Histol Histopathol (1996) 11: 633-640

Histology and Histopathology

Type II alveolar epithelial cells and free alveolar cells after intratumor TNF-α administration

M. Sulkowska[1], S. Sulkowski[1], H.F. Nowak[1] and S. Terlikowski[2]

[1]Department of Pathological Anatomy and [2]Department of Gynecology and Septic Obstetric, Medical School of Bialystok, Poland

Summary. The experiment used Morris hepatoma 5123 series growing in muscles of the Buffalo rats. A suspension of $3x10^6$ neoplastic cells was injected into the right hind leg of the animals. After fourteen days, TNF-α was administered into the tumour in a dose of $1.5x10^4$ U/24 hours in 0.5 ml PBS solution. The group I animals were injected for 4 days and group II for 8 days. Control groups consisted of rats with injected Morris hepatoma which were given PBS solution instead of TNF-α (group III A and B) and animals without the hepatoma, given 4 or 8 TNF-α, respectively (groups IV A and B). In the present study, we have explored the effect of intratumor TNF-α administration on the composition of cells isolated from the lungs through multiple bronchoalveolar lavages (BAL). Ultrastructural evaluation of the pulmonary tissue was done using a transmission electron microscope (TEM), with special attention paid to type II alveolar epithelial cells and free alveolar cells. Examinations in TEM in groups I, II and IV (A and B) found, in the lumen of alveoli, an increase in the number of alveolar macrophages (AM) with morphological features of intensified activity and AM with numerous secondary lysosomes containing material of phospholipid structure. Also, numerous type II alveolar epithelial cells with emptied lamellar bodies were observed. The above mentioned changes were especially marked after eightfold TNF-α administration.

In groups I, II and IV (A and B), compared with group III, a significant increase was found in the total number of cells isolated by BAL as well as in the number of cells with positive reaction in staining according to Beckstead's method. It may indicate that the changes in the parameters mentioned above are related to TNF-α action. The results obtained indicate the possibility of systemic effect of TNF-α after its administration into the experimental Morris hepatoma.

Key words: TNF-α, Morris hepatoma 5123, Pulmonary type II epithelial cells, Ultrastructure

Offprint requests to: Dr. Mariola Sulkowska, Department of Pathological Anatomy, Medical School of Bialystok, ul. Waszyngtona 13, 15-269 Bialystok 8, Poland

Introduction

Tumor necrosis factor-α, (TNF-α) is a pro-inflammatory cytokine produced primarily by cells of macrophage/monocyte lineage in response to endotoxin exposure (Kelley, 1990). TNF-α was first recognized for its ability to induce hemorrhagic necrosis of certain tumors (Beutler and Cerami, 1987). TNF-α is distinct from most of the other cytokines in being strikingly toxic. Intravenous administration of TNF-α injures pulmonary arterioles, venules and capillaries, which results in alveolar epithelial injury, alveolar exudation, alveolar neutrophil accumulations, and capillary endothelial injury (Remick et al., 1987). These are also the histological hallmarks of lung injury associated with sepsis. It has been shown that intravenous administration of large amounts of TNF-α to rats induces pulmonary edema and mechanical changes identical to those seen in adult respiratory distress syndrome (ARDS) (Stephens et al., 1988; Tran van Nhieu et al., 1993). Therefore, TNF-α elaborated on the blood side of the alveolar-capillary barrier may be responsible for a major portion of lung injury.

TNF-α induces a variety of other biological effects in addition to its cytotoxicity. These include modulation of endothelial cell and granulocyte function (Gamble et al., 1985; Nawroth and Stern, 1986), differentiation of myeloid cell lines, growth of B-lymphocytes, angiogenesis, induction of collagenase release from mesenchymal cells, and the metabolic activation of macrophages and osteoclasts (Dayer et al., 1985; Philip and Epstein, 1986; Jelinek and Lipsky, 1987).

The studies up to date have focused on the analysis of pulmonary changes after intravenous (or intraperitoneal) administration of TNF-α. At the same time, there has been a lack of thorough morphological descriptions in literature concerning changes within the pulmonary tissue after TNF-α administration into neoplastic tumours. The aim of the present study was to evaluate the effect of TNF-α on the composition and adherence degree of bronchoalveolar lavage (BAL)-isolated cells from the lungs of rats with experimental Morris hepatoma. Morphological evaluation of the lungs was done using a transmission electron microscope with

special attention paid to alveolar macrophages (AM) and type II alveolar epithelial cells.

Materials and methods

Experimental procedure

The experiment was carried out on female Buffalo rats - of 180-220 g body weight. The animals were maintained in a well sunlight room, at 18-20 °C, on standard granulated diet. A suspension of $3x10^6$ cells of Morris hepatoma (5123 series) was injected into the right hind leg of the animals. After fourteen days, TNF-α was administered into the tumour in a dose of $1.5x10^4$ U/24 hours in 0.5 ml PBS solution. The group I animals were injected for 4 days and group II for 8 days. Control groups consisted of rats with injected Morris hepatoma which were given PBS solution instead of TNF-α (group III A, B) and animals without the hepatoma, given intramuscularly 4 or 8 TNF-α, respectively (groups IV A, B). All experimental animals were sacrificed by intraperitoneal administration of 100 mg sodium pentobarbital solution. A detailed description of the experiment and the results of morphological examinations of the primary focus have been presented in our earlier reports (Terlikowski et al., 1995a,b).

Isolation of lung cells

To obtain cells from the lungs, the multiple broncho-alveolar lavage method of Myrvik, also applied in our earlier studies (Sulkowski et al., 1993), was used. The lungs of 8 animals of each group were perfused with a 0.9% NaCl solution at 4 °C prior to removal from the thorax. Therefore, a cannula was inserted into the pulmonary artery, the left atrium was incised and buffered physiological saline solution administered under 20 cm H_2O pressure until complete pallor of the pleura was observed. To obtain cells from the bronchi and alveoli, PBS was administered intratracheally in 5 doses of 5 ml each. Next, the lungs were massaged gently and washings collected into dishes coated with silicone. All fluid portions obtained in the lung lavage were mixed and centrifuged at 600 g for 5 min at 4 °C: the cells were then resuspended in Parker fluid (medium 199, produced by Serum and Vaccine Plant in Lublin). After the lavage, cell vitality was estimated by means of 1% aqueous solution of trypan blue (93-97% of the cells were intact) and the total number of cells isolated from the lungs was counted in a Thoma counting chamber. Cytological slides were made from the fluid concentrated to have $1x10^6$ cells per 1 ml of the solution. Cell composition was calculated in smears stained with H-3 method, following the AS-D naphtol esterase cytochemical reaction according to Leder (1970). In order to visualize alkaline phosphatase-positive cells isolated by BAL from the lungs, the method described by Beckstead et al. (1981) was applied.

Quantitative study

Quantitative analysis of neoplastic metastases to the pulmonary tissue was done based on histological preparations (8-10 μm thick) stained with hematoxylin and eosin. The lungs for the morphometric examinations, taken out of the thorax, were distended using 10% neutral formalin at a pressure of 25 cm. After 24 hours, the lung volume was estimated and then each lobe was cut into two horizontal planes from edges to the hilum, thus obtaining three complete cross-sections of the lung. Only neoplastic cell aggregates localized outside blood vessels were considered to be metastatic foci. The morphometric evaluation of the lungs based on Dunnil's method (Dunnil, 1964) determined the ratio between pulmonary tissue not involved in the neoplastic process and neoplastic metastases. Absolute volume (in cm^3) of metastatic foci in the lungs was calculated from the formula V=100%=P+Q, where V is the absolute lung volume, P and Q stand for proportional size of pulmonary tissue not involved in the neoplastic process (P) and of metastatic neoplastic foci (Q).

All values are presented as means from five assays ± standard deviation (SD). Results of the investigations were analysed with the student t-test using the computer programme, Excel 5.0. The p-value<0.05 was considered significant.

Morphological study

For ultrastructural analysis in the transmission electron microscope (TEM), sections were collected from those parts of the lungs in which no neoplastic metastases were macroscopically observed. Small blocks of 1 mm^3 were cut of the lungs and refixed in cold 2.5% glutaraldehyde solution and osmium tetroxide. 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.

Results

The absolute numbers of cells obtained from the lungs by BAL method and their proportional composition have been given in Fig. 1. In all groups macrophages were a remarkably dominant population. No significant differences were noted in groups I and II with regard to the number of macrophages and neutrophils isolated from the lungs compared with group IV. Such differences, however, were observed compared with group III (A and B). In groups I, II and IV (A and B) a significant increase in the number of cells with positive reaction in staining according to Beckstead's methods was also found. Cytochemical alkaline phosphatase staining according to Beckstead exhibited various reaction intensities in the cells (Figs 2, 3). Cells with focal granular reaction of dark blue and also with a very strong reaction, quite regularly spread within the whole cell, were noted. Phosphatase-positive

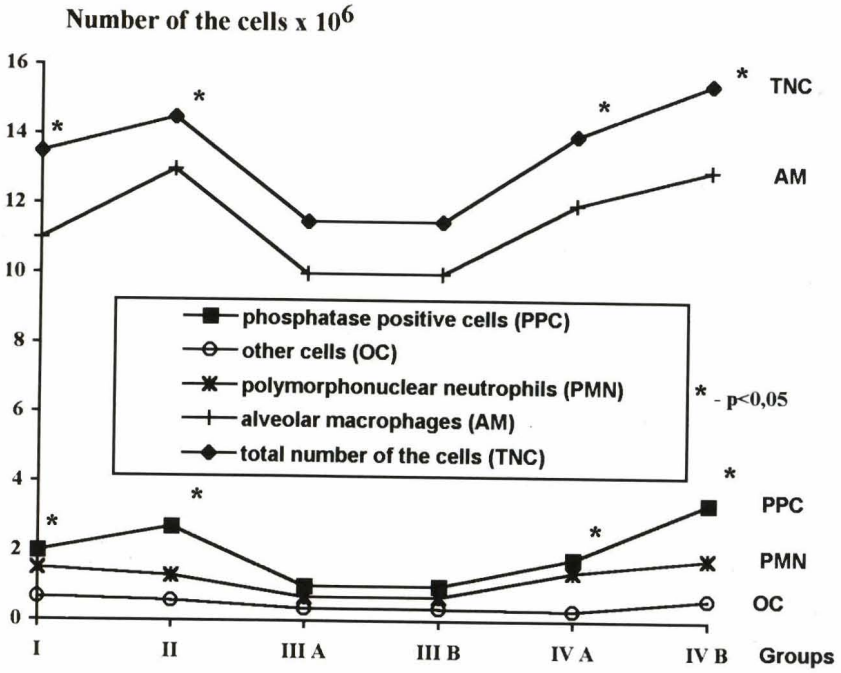


Fig. 1. Cell content of BAL.

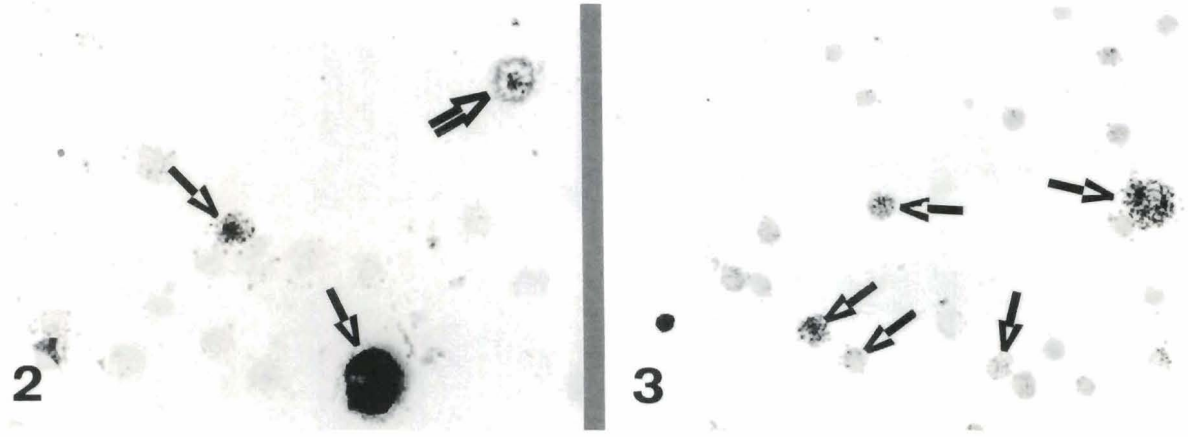


Fig. 2. Reaction to alkaline phosphatase according to Beckstead in cells isolated by BAL from the lungs of rats from group II. Cells with highly positive granular (single arrow) and diffusive reaction (single arrow), and neutrophil (double arrow). x 600

Fig. 3. Reaction to alkaline phosphatase according to Beckstead in cells isolated by BAL from the lungs of rats from group II. Cells with positive reaction to alkaline phosphatase of various degree of intensity. Most cells with pink-stained cytoplasm and few blue granules are the macrophages (arrow). x 400

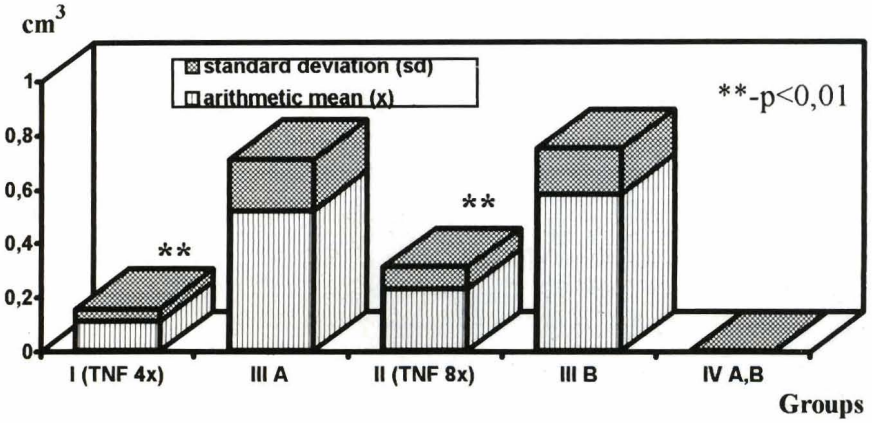
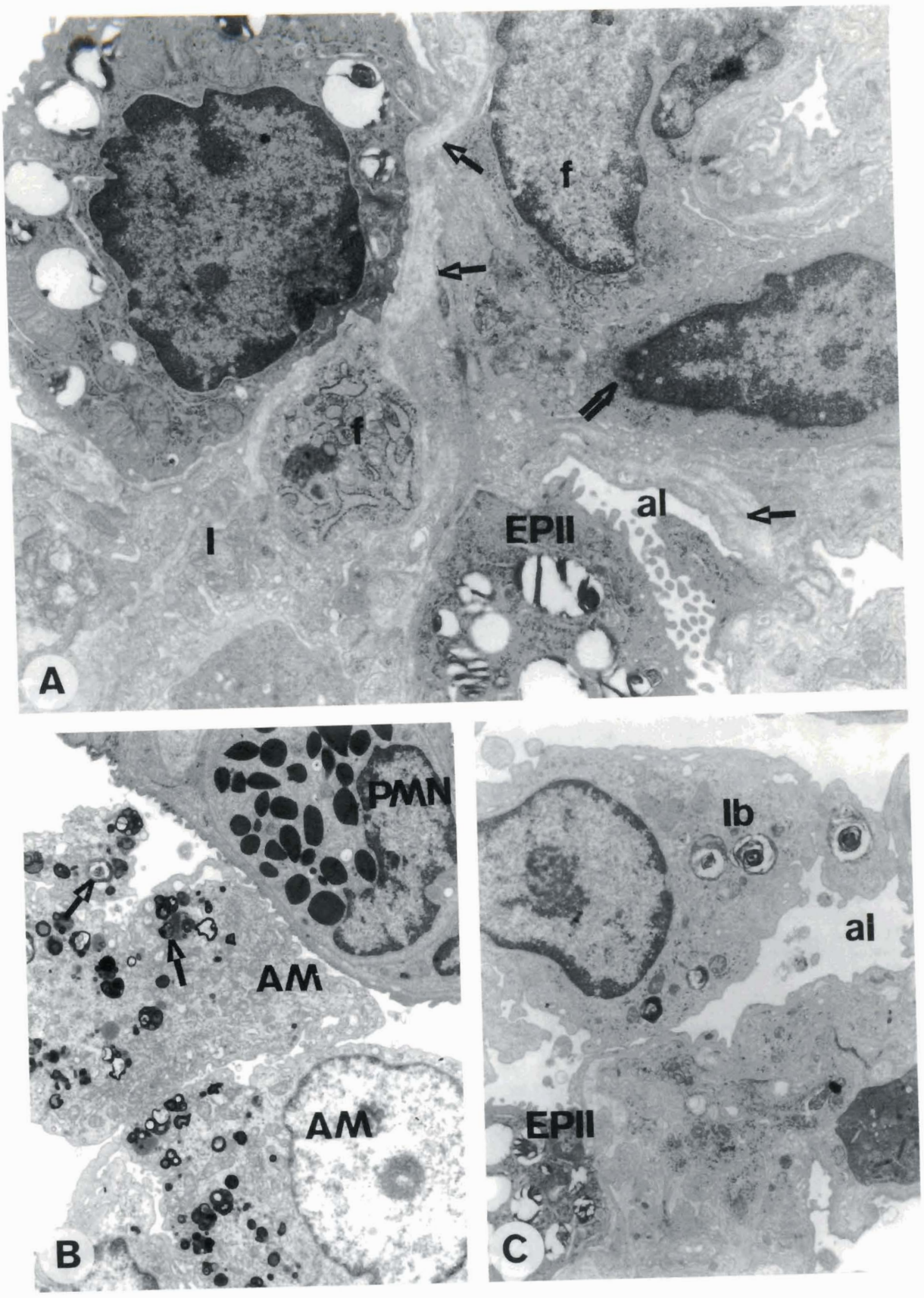


Fig. 4. The mean volume (in cm^3) of pulmonary metastatic tumors.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reaction was demonstrated by some mononuclear cells and neutrophils, the latter being relatively easy to recognize due to distinctive shape of the nucleus. The reaction was negative in a significant majority of cells.

Results of the quantitative analysis of neoplastic metastasis in the lungs of the animals of each experimental group have been given in Fig. 4.

Ultrastructural examinations in TEM in the present study have focused on type II alveolar epithelial cells and free alveolar cells. Among the latter, alveolar macrophages (AM) were found to dominate in all the groups. Granulocytes, sporadically observed in the lumen of pulmonary alveoli, were frequently seen in the lumen of blood vessels, especially in TNF-α-treated animals (Figs. 5B, 7A). The AM population was strongly differentiated in all the experimental groups; however, certain predominant AM subpopulations could be observed.

In all the experimental groups (I-IV), in the lumen of alveoli, AM with numerous secondary lysosomes filled up with phospholipid-like material or/and containing lamellar bodies were found (Figs. 5B, 7B). These cells were irregularly distributed in respective areas of the pulmonary tissue. They were most abundant and found in the largest aggregates in groups II and IVB. These groups (group IVB in particular) also presented the pictures of type II pneumocytes with emptied lamellar bodies (Figs. 5A, C, 7A, C). An interesting phenomenon was blending of partly emptied lamellar bodies with one another and also with type II cellular cytoplasm (Fig. 7A, C). The lumen of alveoli showed loosely lying lamellar structures (Figs. 6B, 7A). It should be noted here that traits of severe damage to alveolar epithelial cells (necrosis inclusive) were only focally observed. Sporadically, in the alveolar lumen, cells containing a variable number of lamellar bodies were seen. These cells may correspond to desquamated II type pneumocytes or AM which phagocytized lamellar bodies ejected from pneumocytes (Fig. 5C). In groups I, II and IV (A, B) a large AM subpopulation with well developed cytoplasmic membranes which formed numerous microvilli was observed in the lumen of alveoli. These cells were especially frequent in group IVB (Fig. 7B). Intensified fibroplasia processes were observed in group III in the interstitium of alveolar septa in TNF-α-treated animals and in the vicinity of neoplastic metastases (Fig. 6D).

Discussion

Cytokines produced by monocytes/macrophages such as tumor necrosis factor α (TNF-α) as well as interleukin-1 alpha (IL-1 α) and interleukin-1 beta (IL-1ß) are in vitro chemotactic for neutrophil granulocytes (Gamble et al., 1985; Nawroth and Stern, 1986). The lungs have been found to be an organ particularly subjected to TNF-α action (Remick et al., 1987). Only few studies have revealed the presence of TNF-α in the serum after its intratumor administration (Mittelman et al., 1992). Thus, the fact of an increased number of BAL-isolated cells from the air passage in the TNF-α-treated animals is interesting. In our studies, both the macrophage and granulocyte numbers were on the increase; statistically significant differences in the number of cells with positive reaction in staining for alkaline phosphatase according to Beckstead were also observed. Possibly, most cells demonstrating intensive granular or diffusive reactions in this staining are type II pneumocytes, subjected to desquamation. It is not unlikely, however, that some of these cells, especially those with poor of focal granular reaction, belong to a subpopulation of alveolar macrophages containing a great number of phagocytized lamellar structures (Sulkowski et al., 1993). This assumption has been confirmed by the discovery of alkaline phosphatase activity both in lamellar bodies and in lysosomes of cells isolated from the lungs (Hook and Gilmore, 1982). However, although alkaline phosphatase activity in lysosomes and lamellar bodies was detected with sensitive biochemical methods, this fact cannot be excluded from the discussion upon the results obtained by means of cytochemical methods. Also, the results of ultrastructural examinations in TEM confirm the assumption that the increase in the number of phosphatase-positive cells observed in BAL, in groups II and IV in particular, may be related to the increase in the percentage of macrophages which contain numerous secondary lysosomes. The material they contain is likely to originate from type II epithelial cell granules or from damaged extracellular lining alveolar layer. In TEM examinations we observed neither severe damage nor desquamation of type II cells. However, we found features of delamelation as well as slight focal damage to these cells. The above changes were especially frequent in groups II and IVB, which may partly justify the obtained results of BAL analysis, especially those referring to cells with phosphatase-positive reaction in Beckstead method. We could not find out if delamelation changes correspond to TNF-α administration, since delamelation pictures were also occasionally observed in group III (without TNF-α). As has already been mentioned, in group III pulmonary tissue, sections were collected for the investigations from macroscopically metastase-free regions. However it cannot be, excluded

Fig. 5. Group II. TEM. **A.** Lung parenchyma with distinct fibroplasia processes within the interstitium (I). Fragments of cells, probably fibroblasts (f) with well developed rough endoplasmic reticulum and a poorly differentiated cell (double arrow) are seen. Basement membranes are thickened (single arrow), with focal collagen accumulation. Type II epithelial cells with partly emptied lamellar bodies by the wall of extended interalveolar septum; one of the cells (EPII) is localized within a collapsed alveolus (al). x 4,400. **B.** In the lumen of an alveolus, macrophages (AM) with relatively poorly developed cellular membranes contain numerous secondary lysosomes filled up with phospholipid-like material; lamellar structures can be seen in some of the lysosomes (single arrow). The lumen of a blood vessel of the alveolar septum is filled with granulocyte (PMN). x 3,000. **C.** In the lumen of an alveolus (al), a cell containing few lamellar bodies (lb). In its vicinity, a type II pneumocyte (EPII) with partly emptied lamellar bodies. x 4,400

that delamelation of type II pneumocytes observed in group III is related to the presence of hepatoma micrometastases to the pulmonary tissue. Metastatic foci being formed, by pressing the adjacent lung parenchyma, produce atelectasis foci, which results in increased ejection of lamellar structures from type II cells. On the other hand, since delamelation phenomenon was also observed in group IV (TNF

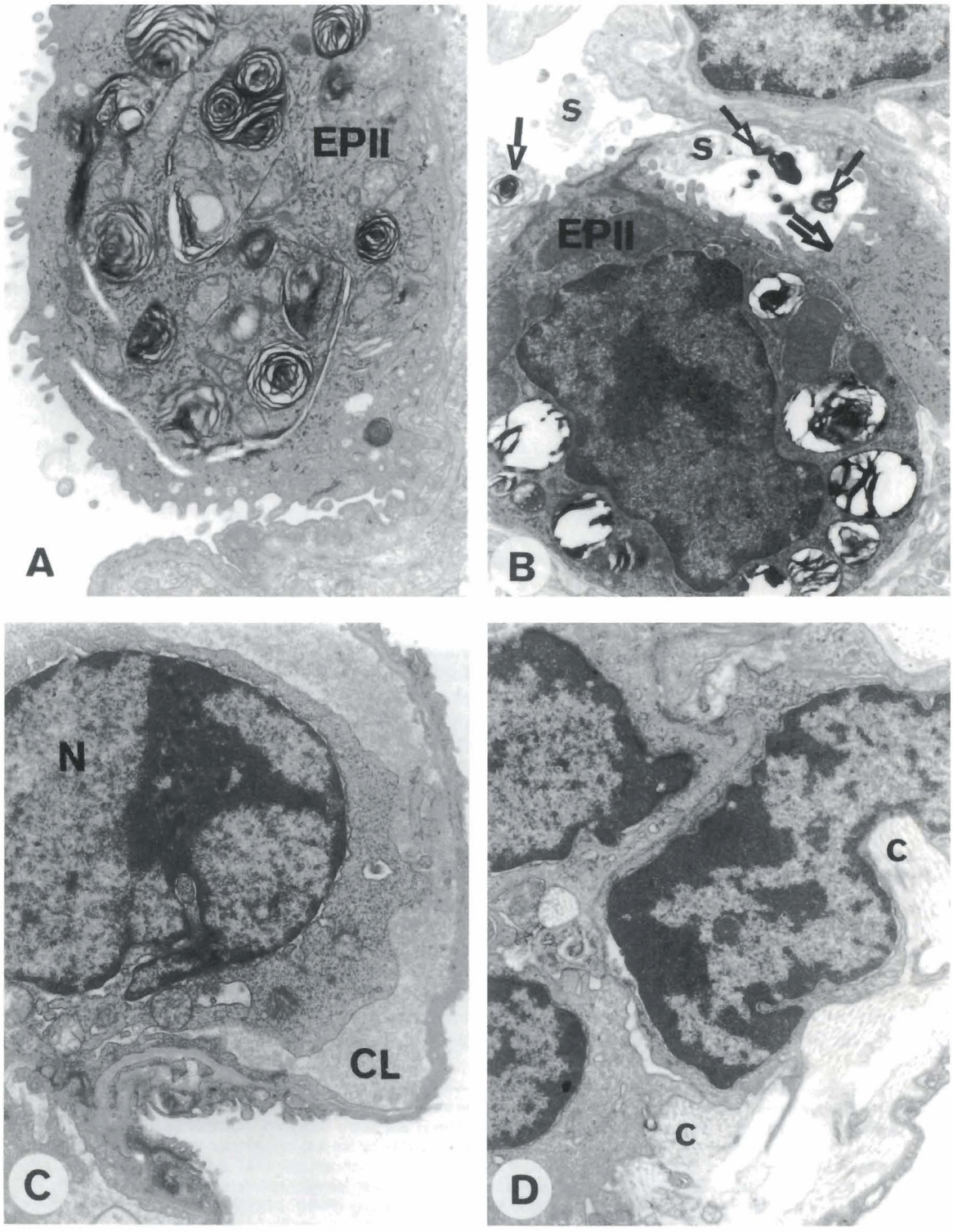

Fig. 6. Group III. TEM. **A.** Ultrastructural picture of a typical type II alveolar epithelial cell (EPII) most frequently observed in the lungs of group III animals. **B.** A type II epithelial cell (EPII) with partly emptied lamellar bodies. In its vicinity, a fragment of another EPII (double arrow) with characteristic microvilli as well as numerous lamellar structures (single arrow) and surfactant element(s). **C.** In the lumen of a blood vessel (CL), a neoplastic cell with irregular distribution of nuclear chromatin (N), scanty cellular organelles and numerous free ribosomes. **D.** In the vicinity of neoplastic cells, collagen accumulation (c) is visible. x 7,000

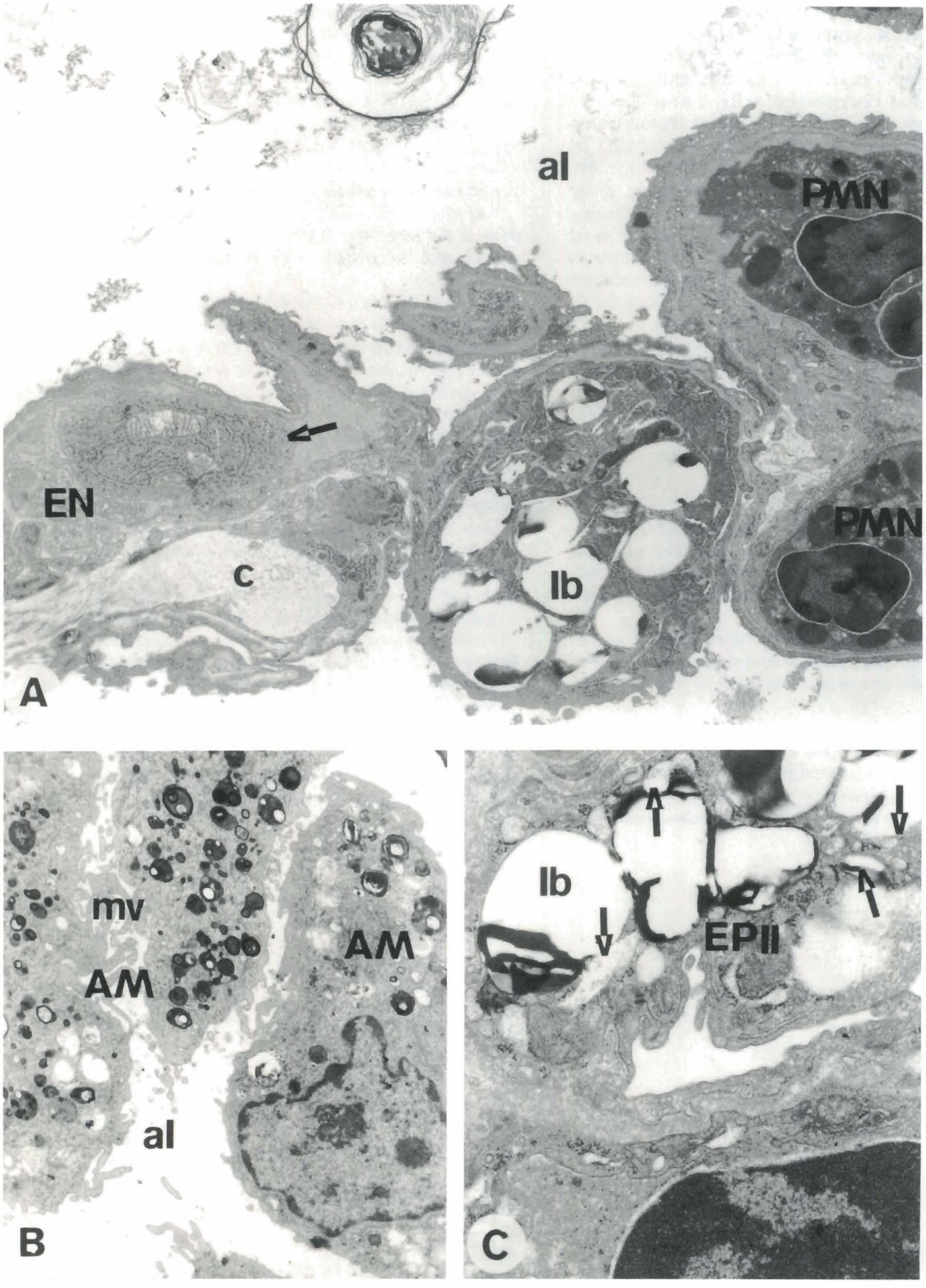

Fig. 7. Group IVB. TEM. **A.** Fragment of the interalveolar septum with focal collagen accumulation (c). In the vascular lumen, granulocytes (PMN) and a fragment of cell (arrow) with well developed rough endoplasmic reticulum; in its vicinity, endothelium (EN) showing stimulation. By the alveolar wall, a type II pneumocyte with emptied lamellar bodies (lb). In the alveolar lumen (al) loosely lying lamellar bodies. x 3,300. **B.** In the lumen of an alveolus (al), a group of macrophages (AM) of differentiated degree of cellular membrane development; an AM forms numerous microvilli (mv). In the cellular cytoplasm, numerous secondary lysosomes filled up with material of electron density corresponding to that of lipids or/and phospholipids. x 12,000. **C.** Fragment of a type II epithelial cell (EPII) with partly emptied lamellar bodies (lb). Blending of emptied bodies with one another and with the pneumocyte cytoplasm can be observed (arrow). x 3,000

without hepatoma), delamelation processes in groups I and II (TNF + hepatoma) may be related to the TNF-α effect. It seems possible that the effect of TNF-α is indirect-connected with the flow of inflammatory cells to the pulmonary tissue, granulocytes in particular.

TNF-α affects target cells through specific receptors p-55 and p-75. Main target cells include granulocytes, fibroblasts, vascular endothelial cells, astrocytes and monocytes/macrophages. Through nuclear transcription factors (NFkB). TNF-α stimulates the production of cytokines, hormones and proteins. The following are produced: ACTH; acute phase proteins; collagen; tissue thromboplastins; and a number of cytokines, including IL-1, IL-6, IL-8; TGF-ß, GM-CSF. The production of TNF-α is also stimulated, as well as the production of metaloproteinases, collagenases and free oxygen radicals in the cells of inflammatory reaction, which secondarily is responsible for tissue damage (Dunnil, 1964).

The results obtained in the present study indicate that the systemic effect of TNF-α may be a cause of changes observed in the pulmonary tissue after administration of this cytokine into the experimental Morris hepatoma.

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Accepted February 5, 1996