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Alveolar cells in cyclophosphamide-induced lung injury. An ultrastructural analysis of type II alveolar epithelial cells in situ

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Summary. Recent studies have brought rich evidence in favour of the significant contribution of the surfactant system-forming structures to morphogenesis of many pulmonary disorders. The aim of this study was to evaluate the effect of intraperitoneal cyclophosphamide administration on changes within this system.

The experiments used 40 Wistar rats, of 170g body weight. The animals were divided into two experimental groups. Group I animals were given cyclophosphamide (Endoxan-ASTA) in a single intraperitoneal dose of 150mg/1kg b.w./1ml PBS/. Group II (control) received 1ml PBS. All the animals were sacrificed after 1, 3, 7 and 28 days following intraperitoneal cyclophosphamide or PBS administration. Morphological examinations of pulmonary tissue were based on ultrastructural analysis in the transmission electron microscope.

The study revealed that a single intraperitoneal cyclophosphamide injection caused damage to all elements forming the surfactant system, particularly to type II alveolar epithelial cells. Rebuilding processes in pulmonary tissue, coexisting with destructive changes, occurred with a significant contribution of type II alveolar epithelial cells. These cells are likely to take an active part in pulmonary fibrosis processes observed after the action of cyclophosphamide.

Key words: Lung, Type II pneumocyte, Cyclophosphamide, Ultrastructure

Introduction

Pulmonary fibrosis is caused by many environmental and therapeutic agents, but the molecular events responsible for the pathogenesis of the disease have not been completely defined. Studies of experimental pulmonary fibrosis induced in animals by the commonly used antineoplastic agents, cyclophosphamide (CP) and bleomycin, have proven useful in characterizing the essential molecular features of the disease (Hoyt and Lazo, 1990). CP is a widely used antineoplastic and immunosuppressive agent. Although it is somewhat less toxic than other nitrogen mustards, several reports have associated the therapeutic use of CP in man with diffuse interstitial pulmonary fibrosis (Morse et al., 1985). Until now, little attention has been paid to type II pneumocytes and their significance in CP-induced lung injury. These cells are absolutely essential to the synthesis and secretion of pulmonary surfactant and take part in regeneration of alveolar epithelial cells (Chander and Fisher, 1990; Low et al., 1990; Sulkowski et al., 1993, 1994; Sulkowska et al., 1996b). The aim of the present study was to investigate changes observed within type II alveolar epithelial cells in pulmonary rebuilding processes related to CP effect.

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Material and methods

The experiment used 40 male Wistar rats of 160-180g body weight. The animals were maintained in a well sunlighted room, at 18-20 °C on standard granulated diet. They were divided into two groups. Group I - (20 animals) were given a single intraperitoneal (i.p.) dose of 150mg CP/1kg b.w./1ml PBS. Group II - (20 animals) were given a single i.p. dose of 1ml PBS. All experimental animals were sacrificed by an intraperitoneal administration of 100mg sodium pentobarbital solution after 1, 3, 7 and 28 days of CP (or PBS) treatment.

For ultrastructural analysis in the transmission electron microscope (TEM) small blocks of 1 mm^3 were cut out of the lungs and fixed for 3 hours in cold (4 °C) 2.5% glutaraldehyde solution in 0.1M Na cacodylate buffer at pH 7.4. Fixed tissue samples were washed with 0.1M cacodylate buffer (pH 7.4), and postfixed in 1% osmium tetroxide in 0.1M cacodylate buffer for 1 hour and washed in buffer again. After dehydration in alcohol-acetone series and embedding in epon, they were sectioned and contrasted with lead citrate and uranyl actetate and examined in an Opton 900 PC electron

transmission microscope.

Results

Control subgroups II-1 - II-28

In these groups no significant differences were noted in the morphological picture of the lungs. Ultrastructural examinations showed the walls of the alveoli lined with markedly flattened type I epithelial cells, among which a few type II epithelial cells were seen. Short microvilli were found within the free area of these cells and a small number of lamellar bodies were observed in the cytoplasm (Fig. 1). Sporadically, alveolar macrophages were found in the lumen of the alveoli. They showed a poorly developed cytoplasmic membrane which formed not very numerous processes and rare secondary lysosomes.

Experimental subgroups

In subgroup I-1, focal damage to extracellular alveolar lining by oedematous fluid accumulating at the alveolar walls (Fig. 2) was observed. The greatest alterations, however, were found in type II epithelial cells. Most of the cells displayed damage of varying degree and/or emptiness of lamellar bodies, and disorders in the formation of their content (Fig. 3). Damage to mitochondria (Fig. 3), and, rarely, severe damage to type II cells, including necrosis, were observed. Focal damage to type I cells was also seen. The borderline between type II pneumocytes and interstitium was clear and was usually formed by regular basement membranes.

In subgroup I-3, necrosis of type I and II cells was more frequently found when compared to the previous group. An interesting, although sporadic phenomenon was "going out" of type II cell nuclei to the alveolar lumen with the breaking of cytoplasmic membrane and lack of features of considerable damage to the remaining cellular structures (Fig. 4). Oedematous changes were more intensified when compared with subgroup I-1; however, they occurred focally and did not occupy the whole area of pulmonary tissue. Sites of oedematous fluid accumulation showed numerous lamellar structures (Fig. 5), most probably ejected from type II cells in response to atelectasis development. In the alveolar lumen, macrophage accumulation was found, usually containing numerous secondary lysosomes (Fig. 6). Sporadically, polynuclear cells and neutrophilic granulocytes were seen.

In subgroup I-7, the areas of lung parenchyma exhibiting domination of macrophage accumulation adjoined atelectatic areas. In places, particularly within the atelectatic areas, pulmonary alveolar epithelial lining contained poorly differentiated cells, having no characteristic morphological features. These cells accumulated in layers, thus narrowing the alveolar lumen. Some of them had a small number of short, digitate processes, which may suggest their origin from type II cells in response to alveolar epithelium damage. They were frequently found in the immediate vicinity of mature type II cells, and sporadically young and mature type II pneumocytes filled up the alveolar lumen (Fig. 7).

The changes described above were particularly well pronounced in subgroup I-28. The site of atelectated alveolar lumen showed, apart from type II cells, loosely lying lamellar structures, alveolar macrophages and collagen-like material (Fig. 8, 9, 10). In the vicinity of collagen fibres, numerous cellular processes were observed, some of them being type II cells. Also the borderline between collagen and type II pneumocytes was blurred in places, and collagen fibres seemed to penetrate into the cytoplasm of type II cells.

Discussion

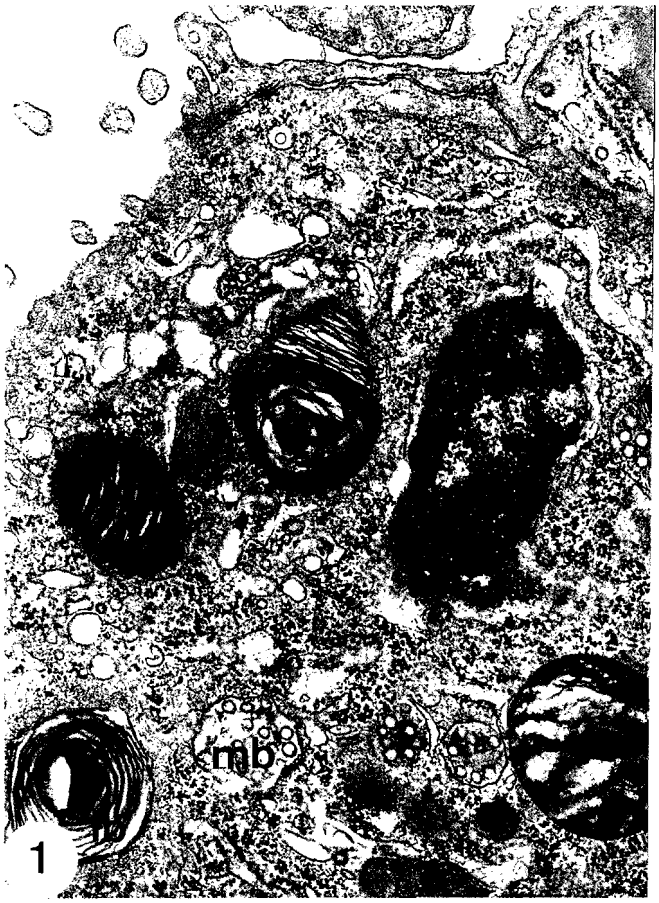
The morphological analysis of pulmonary tissue has revealed phasic character of changes observed in the structures included in the surfactant system. The early stage (subgroups I-1, I-3) was characterized by predominance of destructive changes. Defected extracellular alveolar lining and type II alveolar epithelial cells were observed. Damage to the latter type of cells seems particularly important, as these cells are not very sensitive to the action of destructive factors (Groniowski, 1983). However, severe damage to type II cells, including necrosis, was rare. More frequently, features of damage were found within the respiratory system of the cell (mitochondria), as well as disorders in the structure of lamellar bodies of type II pneumocytes. The accumulation of lamellar structures was noted within atelectatic alveoli and was frequently accompanied by the presence of oedematous fluid. Thus, it seems that the emptiness of lamellar bodies observed in a number of type II pneumocytes may either depend on the CP-induced cell damage itself (evidence in favour of this reasoning is simultaneous damage to mitochondria) or be the result of lamellar body content ejection to the alveolar lumen, in response to atelectasis induced by the accumulation of oedematous fluid, with subsequent damage to the extracellular alveolar lining layer.

Destructive changes in pulmonary surfactant and

Fig. 1. A normal type II epithelial cell. Characteristic lamellar bodies (lb) and multivesicular body (mb) are visible. Control group. TEM. x 12,000

Fig. 2. Oedematous fluid (oe) and damaged surfactant (s) in the alveolar lumen. Group I-1 (1 day after CP administration). TEM. x 7,000

Fig. 3. Cell with strongly pronounced damage traits of lamellar bodies (lb) and mitochondria (→). Group I-1. TEM. x 7,000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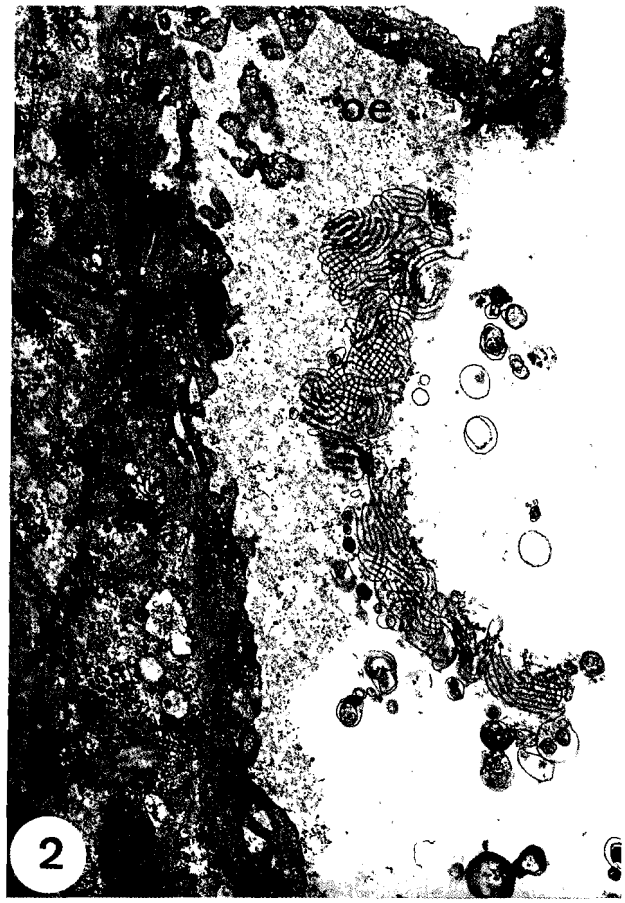
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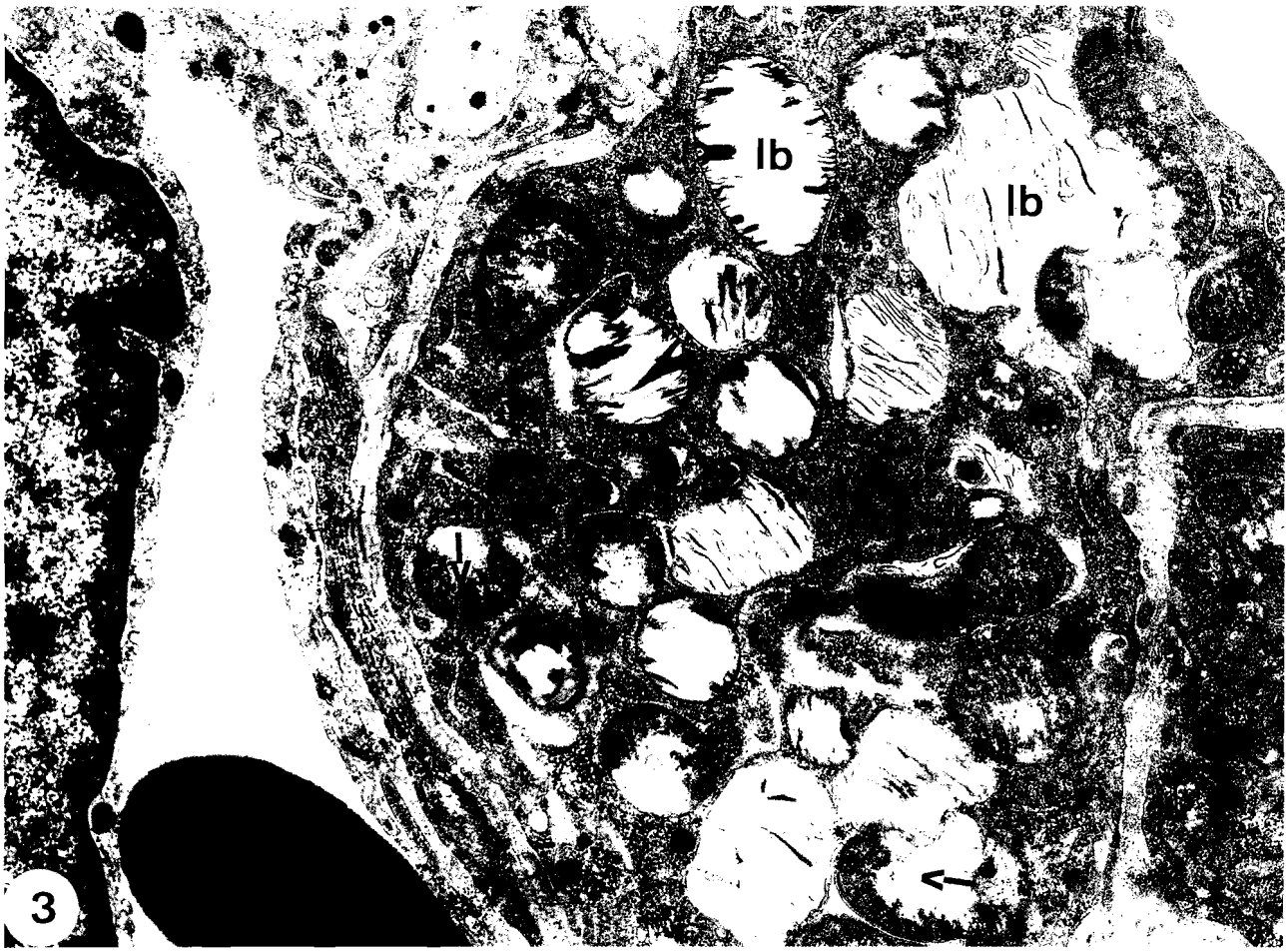
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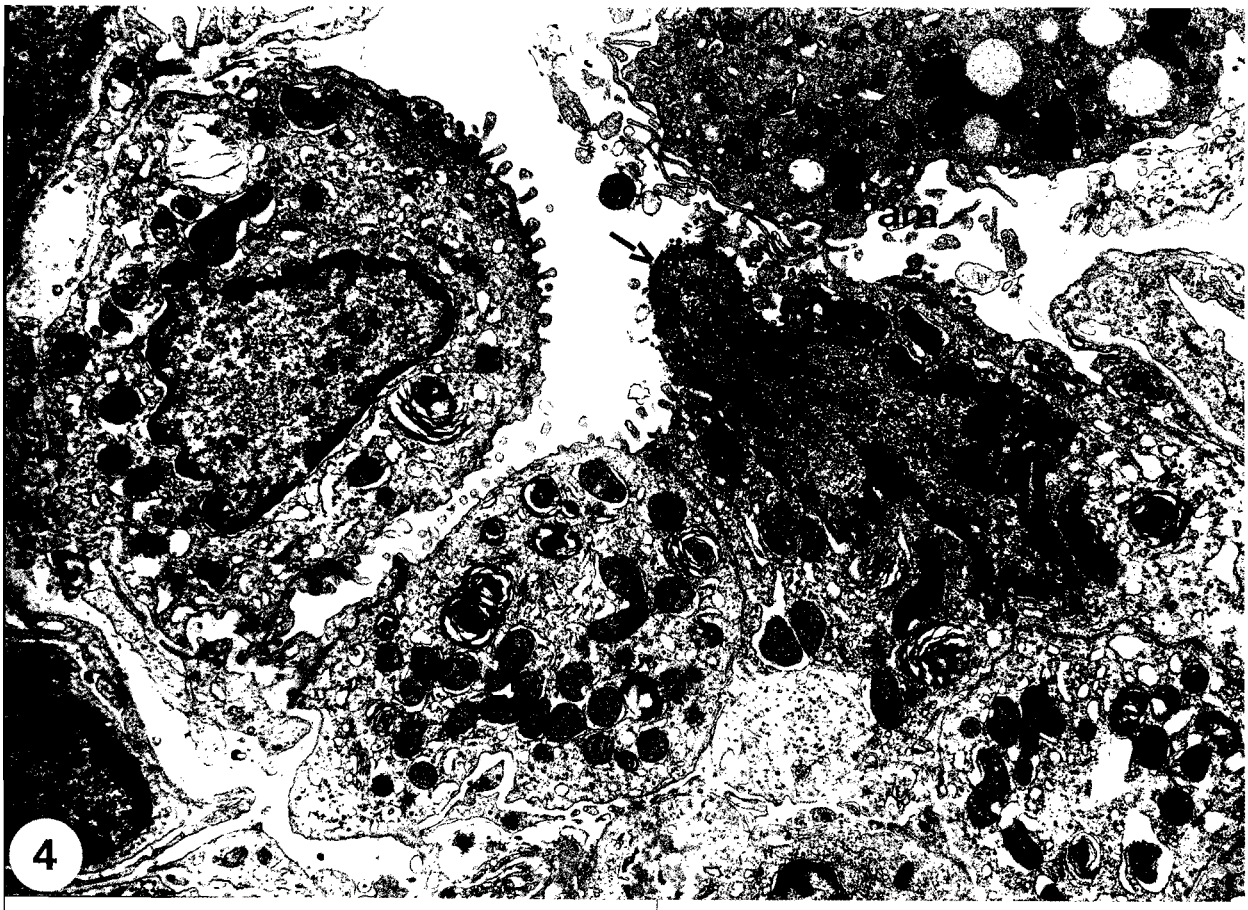
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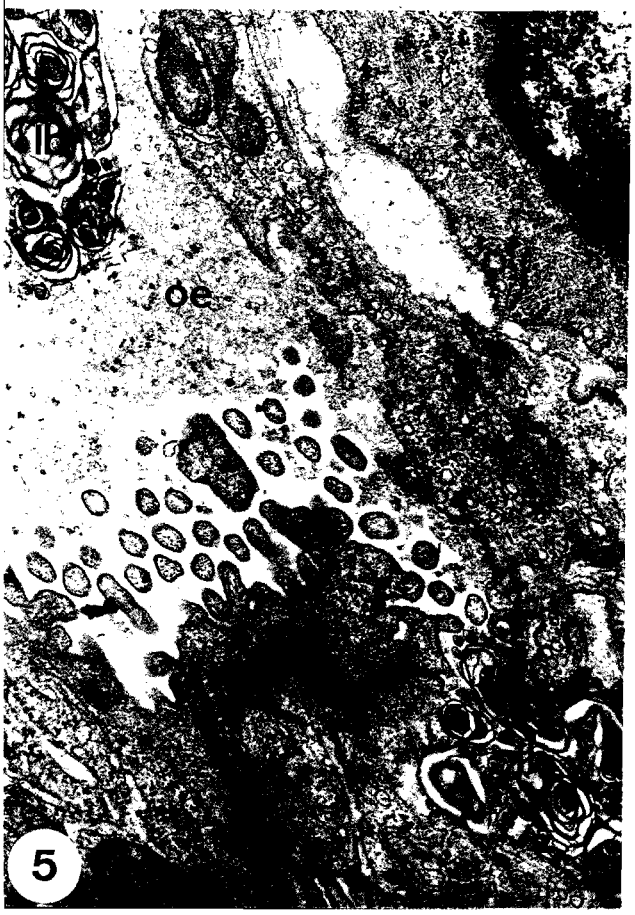
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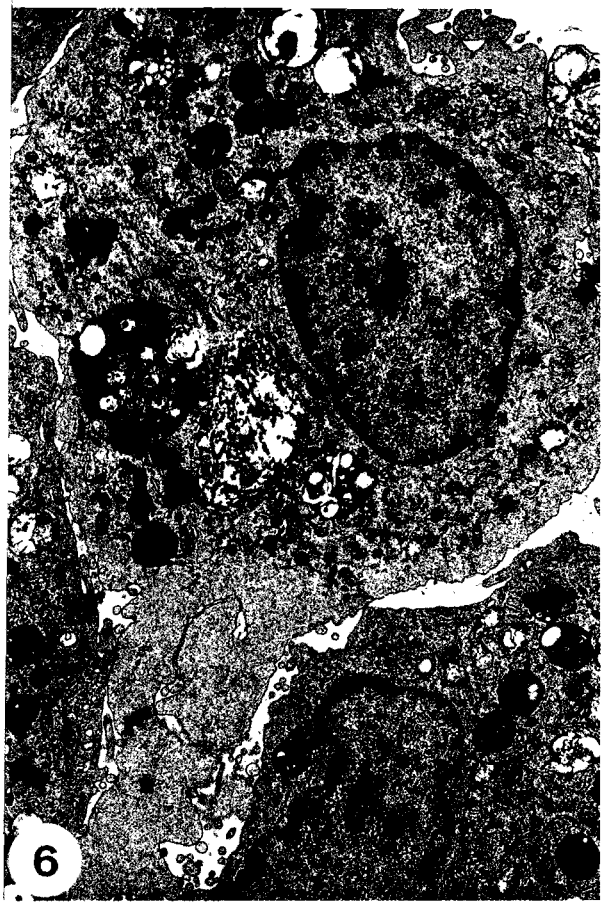
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alveolar epithelial cells were accompanied by the accumulation of phagocytes, mainly mononuclear ones (macrophages). Thus, the morphological changes observed are likely to result not only from the direct CP effect on the surfactant-forming structures, but may also be caused by inflammatory cells flowing into pulmonary tissue. Analysis and interpretation of these changes is the most difficult as no detailed studies have been performed on the surfactant system in pulmonary tissue damage in relation to CP action. The only detailed observations, published in 1988 by Kumar et al. are not fully consistent with the results of the present study. The greatest differences refer to the time of destructive changes within type II alveolar epithelial cells. In our experiments, features of damage, including emptiness of lamellar bodies of type II pneumocytes, was observed immediately after CP administration (subgroups I-1 and I-3). In the studies by Kumar et al. (1988), in a similar period, only damage to type I epithelial cells was observed, while emptiness of lamellar bodies was noted after 8 weeks following CP administration. This is even more suprising as the authors applied a dose twice as high (300mg/kg b.w.). At the later stage (after 2 weeks), Kumar et al. (1988) found macrophage accumulation within pulmonary tissue. Simultaneous with macrophage accumulation was type II epithelial cell proliferation, which, in respect to time, is similar to the results obtained in the present study. It cannot be excluded that the above discrepancies are related to the type of experimental animals. The studies by Kumar et al. (1988) used female BALB/c mice, while the present study was performed on rats.

All studies dealing with the effect of CP on pulmonary tissue have revealed intensification of fibroplasia processes with fibrosis within the inter-alveolar septa and/or intraalveolar fibrosis (Collis et al., 1980; Morse et al., 1985; Hoyt and Lazo, 1990; Walker Smith, 1990; Usui et al., 1992). Our present observations confirm the findings of other authors. They indicate, however, the possibility of a significant contribution of

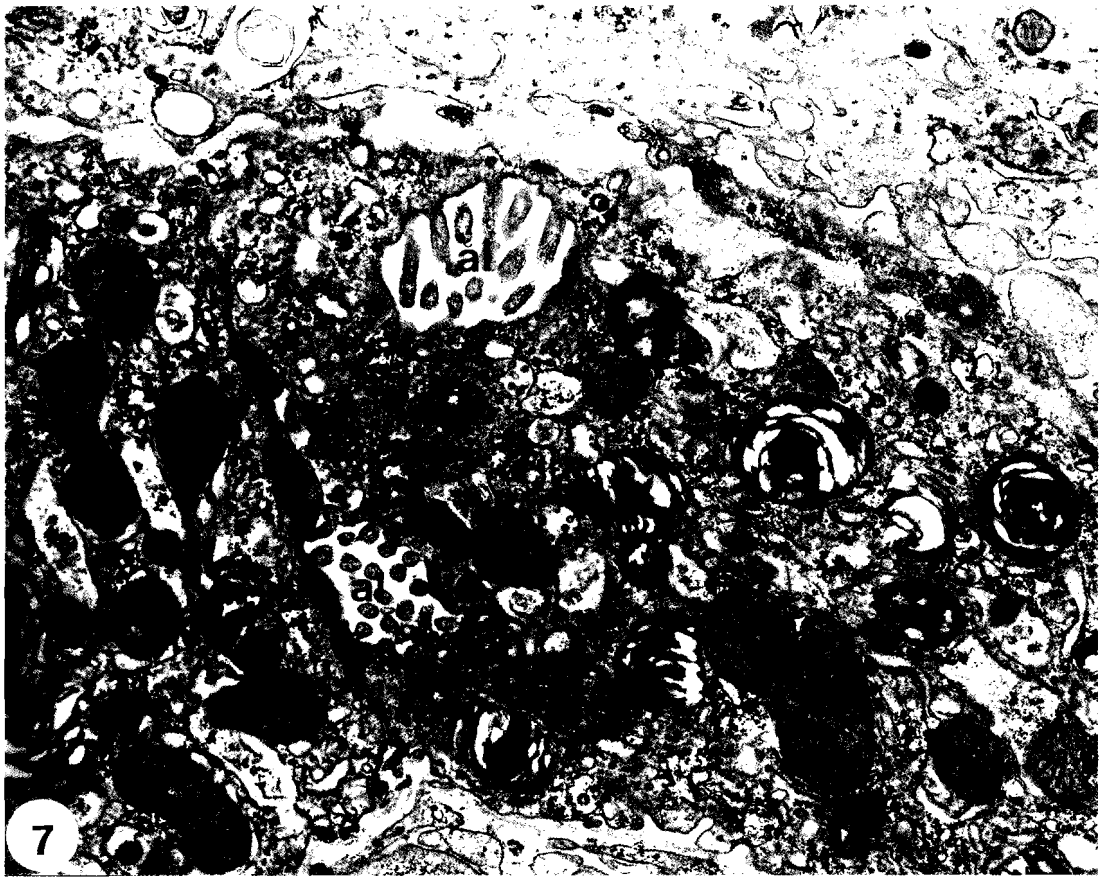

Fig. 7. Type II epithelial cells fill a collapsed alveolus (al). Group I-7. TEM. x 7,000

Fig. 4. A cellular nucleus "goes out" of a type II cell (→). In its vicinity a fragment of an alveolar macrophage (am). Group I-3. TEM. x 3,000

Fig. 5. Lamellar bodies (lb) and oedematous fluid (oe) in the alveolar lumen. Group I-3. TEM. x 12,000

Fig. 6. Accumulation of alveolar macrophages containing numerous secondary lysosomes and phagocytic vacuoles filled with lipid- and/or lipoproteid-like material. Group I-3. TEM. x 3,000

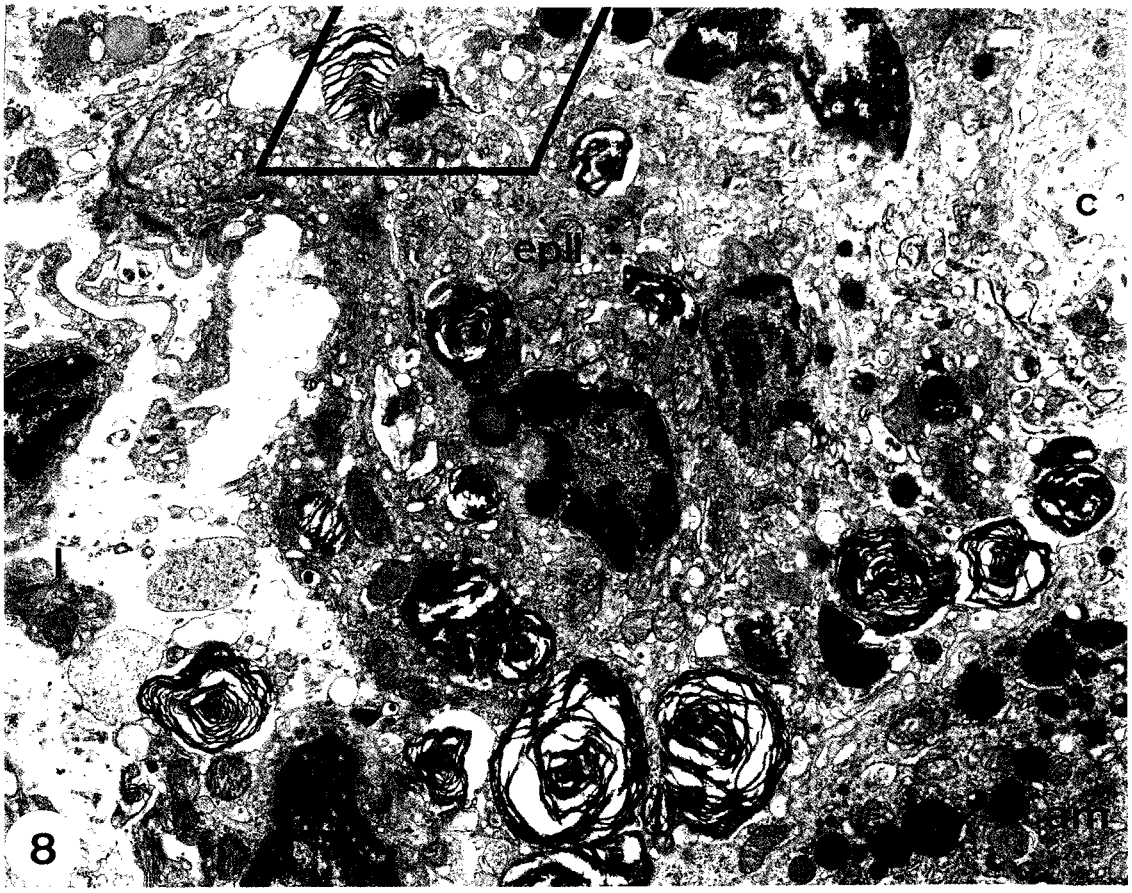

Fig. 8. Fragment of strongly rebuilt lung parenchyma. Type II epithelial cells (ep II) and collagenic fibres (c) are localized within the atelectated alveolar lumen. The bottom right corner shows a fragment of an alveolar macrophage (am). Collagen accumulation is visible from the side of the interstitium (I) of the interalveolar septum. Group I-28. TEM. x 3,000

Fig. 9. Presents a fragment of Fig. 8 (framed). Lack of clear borderline between collagen and type II cells. Parabasilar parts of type II cells (ep II) are strongly developed. In their vicinity processes, most probably belonging to fibroblasts, are seen (f). Group I-28. TEM. x 12,000

type II alveolar epithelial cells to fibroplasia processes, while earlier analyses focused on macrophages and fibroblasts as the only cells participating in fibrosis-dependent pulmonary tissue rebuilding. Strict correlation has been known for a long time between fibrosis processes observed in pulmonary tissue and the increase in the number of type II cells (Rannels and Rannels, 1989; Lwebuga-Mukasa, 1991; Sugiyama and Kawai, 1993; Sulkowski et al., 1993/1994). We know little, however, about active participation of these cells in the synthesis of connective tissue fibres (Crouch et al., 1986). Most studies are based on cell cultures (Crouch et al., 1987; Federspiel et al., 1991; Simon et al., 1993), while there are hardly any reports on studies in situ (Sulkowska et al., 1996a). Adamson et al. (1988) reveal the possibility of active participation of type II alveolar epithelial cells in fibroplasia processes observed in the lungs and related to alveolar epithelium damage. These authors have found a close relationship between alveolar epithelium damage degree and fibroplasia intensification degree in the lungs in mouse pulmonary tissue cultures, in blood-free environment. Their findings seem the more important as the experimental model they applied eliminated inflammatory cell inflow to pulmonary tissue, which usually accompanies destructive processes in the respiratory tract epithelium. Recent studies of pulmonary fibrosis processes have paid special attention to fibroblast growth factors coming from the blood or produced within pulmonary tissue by lymphocytes, blood platelets and macrophages in particular (Bitterman et al., 1983; Goldstein and Fine, 1986; Reiser and Last, 1986; Wahl, 1986). Adamson (1988) reports the possibility of fibroblast proliferation control (and thus, processes of fibrosis) by alveolar epithelial cells. Most probably, severe damage to pulmonary tissue causes permanent or long-lasting disorders of normal intercellular relationship, resulting in enhanced production of connective tissue fibres. Epithelial control of mesenchymal cell proliferation has been reported in other papers (Terzaghi et al., 1978; Smith and Post, 1989; Sulkowski, 1994; Sulkowska and Sulkowski, 1997). Also, results of the present study, particularly ultrastructural analysis in TEM, seem to confirm the above observations. Moreover, in subgroup I-28, pictures were observed suggesting the possibility of active participation of type II alveolar epithelial cells in the development of fibrosis. The type of the experiments performed does not allow a precise definition of the nature of this contribution; however, results of the studies carried out on alveolar cell cultures suggest that type II cells are likely to produce collagen and other elements of extracellular matrix (including proteo-

Fig. 10. The alveolar lumen (al) is filled with collagen-structured material (c) and lamellar bodies (lb). Group I-28. TEM. x 12,000

glycans), which constitute a framework for new connective tissue fibres, and thus enable their formation (Sage et al., 1983; Rannels and Rannels, 1989; Rannels at al., 1992).

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