

Alveolar cells in cyclophosphamide-induced lung injury. II. Pathogenesis of experimental endogenous lipid pneumonia

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Summary. An ultrastructural and histological study was made to analyse the structural and cellular features of the pulmonary lesions produced in Wistar rats by intraperitoneal (i.p.) administration of cyclophosphamide (two i.p. doses of 150 mg CP/1 kg bw/1 ml PBS). Rats exposed to cyclophosphamide (CP) developed a condition whose morphological picture corresponded to endogenous lipid pneumonia and/or pulmonary alveolar proteinosis-like changes. Damage to the endothelium and neutrophil accumulation in lung vascular bed were found to be potential initiators of endogenous lipid pneumonia-type changes. The possibility of the evolution of the acute lung injury into endogenous lipid pneumonia-type changes and into alveolar proteinosis-like changes was demonstrated. The results of the study supplement the existing theories of pulmonary alveolar proteinosis pathogenesis.

Key words: Lung damage, Cyclophosphamide, Ultrastructure

Introduction

Pulmonary alveolar proteinosis (PAP) and endogenous lipid pneumonia (ELP) are regarded as two morphologically and casually different conditions with endogenous fat accumulation in the lung. PAP is characterized by the intra-alveolar accumulation of phospholipids and amorphous proteinaceous material (Claypool et al., 1984). In ELP, the alveoli are filled with foamy macrophages with their cytoplasm dotted with ultrastructurally amorphous lipid droplets (Corrin and King, 1970; Verbeken et al., 1989). Our earlier studies of the ELP and PAP-type changes accompanying primary non-small cell lung carcinomas demonstrated the possibility of their coexistence and suggested a potential transformation of ELP into PAP-type alterations. We

also set up a hypothesis explaining possible mechanisms of the development of ELP-type changes in the vicinity of neoplasms (Sulkowska et al., 1997; Sulkowska and Sulkowski, 1998b). The aim of the present study was to confirm those hypotheses. Therefore, we applied a modified model of lung damage induced by cyclophosphamide (CP), which had been already used in our earlier studies.

Materials and methods

Design of the study

The experiment used 40 male, pathogen-free, Wistar rats of 160-180 g body weight. The animals were maintained in a well sunlit room, at 18-20 °C on standard granulated diet. They were divided into two groups. Group I: (25 animals) was given two intraperitoneal (i.p.) doses of 150mg CP/1kg bw/1ml PBS. Group II: (15 animals) was given two i.p. doses of 1ml PBS. Doses and mode of CP administration, as well as observation periods were established on the basis of our earlier studies (Sulkowska and Sulkowski, 1998a) and pilot research. All experimental animals were sacrificed by intraperitoneal administration of 100mg sodium pentobarbital solution after 1, 3, 7, 14 and 28 days of second dose of CP, so each group consisted of five subgroups labeled I(II)-1, I(II)-3, I(II)-7, I(II)-14, I(II)-28, respectively.

Morphological study

The routine paraffin method was used to prepare sections for histological analysis. After 24 hours of fixation in 4% neutral formalin each lobe was cut into two horizontal planes from edges to the hilum, thus obtaining three complete cross-sections of the lung. The sections were stained with hematoxylin-eosin (HE), periodic acid-Schiff (PAS), impregnated with silver salts according to Gomori and then examined for several parameters. Histological analysis focused on the presence of ELP- and PAP-like changes. We specifically looked for desintegration and proliferation of type II

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Experimental endogenous lipid pneumonia

alveolar epithelial cells, thickening and infiltration of alveolar septa by monocytes and neutrophils as well as presence of foamy macrophages and amorphous debris in alveolar lumen. These parameters were graded from (\pm), to (+++), thus: (+++), changes were found in all animals of a subgroup, and in all or most of the preparations examined; (++) the changes occurred in all animals, but not in all samples examined; (+), the changes were noted only in some of the animals of a given subgroup; (\pm), morphological changes were rare; and (-), no changes.

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 ($+4\text{ }^\circ\text{C}$) 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), 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 acetate, and examined in an Opton 900 PC electron transmission microscope. Sections for TEM analysis were collected from the central parts of both lobes of the upper lungs prior to their fixation in formalin.

Results

Histological study

Light microscope pictures showed foci of slightly intensified congestion and/or oedema of the lungs after 1

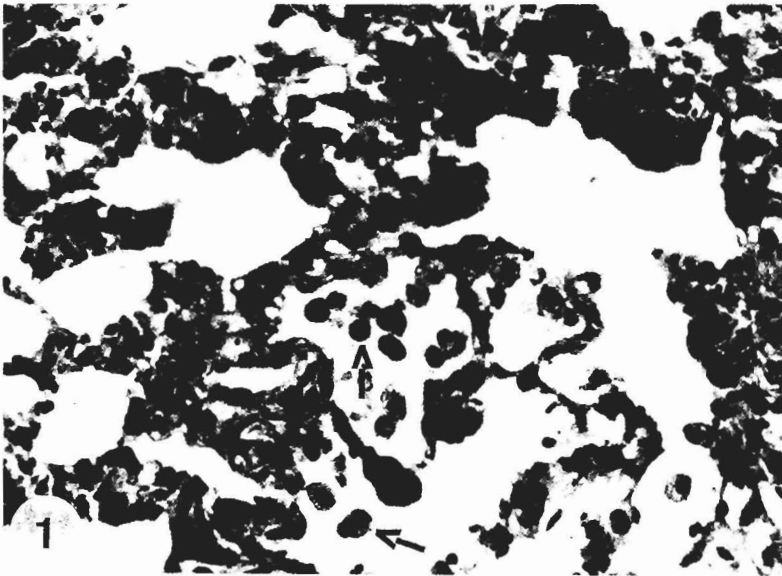


Fig. 1. Early ELP-type changes. Interalveolar septa are infiltrated with inflammatory cells. The alveolar lumen shows focal macrophage agglomerations (arrow). Subgroup I-3. HE, x 240

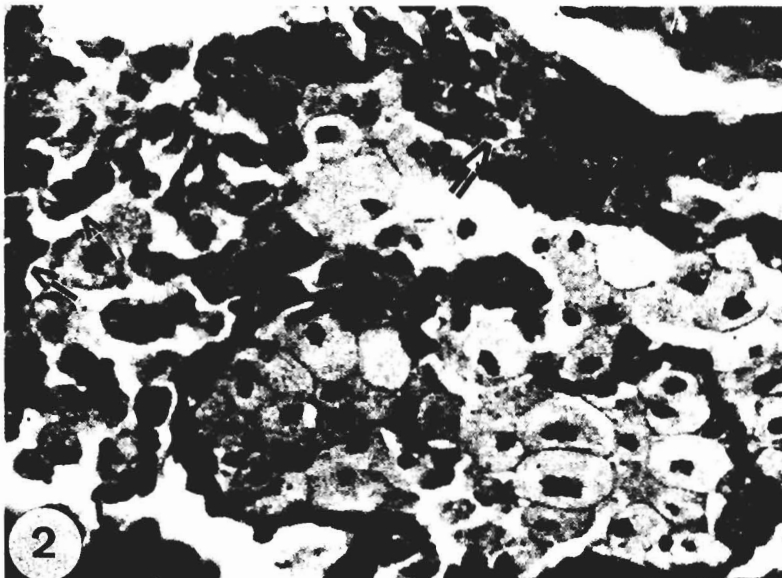


Fig. 2. The alveolar lumen is filled with "foamy macrophages". In places, the alveolar wall is lined with large cuboidal cells corresponding to young type II pneumocytes (arrow). Morphologically similar cells form a several-level layer (double arrow). Subgroup I-14. HE, x 240

day following CP administration (subgroup I-1). After 3 days (subgroup I-3) these changes were much more pronounced. The walls of the alveoli were focally

thickened. Within them, monocytes and neutrophilic granulocytes were accumulated. In certain parts of the lungs, the alveolar lumen showed an increased number

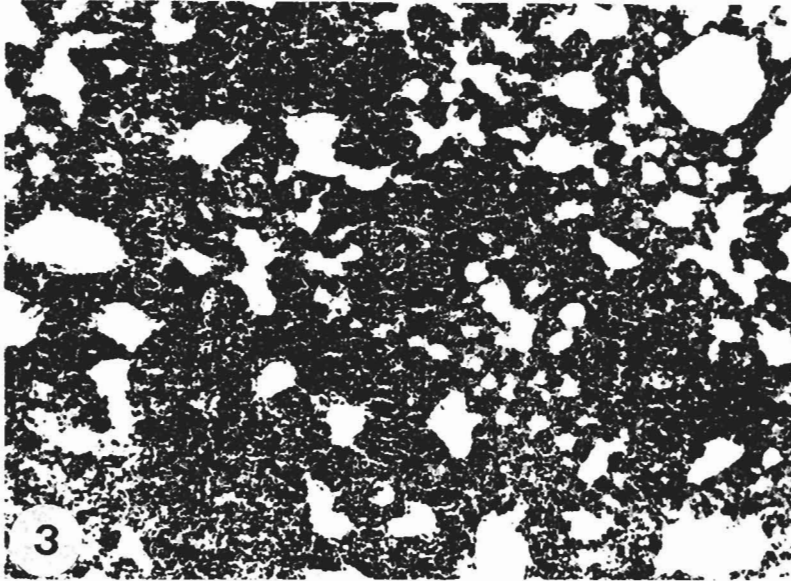
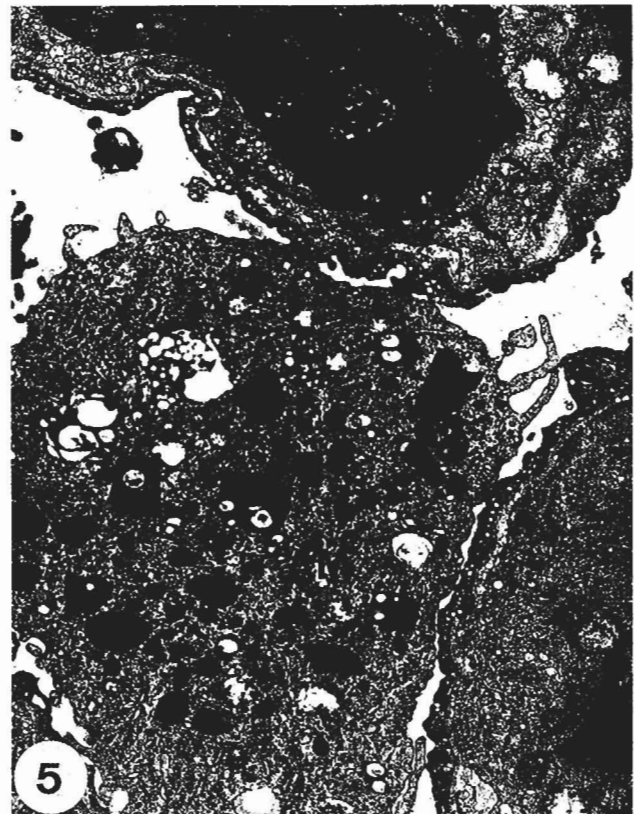
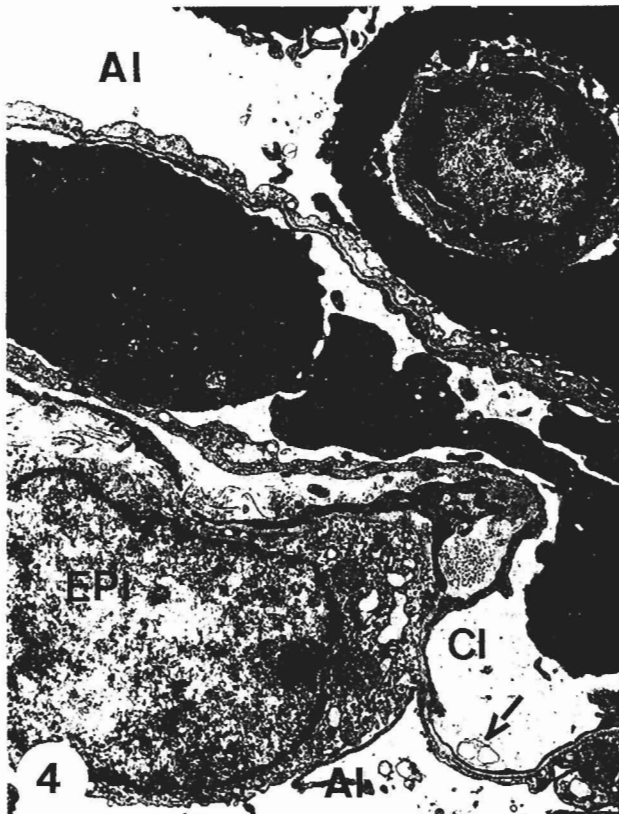


Fig. 3. ELP/PAP-like changes fuse together to cover considerable areas of lung parenchyma. Subgroup I-28. HE, x 80



Figs. 4, 5. Ultrastructural pictures of interalveolar septa of the lungs from subgroup I-1 (Fig. 4) and I-3 (Fig. 5). The capillary lumen (Cl) shows numerous inflammatory cells, which in subgroup I-3 adhere to damaged endothelium (arrow). The alveolar lumen (Al) exhibits fragments of macrophages - one of the cells (AM) contains a number of secondary lysosomes (Fig. 5). A pulmonary alveolar epithelial cell, probably of type I (EP I) shows features of damage (Fig. 4). TEM. Fig. 4, x 3,000; Fig. 5, x 4,400

Experimental endogenous lipid pneumonia

of macrophages, without profuse light cytoplasm. After 7 days (subgroup I-7) following CP administration light microscope pictures revealed more intensified changes of lung parenchyma, compared with the previous groups. The areas of atelectatic lung parenchyma alternated with the areas of oedema; mostly interstitial. The latter showed considerable thickening of interalveolar septa with an accumulation of numerous inflammatory cells,

mainly monocytes and neutrophilic granulocytes. Thickened walls of the pulmonary alveoli were focally lined with large cuboidal cells resembling young type II alveolar epithelial cells, while the alveolar lumen showed large macrophages with profuse foamy-like light cytoplasm - "foamy macrophages". The changes described above were particularly well pronounced in subgroups I-14 and I-28. The results of histopathological

Table 1. Incidence and intensification of histopathological changes observed in ELP- and PAP-like changes

GROUPS	TYPE OF CHANGES						
	Neutrophils and/or monocytes within alveolar septa	Macrophages in alveolar lumen	Damage to alveolar epithelial cells	Proliferation of type II cells	Foamy macrophages in alveolar lumen	Amorphous debris in alveolar lumen	Thickening of alveolar septa
I-1	++	+	+	-	-	-	-
I-3	+++	++	++	-	-	-	±
I-7	+++	+++	+	++	+	-	+
I-14	++	++	+	++	++	±	++
I-28	+	+	+	+	+++	+	++
II 1-28	±	±	-	-	-	-	-

For grading system (±) to (+++) see Material and Methods

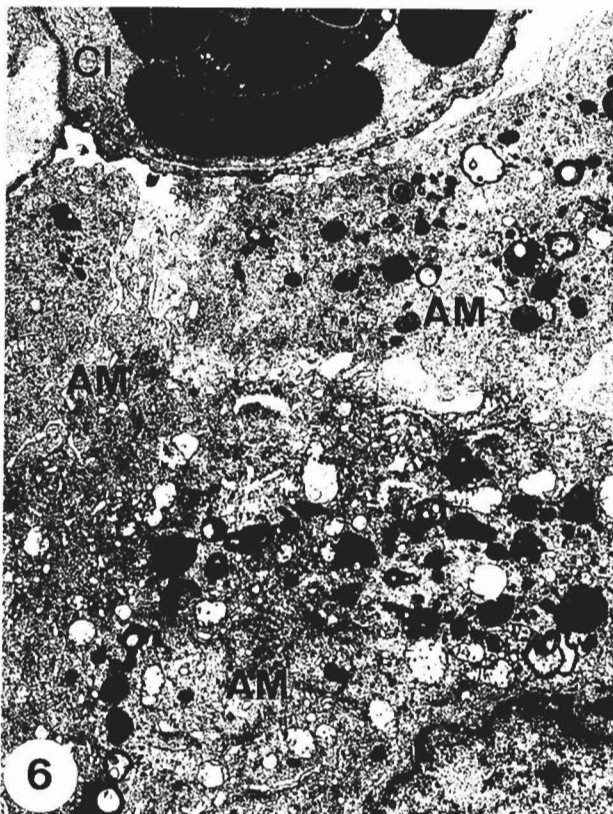


Fig. 6. The alveolar lumen shows macrophages (AM) with numerous secondary lysosomes containing phospholipid-like material. Fragment of a neutrophil visible in the capillary lumen (CI). Subgroup I-7. TEM, x 3,000

Fig. 7. The lumen of an atelectatic alveolus (AI) is filled with mature pneumocytes (EP II) showing differentiated damage degree. Part of the alveolar epithelial lining contains young flattened cells, with no lamellar bodies, which differentiate into type II pneumocytes (EP). Subgroup I-14. TEM, x 4,400

Experimental endogenous lipid pneumonia

examinations of the lungs are shown in Table 1 and figures 1-3.

Ultrastructural study

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. Sporadically, alveolar macrophages were found in the lumen of the alveoli. They showed poorly developed cytoplasmic membrane which formed not very numerous processes and rare secondary lysosomes. The vascular lumen was filled up with plasma and erythrocytes; sporadically, neutrophils, lymphocytes, monocytes and blood platelets were seen.

Experimental subgroups

In subgroup I-1 and I-3, focal damage to alveolar

epithelial cells was observed (Fig. 4). The greatest alterations were found in type II epithelial cells. Most of the cells displayed damage of a varying degree and/or emptiness of lamellar bodies, and disorders in the formation of their contents. In the alveolar lumen, macrophage accumulation was found, usually containing numerous secondary lysosomes (Fig. 5). The lumen of blood vessels showed monocytes and neutrophils, attached to endothelium (Figs. 4, 5). Features of oedema and/or focal endothelial damage were also observed. In subgroup I-7, the areas of lung parenchyma exhibiting domination of macrophage accumulation (Fig. 6) adjoined atelectatic areas. In places, particularly within the atelectatic areas, pulmonary alveolar epithelial lining contained poorly differentiated cells, having no characteristic morphological features. 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 showing features of damage, and sporadically young and mature type II pneumocytes filled up the alveolar lumen. The vascular lumen revealed an accumulation of inflammatory cells, particularly neutrophils and focal endothelial cell damage. The changes described above

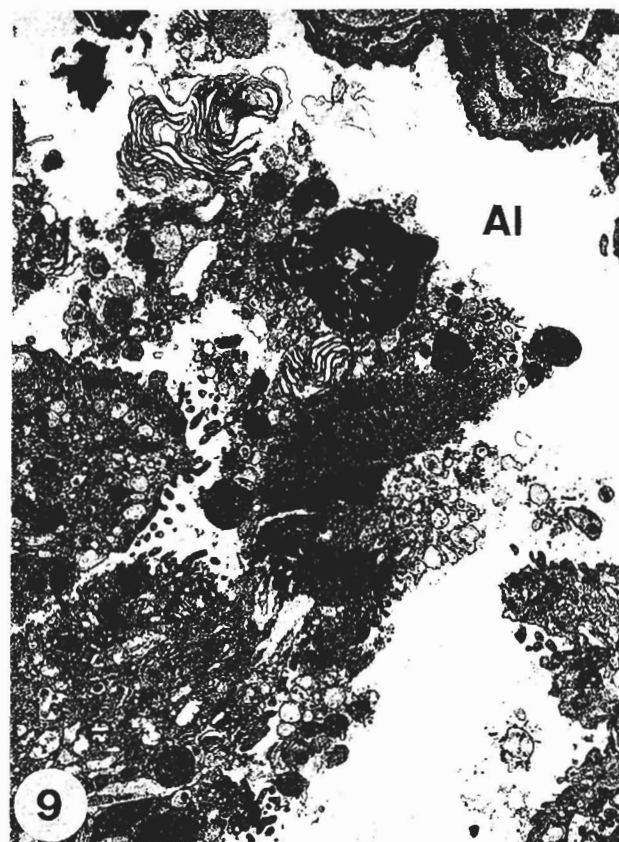


Fig. 8. Severely damaged type II epithelial cells (EP II) release lamellar bodies to the alveolar lumen (Al) (arrow). Subgroup I-14. TEM, x 3,000

Fig. 9. Fragments of disintegrated cells form amorphous debris in the alveolar lumen (Al). Subgroup I-28. TEM, x 3,000

Experimental endogenous lipid pneumonia

were also observed in subgroup I-14 and I-28. The site of atelectated alveolar lumen showed, apart from type II cells with different degrees of damage (Figs. 7, 8, 10), loosely lying lamellar structures and amorphous debris (Fig. 9) as well as lipoprotein-like material (Fig. 10) and numerous alveolar macrophages with secondary lysosomes containing lipoprotein-like material (Fig. 11). The changes within blood vessels did not differ significantly from those observed in subgroup I-7. Some of the capillaries showed only an increased number of blood platelets and occasionally larger fragments of cytoplasm from disintegrated megakaryocytes.

Discussion

Many hypotheses have been proposed to explain the pathogenesis of PAP. The earliest theory of Rosen et al. (1958) supported by the studies of Kuhn et al. (1966) assumed that desquamation and disintegration of pulmonary alveolar epithelial cells, mainly type II cells, is the main cause of the lipid-protein deposit accumulation in pulmonary alveoli. Further studies have

demonstrated that certain proteins present in the masses that fill up pulmonary alveoli originate from blood serum (Rupp et al., 1973), although it is the surfactant elements that are the main source of intra-alveolar deposits, mainly the lipid ones (Sahu et al., 1976; Bedrossian et al., 1980; Claypool et al., 1984). In 1965 Larson and Gordinier established a theory explaining the cause of PAP development as hypersecretion of type II pneumocytes. In subsequent years, this theory was supplemented with alveolar macrophage contribution to PAP pathogenesis. Golde et al. (1976) were concerned with functional insufficiency of alveolar macrophages observed in PAP. According to them, a decrease in surfactant clearance with its increased secretion by type II pneumocytes would create conditions for the pathological accumulation of lipoprotein masses in the alveoli. It seems, however, that macrophage insufficiency is a secondary alteration which depends mainly on their excessive loading with phagocytized surfactant elements. Claypool et al. (1984) suggest that factors predisposing to PAP (occupational dust exposure, hematological neoplasms or virus infections and others)

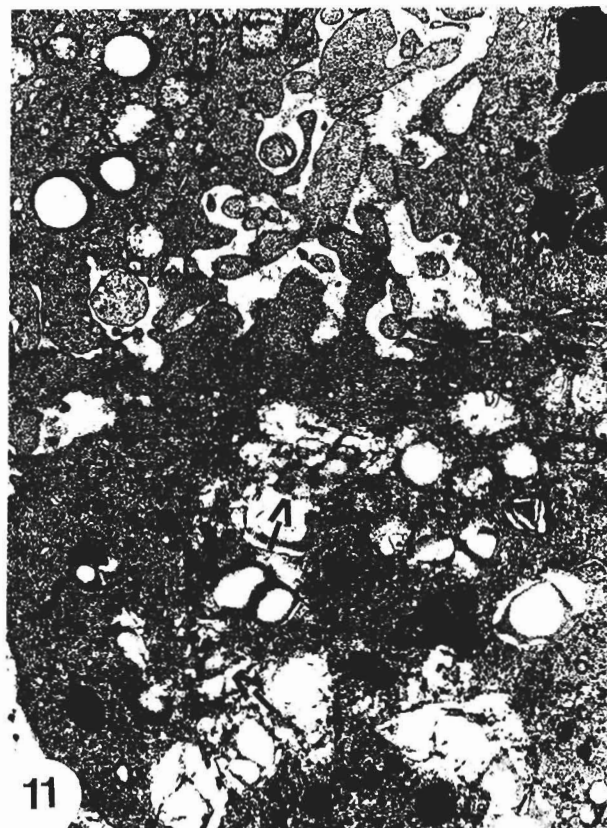
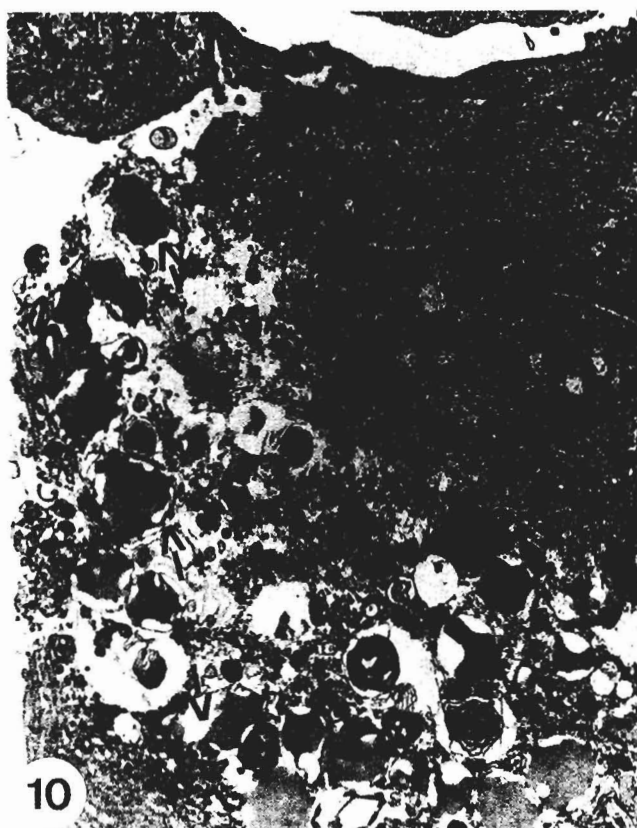


Fig. 10. The alveolar lumen is filled with lipoprotein-like material (arrow), amorphous debris and lamellar bodies from disintegrated cells. The ultrastructural picture of these changes (and changes presented in Fig. 9) resembles those observed in alveolar lipoproteinosis. Subgroup I-28. TEM, x 3,000

Fig. 11. Alveolar macrophages containing numerous secondary lysosomes partially filled with lipoprotein-like material (arrow). Subgroup I28. TEM, x 4,400

could change the receptors of type II cellular membrane of the surfactant appoproteins and thus contribute to a reduction in the reversible reabsorption of damaged surfactant or its excess. This theory, although very interesting, is not fully proved and requires further studies. The concept of Hook et al. (1978) which considers the significance of hydrolases released from the disintegrating alveolar macrophages seems to be a supplement to the above hypotheses. Hydrolases released in excess may provide conditions for desquamation and disintegration of alveolar epithelial cells. The theory is supported by recent studies on molecular mechanisms governing type II cell adhesion. Preliminary data suggest that type II cells express a wide variety of integrins, including α_1 , α_2 , α_3 , α_5 , α_6 , α_v , and β , but their regulation is not well characterized. It seems that all factors which can upregulate specific cell adhesion molecules and integrins will influence the differentiation of EP II as well their adhesion to the extracellular matrix. One of those factors could be proteolytic enzymes (Honn et al., 1994; Wu, 1997).

The results of morphological examinations of the present study confirm the significance of type II pneumocytes and alveolar macrophages in the morphogenesis of PAP-type changes, although at the same time they indicate the role of neutrophils in the morphogenesis of ELP- and PAP-type changes. The accumulation of these cells in the vessels of the interalveolar septa of the lungs was particularly pronounced in the early stage of their cyclophosphamide-induced damage. Damage to pulmonary alveolar epithelial cells and accumulation of alveolar macrophages were also observed in that period. The above pattern corresponds to the early ELP-type changes. Subsequent stages revealed the proliferation of type II cells and the presence of large foamy macrophages in the alveolar lumen. At the same time, the interstitium of the interalveolar septa showed the intensification of fibroplasia processes, and the lumen of blood vessels exhibited an increased number of inflammatory cells, including neutrophils. Simultaneous ultrastructural examinations revealed that some of the foamy macrophages present in the alveoli and type II cells showed features of damage and/or disintegration. This resulted in the appearance of amorphous debris containing damaged cellular organelles and lamellar bodies in the alveolar lumen, corresponding to PAP-type changes. It should be stressed that the above changes were focal and of varied intensity in the respective parts of the lungs, which would suggest "secondary alveolar proteinosis". However, with time, these changes tended to blend and occupy larger areas of lung parenchyma, which would indicate a transition of "secondary alveolar proteinosis" into typical PAP-type changes.

The findings of the present study confirm our earlier hypotheses (Sulkowska et al., 1997; Sulkowska and Sulkowski, 1998b) suggesting a possible transition of ELP-type changes into PAP and support a role of neutrophils as a factor that triggers a chain of changes

leading to ELP/PAP-type changes. The inflow and accumulation of neutrophils in the vascular system is associated with damage or activation of vascular endothelium of the interalveolar septa. Neutrophilic granulocytes, and, to a lesser, extent other cells, particularly monocytes adhering to the vascular walls, are the source of proteases, cytokines and/or reactive oxygen radicals which destroy the structures forming the interalveolar septum of the lungs together with the pulmonary alveolar epithelial cells (Sulkowska and Sulkowski, 1997; Sulkowska et al., 1998). Type II cell proliferation is a response to the damage, particularly to type I epithelial cell. The inflow and accumulation of macrophages in the alveoli is a response to early damage to epithelial cells, surfactant and other structures which form the interalveolar septum of the lungs. Macrophages themselves can both produce the agents that directly damage lung structure (proteases, cytokines) and stimulate neutrophil inflow to the lungs through the production of a chemotactic factor. The vicious circle thus triggered may in consequence lead to the development of ELP and next PAP-type changes.

It seems that the above hypothesis does not coincide with the theories of PAP development presented in the first part of the discussion. On the contrary, it is a supplement to these theories, allowing a better understanding of this interesting lung disorder. The study also indicates the possibility of ELP- or/and PAP-type changes in the course of cytostatic therapy.

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