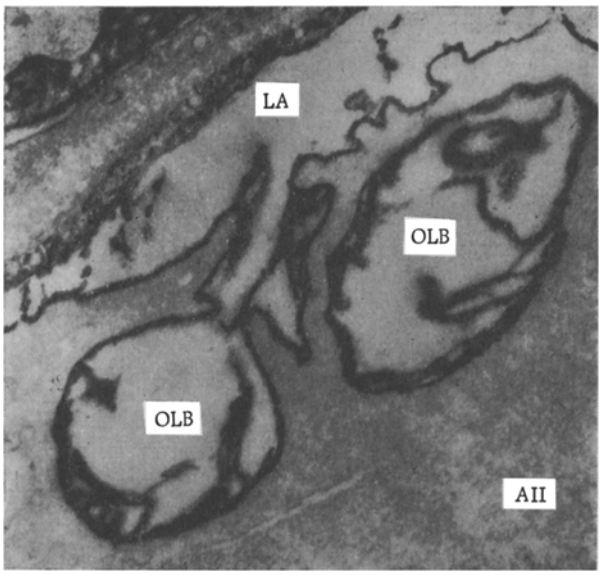

Fig. 2. Finely granular reaction product on membranes and inner surface of smiophilic lamellar bodies of type II alveolocyte at time of liberation of their contents into lumen of alveolus. OLB) Osmiophilic lamellar body; AII) type II alveolocyte. Magnification 45,000 ×.

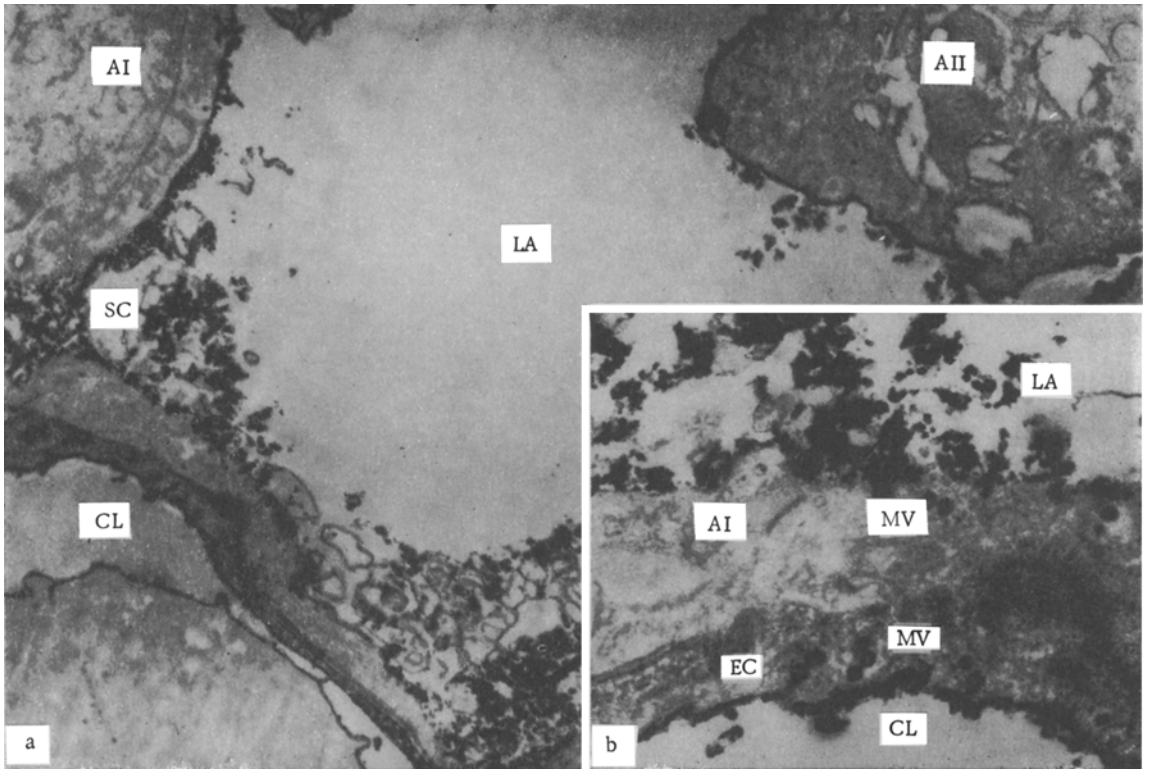

Fig. 3. Localization of reaction product in alveolar wall of guinea pig: a) in composition of structures of surfactant complex preserved in niches between alveolocytes, and in the form of an electron-dense layer on surface of types I and II alveolocytes and capillary endothelium. Magnification 15,000 ×; b) fragment of air–blood barrier, reaction product in composition of micropinocytotic vesicles of alveolar and endothelial cells. Magnification 52,800 ×. MV) Micropinocytotic vesicles.

of rats, the electron-dense material in these animals was found principally in the composition of the outer membrane and in the matrix of the hypophase of the surfactant complex (Fig. 1c).

A similar distribution of specific proteins in the structure of the surfactant complex of rabbits was discovered under the electron microscope by means of an immunohistochemical method [8]. According to the authors cited, these proteins also were located on membranes of the osmiophilic lamellar bodies of type II alveolocytes. In the present investigation the Procion Brilliant Blue H5GS did not penetrate into the cytoplasm of intact cells. However, at the very moment of secretion of the contents of the osmiophilic lamellar body into the alveolar lumen, the finely granular reaction product could be seen on the lamellae and on the inner surface of this structure (Fig. 2). These observations are in agreement with results obtained by other workers [2, 8], who found that type II alveolocytes can produce not only the lipid, but also the protein component of the lung surfactant.

The reaction product was located not only in structures of the surfactant complex, but also directly on the surface of types I and II alveolocytes in the form of globules 10-30 nm in diameter, forming an electron-optically dense layer of uneven thickness (Fig. 3a). Particles similar to them, 15 nm in diameter, have recently been found on freeze-etchings of the plasmalemma of alveolocytes of rapidly frozen rat lung tissue [7], and are regarded by the author cited as protein in nature. In the present investigation a similar finely globular material could be seen not only on the alveolar, but also on the endothelial surface of the air–blood barrier (Fig. 3a). This is evidence of the close connection between the globules thus revealed and the cell membranes and it may reflect interaction of the dye with molecules of the enzyme and transport systems of the barrier. When its structural integrity was disturbed, the dye penetrated into the interior of the cells and the reaction product could be seen actually in the matrix of the cytoplasm and on the intracellular membranes. As a result of this, the electron-optical density of the type I alveolocytes and, in particular, of the endotheliocytes increased considerably (Fig. 1c).

On the surface of the endothelium and in the lumen of the pulmonary capillaries, the Procion Brilliant Blue H5GS could also bind with plasma proteins, some of which still remained in the niches of the endothelial cells after perfusion of the blood vessels with physiological saline. Material of similar structure filled the micropinocytotic vesicles of the alveolar and endothelial cells of the air–blood barrier, probably reflecting the main mechanism for transport of blood proteins to the alveolar surface (Fig. 3b). This method of penetration of plasma albumin and globulin into the hypophase of the alveoli has been reported by workers who have detected these proteins immunohistochemically on the endothelial and epithelial surfaces of the barrier, in its micropinocytotic vesicles, and on surfactant membranes in rats [2].

Two types of proteins thus exist in the alveolar extracellular lining of rats and guinea pigs: specific proteins synthesized by type II alveolocytes, and globular proteins transported to the surface of the alveoli from the blood by micropinocytotic vesicles. The former are probably closely connected with the structure of the surfactant membranes, whereas the latter are located mainly on the plasmalemma of the alveolocytes, in the matrix of the hypophase, and on the surface of the membranes of the grid structures of the surfactant complex.

The new histochemical method suggested by the writers for studying the alveolar extracellular lining, using Procion Brilliant Blue H5GS, preserves the quality of the electron-immunohistochemical method but, at the same time, is able to reveal both types of proteins of the surfactant complex. The method is sufficiently simple and gives results of good reproducibility under experimental conditions; it can therefore be recommended for the study of the structure and localization of lung surfactant proteins under normal and pathological conditions.

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