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ELECTRON MICROSCOPE STUDIES ON THE RELATIONSHIP BETWEEN MITOCHONDRIA AND INCLUSION BODY IN SPHEROID ALVEOLAR EPITHELIAL CELL AFTER VITAL STAINING WITH ACIDIC AND BASIC DYES

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Abstract. Electron microscope observations were conducted on the relationship between mitochondria and inclusion body in mice spheroid alveolar epithelial cells after injection of trypan blue, an acidic dye and Alcian blue 8GS, a basic dye, by vital staining procedures. When both dyes were injected, the mitochondria of the spheroid alveolar epithelial cell became degenerated; however, in injection of only trypan blue, the cristae showed an increase in electron density. In injection of only Alcian blue 8GS, the cristae showed negative contrast. In most cases the trypan blue particles did not enter into mitochondria, whereas particles of Alcian blue 8GS sometimes entered into the mitochondria. When trypan blue particles entered mitochondria, deposits were not evident in the inclusion body, whereas when Alcian blue particles entered mitochondria deposits were seen in the inclusion body. In both of these cases only a few inclusion bodies were formed so that only traces or no inclusion bodies with vacuolar appearance were observed. From these findings it is suggested that mitochondria maybe convert to inclusion bodies.

In vital staining, acidic dyes have been used by many investigators, but basic dyes have rarely been used because of their toxic effects though the reasons for the toxicity are not clearly known. The author (1, 2, 3) previously used trypan blue or a mixture of aniline blue, ponceau PR and orange G as acid dyes and Alcian blue 8GS as basic dye to observe dye migration into the arterial wall and lymph node and dye phagocytosis in the cell. Similarly in the present study the acidic trypan blue and basic Alcian blue were used in vital staining to observe the dye transport or migration in the alveolar wall, and especially to observe the effects of these dyes on the spheroid alveolar epithelial cell.

The autochthonous cells that constitute the alveolar wall in mammals are endothelial cells of the alveolar blood capillary, alveolar septal cells and alveolar epithelial cells. The alveolar epithelial cells consist of two types: one is

the large type of alveolar epithelial cell and the other is the small type of alveolar epithelial cell. Ham (4) called the former the secretory epithelial surface cell and the latter the squamous epithelial surface cell, but the author would like to designate the former as the spheroid alveolar epithelial cell and the latter as the flat alveolar epithelial cell based on morphology.

Specific inclusion bodies exist in the cytoplasm of spheroid alveolar epithelial cell, but according to Rhodin (5) there are two conflicting opinions about such inclusion bodies: one opinion contends that such inclusion bodies are derived from the mitochondria and the other denies such a conclusion so that this point remains unclarified.

As early as in 1923 Fauré-Fremiet and Dragoiu (6) already detected a sulfur compound in the alveolar wall cell of sheep, guinea pig and rat, and called the cell the granulocyte of the lung. In histochemical investigations of the lung of mice and rats the author (7) also detected a granular organic sulfur compound containing various sulfur radicals, such as $-OSO_3H$, -S-S- and -SH and found that such granules were positive to PAS and Irisol-echt BBN stains. Subsequently, the author (8) injected radioisotopes, such as ^{35}S -H_2SO_4, ^{35}S-DL- cysteine and ^{3}H-thymidine into mice and examined the specimens by light and electron microscope radioautography. These radioactive substances were detected only in normal mitochondria and the so-called inclusion body of spheroid alveolar epithelial cells, so that the author concluded that the inclusion body must be a product of degenerated mitochondria.

This report will describe the dye particle pathways via various cells of the alveolar wall after injection of acidic and basic dyes, and how the degeneration of mitochondria induced by these dye particles occurs, as well as how the degenerated mitochondria are converted into inclusion bodies in spheroid alveolar epithelial cells.

MATERIALS AND METHODS

After diluting trypan blue (an acidic dye) or Alcian blue 8GS (a basic dye) to 0.25% with physiological salt solution, each solution was injected intraperitoneally at 0.2 ml/g body weight to adult mice of both sexes once or for 5,10 or 15 times at two day intervals.

Animals given one injection were sacrificed 20 hours after the dye injection, and animals given over 5 injections were sacrificed one hour after the last dye injection. The animals were sacrificed by ether anesthesia. The lung tissue was removed and small pieces of the lung were prefixed in 5% glutaraldehyde solution whose pH was adjusted to 7.4 with 0.1 M phosphate buffer for 1.5 to 2.0 hours. These pieces were postfixed with osmic acid diluted to 1% with 0.1 M phosphate buffer for 1.5 to 2.0 hours. After fixation, the tissues were dehydrated through an ethanol series, embedded in Epon 812 and ultrathin sections were

prepared. These ultrathin sections were double-stained with uranyl acetate and lead citrate by the method of Reynolds (9). A Hitachi electron microscope HU-11 was employed for observation.

RESULTS

Lung Tissue Findings after Injection of 0.25% Trypan Blue Solution to Mouse Peritoneal Cavity

Tissues removed 20 hours after a single injection (Figs. 1 and 2). Most mitochondria of the spheroid alveolar epithelial cells showed indistinct cristae, but the inclusion bodies showed no marked changes.

Tissues removed one hour after the fifth injection (Figs. 3, 4). Mitochondria of spheroid alveolar epithelial cell were practically all degenerated, and hardly any normal inclusion bodies were observed with high electron density. In other words, the vacuoles had an indistinct limiting membrane within which a minimal quantity of residual or lamellar inclusion body was present.

In the transition from the degenerate mitochondria to inclusion bodies and in the transformation of the inclusion bodies (no. 1–7 in Fig. 4), the electron density of the mitochondria matrix and cristae increased initially; the limiting membrane of the cristae was particularly high in electron density and most cristae became fragmented (no. 1). Then, the distinction between the matrix and cristae became uncertain; at the same time they turn into a homogenous degenerated substance of high electron density (no. 2). Within this homogenous substance there appeared a region of especially high electron density (no. 3), and with this region as the center, the homogenous substance began to condense; at the same time interstices (no. 4) appeared so that it looked as if a mass of homogenous substance of high electron density was enveloped in the vacuoles. This substance became a lamellar structure of minute quantity (no. 5), which gradually decreased (no. 6), and finally became a vacuole or nearly a vacuole (no. 7). In more detail the lamellar component that constituted the skeleton of the inclusion body seemed to correspond to the cristae of the degenerated mitochodria (no. 5). The other components were derived from the matrix of the degenerated mitochondria, and they seemed to undergo decomposition and obliterated faster than the lamellar structure.

Moreover, large and small rounded particles of trypan blue were recognized in the cytoplasm of the capillary endothelial cells, alveolar septal cells and flat alveolar epithelial cells.

Lung Tissue Findings after Injection of 0.25% Alcian Blue 8GS Solution to Mouse Peritoneal Cavity

Tissues removed one hour after the tenth injection (Figs. 5, 6). The spheroid alveolar epithelial cells showed only degenerated mitochondria with indistinct

cristae and normal mitochondria were not evident (Fig. 6). Though inclusion bodies were occasionally present (Figs. 5, 6), their numbers were few, and small inclusion bodies were found that were thought to have been formed by the fragmentations of mitochondria (Fig. 6).

Furthermore, high electron dense oval or elliptical particles of Alcian blue 8GS were observed in various cells that constituted the alveolar wall, as well as in macrophages located in the alveolar space (Figs. 5, 6).

Tissues removed one hour after the fifteenth injection (Figs. 7–10). All mitochondria in spheroid alveolar epithelial cells were degenerated, and the degenerated state was specific. The mitochondria matrix was homogenous and showed increased electron density, but the cristae lost the limiting membrane and had almost no electron density, forming slender transparent bands. Such degenerated mitochondria had the appearance of homogenous high electron dense substances of degenerated mitochondria being divided by slender transparent band-like cristae.

In addition, minute particles of Alcian blue 8GS were seen within the mitochondria or on the limiting membrane of mitochondria (Fig. 8). The size of such fine particles of Alcian blue 8GS was about the same as that of the intramitochondrial granules, but the shape was irregular in contrast to the round shape of the intramitochondrial granules. Such fine particles resembled particles formed by the disintegration of the phagolysosomes in size and shape (Fig. 10). However, the deposition of minimal unit particles of Alcian blue 8GS was not often observable (Figs. 7, 9, 10). Consequently, the degeneration of mitochondria seemed to have no direct correlation to the deposition of dye particles in the mitochondria.

The next outstanding aspect was the changes in inclusion body. Hardly any lamellar structure and myelin figure-like substances were evident that were inherent to the so-called inclusion body, and only very slight traces of the inclusion body were observed in the large vacuoles with limiting membrane. Moreover, it is worthy of note that spheroid alveolar epithelial cells contained fine particles of Alcian blue 8GS in both degenerated mitochondria and vacuolar inclusion bodies (arrows of Fig. 8). Namely, this indicates that the mitochondria underwent degeneration and they were then converted to inclusion bodies. In the cytoplasm of spheroid alveolar epithelial cells in which both mitochondria and inclusion bodies showed such specific changes, rounded aggregates of Alcian blue 8GS were always observed.

Such aggregates of rounded particles of Alcian blue 8GS were seen in all alveolar capillary endothelial cells, alveolar septal cells, stromas of alveolar septa and flat alveolar epithelial cells that composed the alveolar wall. In addition, dye particles were recognized in the alveolar space, and in leucocytes

and blood platelets of the alveolar blood capillary.

DISCUSSION

Entrance of dye particles into spheroid alveolar epithelial cells. From observations of acidic dye and basic dye in various cells of the alveolar wall and the alveolar space, three pathways for reaching spheroid alveolar epithelial cells are possible after dye injection into the peritoneal cavity. The first way is for dye particles to enter into the alveolar septum via the endothelial cells of the alveolar blood capillary, then enter into spheroid alveolar epithelial cells. Another way is for the dye to pass through the united basement membrane of both the spheroid alveolar epithelial cell and capillary endothelial cell and entering directly into the spheroid alveolar epithelial cells. The third way is for the dye to pass through the flat alveolar epithelial cells from the intra-alveolar capillary endothelial cells to the spheroid alveolar epithelial cells. Most of dye seem to be transported by the first pathway, and such transport should be a passive transfer. It seems that the dye particles are ultimately ejected into the alveolar space from the spheroid alveolar epithelial cells by exocytosis.

Dye particles in spheroid alveolar epithelial cells. Relatively large particles of the two kinds of dyes wandered freely in the cytoplasm. Some were present in free states while others were phagocytized in lysosomes in the form of phagolysosomes, and some appeared as still finer particles to be released into the cytoplasm. Therefore, both large and small particles were found in the cytoplasm.

Relationship between dye particles and mitochondria in spheroid alveolar epithelial cells. Injection of either the acidic dye or basic dye resulted in mitochondria degeneration. However compared with the acidic dye, basic dye particles were more distinctly deposited in the mitochondria. This may indicate that positively charged basic dye entered into negatively charged mitochondria by electrostatic polar adsorption.

The next characteristic feature was the change in mitochondria, that is, when acidic dye was injected at an early stage of mitochondria degeneration the cristae were relatively clear with the limiting membranes especially showing high electron density. In contrast, when the basic dye was injected, the matrix of mitochondria showed an increase in electron density but the limiting membranes of the cristae were not apparent and the cristae completely lost electron density, appearing in negative contrast, forming slender transparent bands. Thus such transparent bands appeared as if they were sectioning the degenerated mitochondria.

It can be assumed from this specific picture of mitochondria degeneration that Alcian blue 8GS, a basic dye, is a far stronger cytotoxin than acidic dye,

and the basic dye seems to have extremely strong toxicity even on the mitochondria.

Toxicity of acidic dye and basic dye on cytoplasm and mitochondria. The mechanism of vital staining is complex and difficult to generalizeon. However, Höber (1909) stated that the chief components of the living cell are charged negatively, and thus cell components are usually stained with positively charged basic dyes but not with negatively charged acidic dye. A basic dye of positive charge combines electrostatically with the cell membrane of negative charge, and when the basic dye is engulfed into the cytoplasm by endocytosis, the dye undergoes polar adsorption with negatively charged cytoplasm, resulting in strong cell toxicity. On the other hand, an acidic dye does not commonly pass through the cell membrane, but trypan blue dye has a weak charge and also has the property of being readily adsorbed so that it enters into the cell without much resistance. As there are basic proteins of positive charge in the cytoplasm, trypan blue particles of negative charge undergo polar adsorption electrostatically, but such polar adsorption in the cytoplasm occurs more often and more strongly with basic dyes. When such foreign substances as dyes enter into the cell and polar adsorption occurs, it is rather clear that a toxic effect is forced on the cell.

Since mitochondrion is a cell organism that undergoes change most sensitively in cell injury, it is obvious that the degeneration of mitochondria occurs by repeated dye entrance into the cell. In addition, a more marked intracellular deposit of basic dye as compared with acidic dye would induce a greater degeneration of mitochondria, particularly in the case of basic dye entrance into the cell, and the mitochondria cristae disappear or become transparent which completely stops the respiratory functions of mitochondria, attended by a loss of energy generation.

Relationships of dye particles, lysosomes and mitochondria in spheroid alveolar epithelial cells. Lysosomes ingesting dye particles in the spheroid alveolar epithelial cell were converted to phagolysosomes which decomposed dye particles into finer particles. These finer particles appeared to be liberated into the cytoplasm, and a substance similar to these finer particles in size was sometimes seen in the mitochondria. This was particularly marked in the case of basic dye. As Alcian blue 8GS is a water soluble phthalocyanine dye with copper, the electron density of these dye particles was quite high. The author previously described (1, 2, 3) in various cells that peculiar rounded particles of Alcian blue 8GS of 0.2–0.3 μ in diameter were present, but such rounded particles were also seen in the cytoplasm of spheroid alveolar epithelial cells.

These particles were ingested by lysosome in the spheroid alveolar epithelial cell and were converted to the so-called phagolysosome within which there

were finely divided minute particles. There were also fine particles in mitochondria of practically the same size and electron density as these minute particles. These latter particles are apt to be mistaken for intramitochondrial granules, but the fine particles of dye are mostly slender or irregular in shape and not round, so differentiation is possible. For this reason it is thought that the slender or irregularly shaped particles were ejected from phagolysosome into the cytoplasm, then by polar adsorption they adhered to membranes of mitochondria, and subsequently by breakage of both the outer and inner membranes, they entered into the matrix of the mitochondria.

Relationship between degeneration of mitochondria of spheroid alveolar epithelial cells and dye. Particles of dye were always detected in the cytoplasm of spheroid alveolar epithelial cells, but they were not always deposited in the mitochondria. However, because mitochondria degeneration of spheroid alveolar epithelial cell was the same irrespective of the absence or entrance of dye particles into mitochondria so far as the dye particles were present in the cytoplasm, it is assumed that the mitochondria did not degenerate by the direct action of the dye but the entrance of the dye itself into cytoplasm elicited cytotoxicity, and the mitochondria were damaged, underwent degeneration and were finally destroyed.

The finding that the basic dye penetrates into mitochondria while the acidic dye does not may be explained by the limiting membrane of mitochondria having a negative charge equal to the cell membrane. The acidic dye of negative charge had difficultly entering into the mitochondria, but the basic dye of positive charge bound readily with the limiting membrane, hence it is easier for basic dye to enter into mitochondria.

Effect of mitochondria degeneration on formation of inclusion body in spheroid alveolar epithelial cells. In spheroid alveolar epithelial cells with marked degeneration of mitochondria due to the entrance of dye into the cytoplasm, very few inclusion bodies were formed and there were only traces of inclusion bodies in the large vacuoles. Moreover, in cases where fine particles of dye were observed in degenerated mitochondria, the same dye particles were recognized relatively easily in large vacuolar inclusion bodies. This signifies that the so-called inclusion body was formed by the degeneration of mitochondria.

As already mentioned the author (7) histochemically detected sulfur radicals such as $-SO_3H$, -S-S- and -SH in spheroid alveolar epithelial cell, and the author (8) also pointed out that the formation of inclusion body was due to the degeneration of mitochondria from the presence of ^{3}H -thymidine, ^{35}S -H_2SO_4 and ^{35}S -D-L-cysteine in both normal mitochondria and in the inclusion body of spheroid alveolar epithelial cell, and even in the present experiment it was demonstrated that the inclusion body was formed by the degeneration of

mitochondria.

However in the case of the inclusion body being formed by mitochondria degeneration, normal mitochondria were converted to inclusion bodies after degeneration, while mitochondria damaged by a foreign substance, such as dye, rarely changed to inclusion bodies; namely, in vital staining with Alcian blue dye that elicited marked mitochondria degeneration and marked damage to cristae, it was difficult to observe inclusion body formation.

Mechanism of inclusion body formation from mitochondria in spheroid alveolar epithelial cells. It is known that various substances, including various proteins, phospholipids, fatty acid, amino acids, hippuric acid, citrulline and glutamine are synthesized in mitochondria, but the structural component of mitochondria responsible for the synthesis is not very clear. However, when the cristae become markedly degenerated, namely when damaged, the formation of inclusion bodies was hardly observable. From this observation it is probable that the cristae play an important role in the formation of inclusion bodies. Actually, the limiting membrane of cristae is probably the important site where oxidative phosphorylation is performed in the synthesis of ATP from ADP and inorganic phosphates.

Consequently, when the membrane of mitochondria cristae is injured, mitochondria rapidly die, and the synthesis of organic sulfuric compounds can no longer be carried out. Thus, no formation of inclusion body occurs. Among these organic sulfuric compounds, particularly important is acid mucopolysaccharide.

Recently the author (8) stated that organic substances having $-OSO_3H$ radical in the inclusion body were formed by the mechanism of sulfate activation and transfer described by Lipmann (10) within the mitochondria. Namely, by the transfer of SO_4 radical of PAPS, a donor, that was activated by binding of sulfuric acid ions with bimolecular ATP to an acceptor, such as the steroid, amino group, phenol and hydroxy group of various compounds, organic substances containing the OSO_3H radical were formed.

The only *in vivo* cell in which such a specific inclusion body is formed from mitochondria is the spheroid alveolar epithelial cell. Therefore, the mitochondria of the spheroid alveolar epithelial cell may have functional structures different from other cells.

It is known that the outer membrane of mitochondria does not allow the passage of macromolecules but it readily allows the passage of small molecules, such as sugar, adenine and nucleotide; however, there is a selective permeability in the inner membrane of mitochondria, and sugar and AMP are usually hardly permeable. Nonetheless, the acceptors of the sulfate activation system, such as hexosamine derivatives, galactose, steroids, phenols, hydroxy groups,

SO_4^{2-} ion, HPO_4^{2-} (phosphoric acid ion), ADP and ATP may be able to permeate through this inner membrane of mitochondria. It is thought that the synthesis of ATP by oxidative phosphorylation is conducted in the inner membarne of mitochondria composed of a close linkage with the electron transfer system and the oxidative phosphorylation system and that this ATP is liberated into the mitochondria matrix where sulfate activation is conducted by ATP and SO_4 ions, thereby organic sulfates containing OSO_3H radical are produced. When the organic sulfates containing OSO_3H radical thus produced accumulate and fill up the matrix, the oxidative phosphorylation ceases; thereby the degeneration of the mitochondria starts, then the transfer from the degenerated mitochondria to inclusion body follows. As already stated, in mitochondria with cristae damaged by dye, the inevitable formation of inclusion body seems to no longer occur.

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Legends to Figures (1-10)

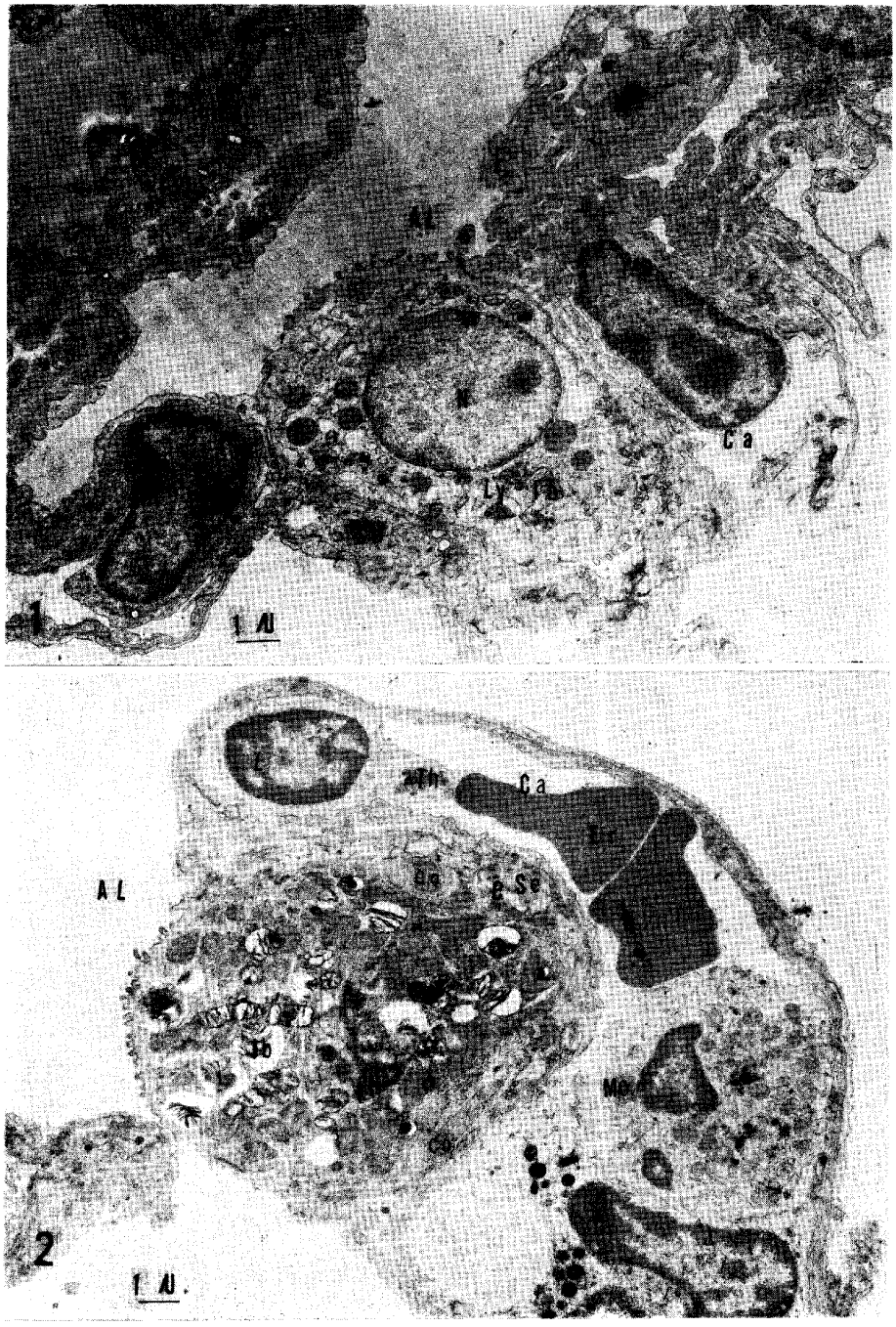
a, Particles of dye. In Figs. 1-4 the dye is acidic and in Figs. 5-10 the dye is basic. AL, alveolar space; Ca, alveolar blood capillary; co, collagenous fibers in the alveolar septum; dM, degenerated mitochondria of spheroid alveolar epithelial cell; e, elastic fibers of alveolar septum; Er, erythrocyte; F, flat alveolar epithelial cell; Ib, inclusion body of spheroid alveolar epithelial cell; L, lymphocyte; Ly, lysosome; Ma, macrophage in the alveolar space; Mo, monocyte; N, nucleus of spheroid alveolar epithelial cell; NL, neutrophile leucocyte; Th, thrombocyte; Se, alveolar septal cell. No. 1-7 show the process of transfer from degenerated mitochondria to inclusion body and the transformation of the inclusion body. Arrows point out the localization of the acidic or basic dye particles.

Figs. 1, 2. A single intraperitoneal injection of 0.25% trypan blue solution to mouse. Cristae of mitochondria of spheroid alveolar epithelial cell have become indistinct and degenerated mitochondria have increased. ×5,490.

Figs. 3, 4. After five intraperitoneal injections of 0.25% trypan blue solution to mouse. All mitochondria of spheroid alveolar epithelial cell are degenerated. Only a few inclusion bodies are formed showing an appearance close to vacuoles. The numbers point out the conversion of mitochondria to inclusion body, and the transfer process of the inclusion body itself. Dye particles of various sizes are observed in cells constituting the alveolar wall. ×6,700.

Figs. 5, 6. After 10 intraperitoneal injections of 0.25% Alcian blue 8GS solution to mouse. All mitochondria of the spheroid alveolar epithelial cell are degenerated, and the quantity of components in the formed inclusion body is small. Rounded particles of Alcian blue 8GS are observed in cells composing the alveolar wall and in alveolar macrophages. ×5,300.

Figs. 7-10. After 15 intraperitoneal injections of 0.25% Alcian blue 8GS solution to mouse. The degeneration of mitochondria of spheroid alveolar epithelial cell is specific, in which the cristae present negative contrast, and the cristae appear to partition the degenerated mitochondria of high electron density. The quantity of components in the inclusion body is extremely minimal, appearing like a vacuole. In Fig. 8 small elliptical particles are observed to be dye particles of Alcian blue 8GS in mitochondria (arrows), and similar substances are also seen in the vestigial inclusion body of vacuolar appearance (arrows). In Fig. 10 fine particles of Alcian blue 8GS are found in the lysosome (arrows). There are also rounded particles of Alcian blue 8GS in cells constituting the alveolar wall. Fig. 7, ×5,310; Fig. 8, ×8,640; Figs. 9, 10, ×5,350.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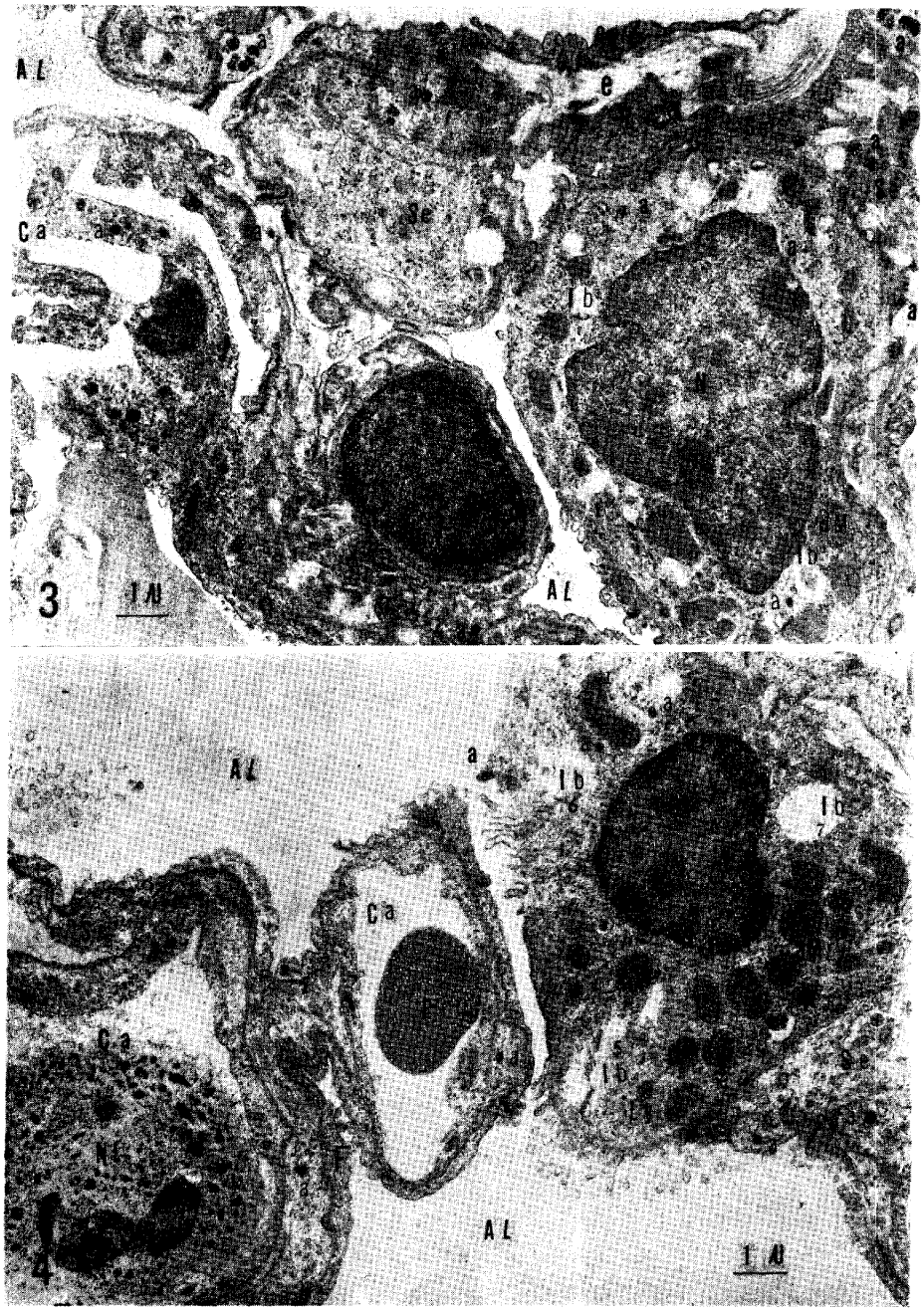
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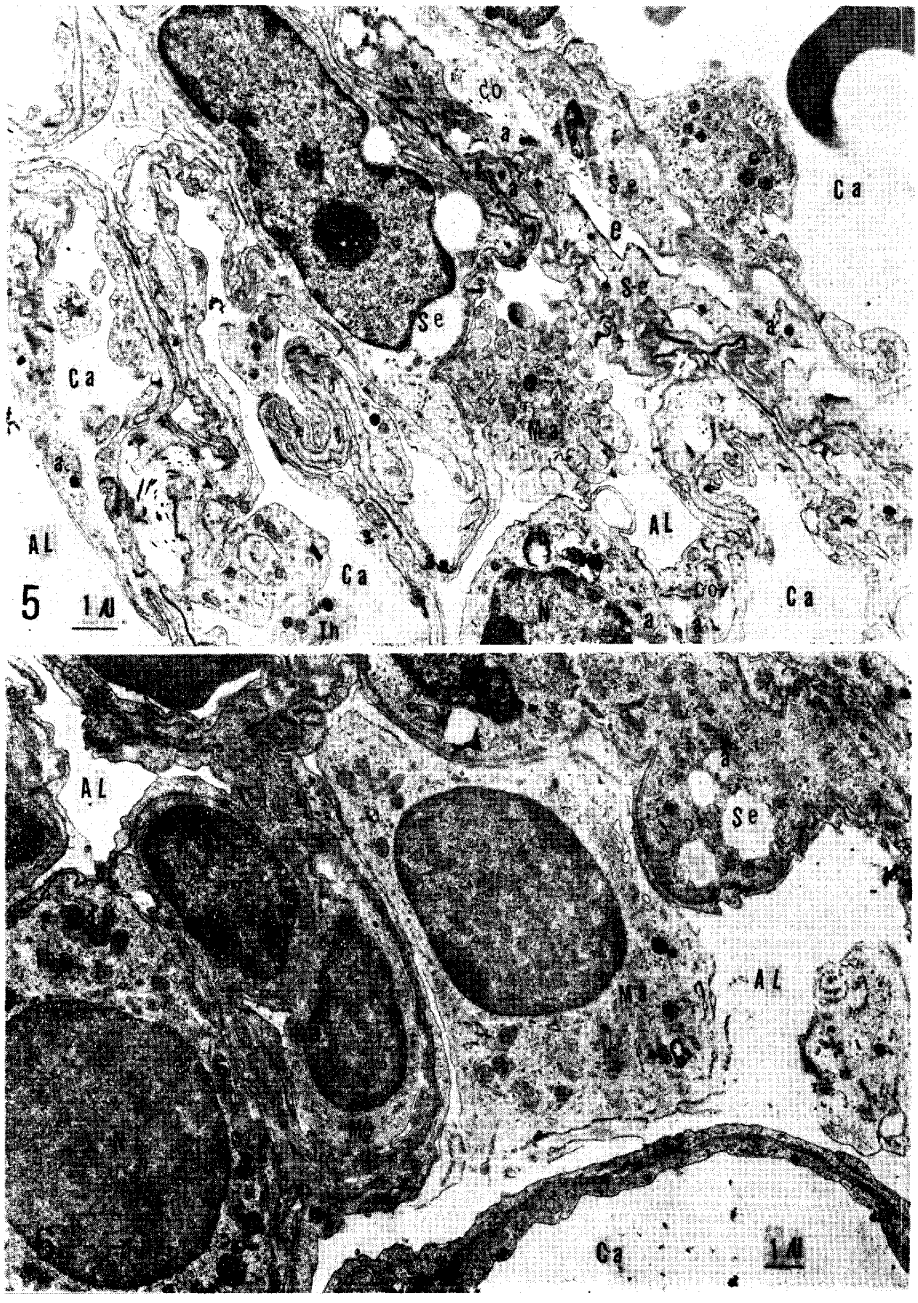
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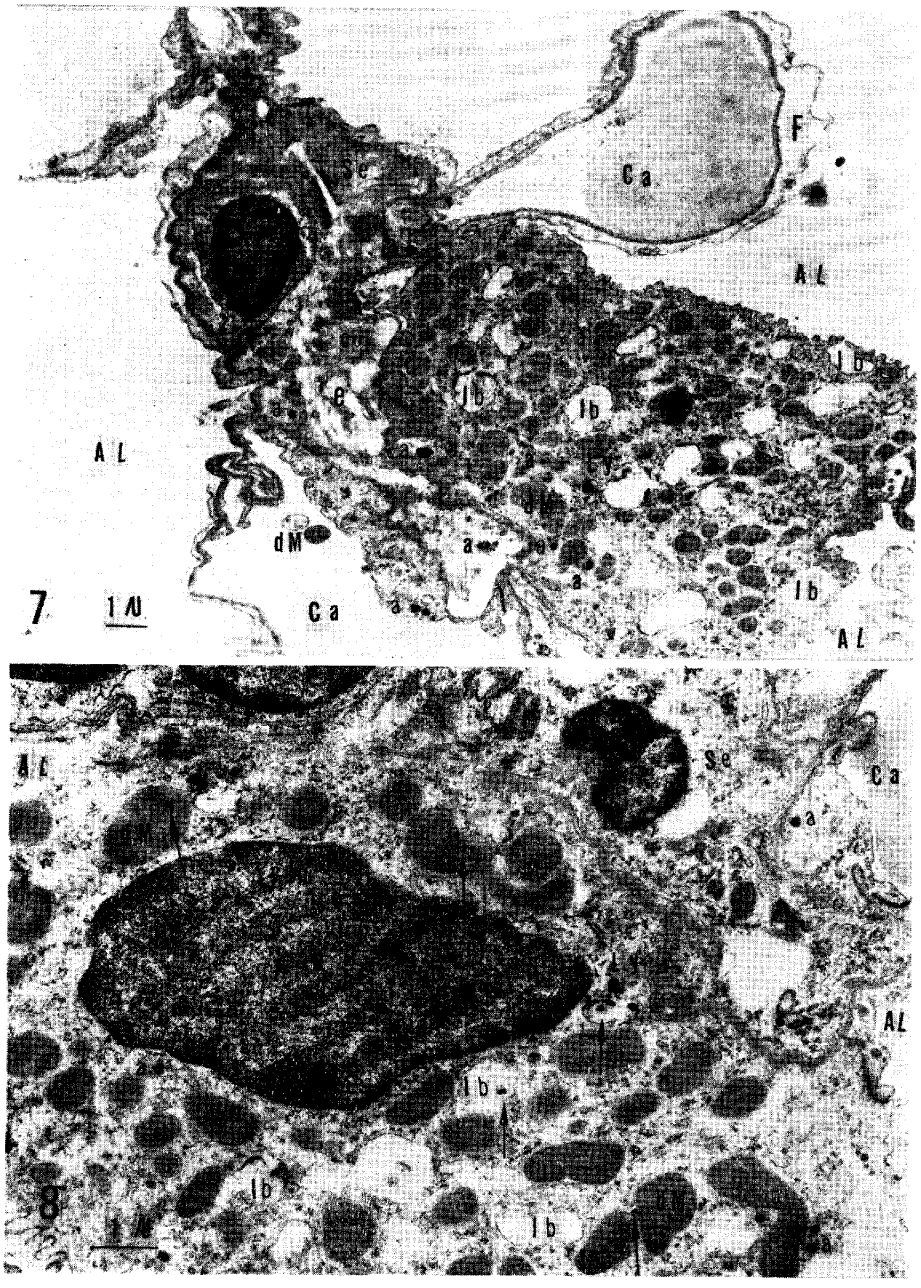
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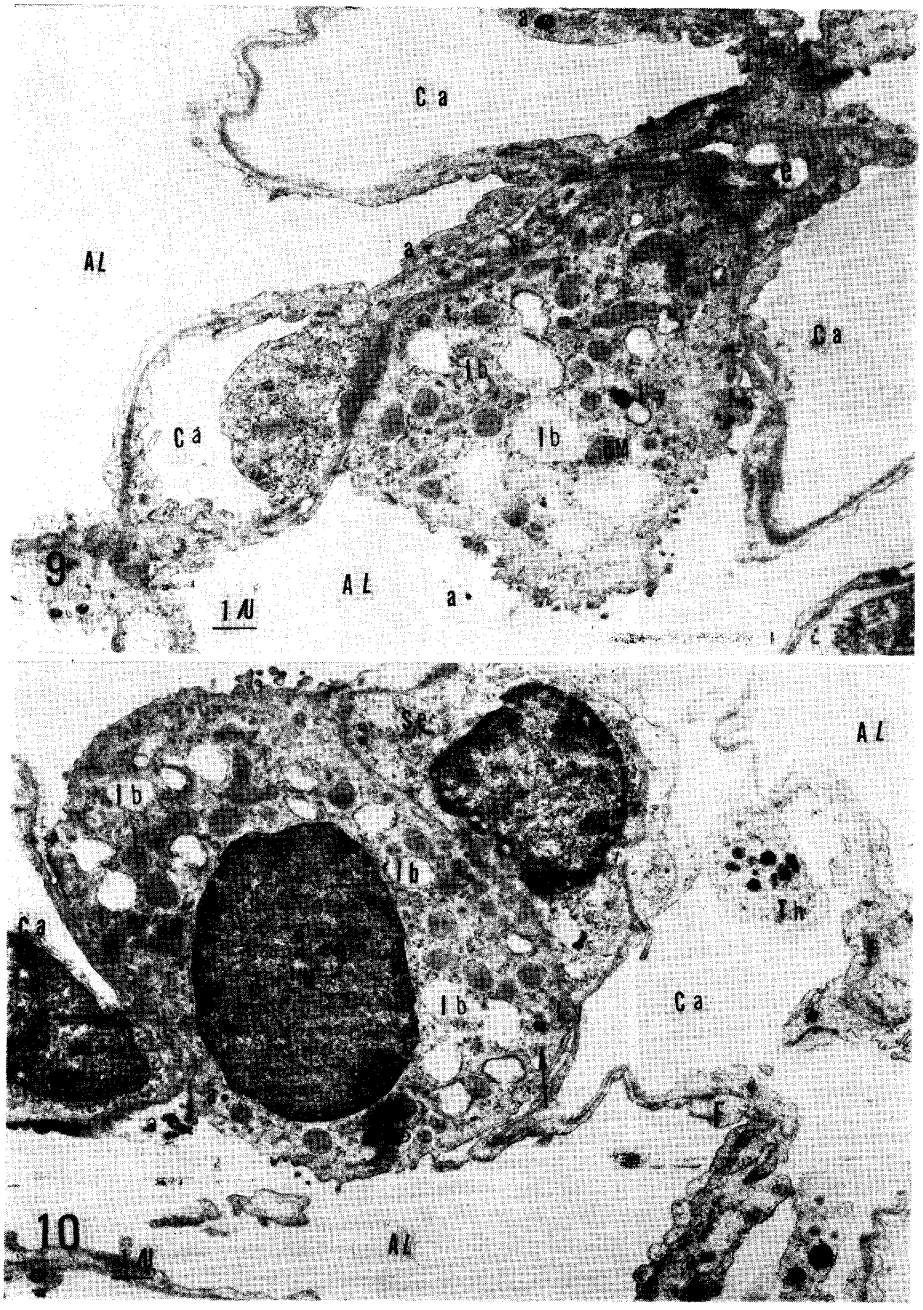
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