

The effects of cyclophosphamide on pulmonary thrombopoiesis in rats

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Summary. Cyclophosphamide (CP), an antineoplastic and immunosuppressive agent, even when administered in large doses, slightly affects the quantity of blood platelets. The aim of the present study was to analyse the effect of single intraperitoneal administration of CP (150mg/kg b.w.) on the quantitative changes in platelets obtained from the left and right ventricle of the heart, as well as to evaluate the occurrence of megakaryocytes in lung tissue depending on the period of time that passed from CP administration.

In control subgroups, fewer platelets were found in the blood collected from the RV compared with the left ventricle at all time intervals. After 1 and 3 days following i.p. administration of CP, a decrease was observed in the number of platelets both in the blood from the right ventricle and left ventricle when compared with control. However, after 14 days, the number of platelets in the blood from the left ventricle was higher, compared with the left ventricle and right ventricle of control animals, and significantly higher ($p < 0.001747$), compared with their number obtained from the right ventricle of CP-receiving animals.

Simultaneous ultrastructural examinations with transmission electron microscopy revealed the increased number of platelets in the lung vascular bed of CP-receiving rats at all time intervals. However, megakaryocytes were found 7 and 14 days after administration of CP. The findings clearly indicate that the lungs could be a major place of thrombopoiesis following therapy with a single large dose of CP.

Key words: Lung, Megakaryocytes, Platelets, Cyclophosphamide

Introduction

The theory of Wright, of 1910, ascribing platelet production to the bone marrow has been generally accepted. Platelets are produced via fragmentation of the cytoplasm of megakaryocytes. Megakaryocytes occur in

the vicinity of vascular sinuses of the marrow and produce two types of cytoplasmic processes (Tavassoli and Aoki, 1989; Young, 1989). One type, derived from the external layer of megakaryocyte cytoplasm, is devoid of cellular organelles. The other type, rich in cytoplasmic organelles and containing alpha granules, is a source of platelets. Already in 1914, Oelhaffen investigated the presence of megakaryocytes in peripheral venous blood. Later studies revealed that megakaryocytes pass with blood from the marrow to a number of organs, particularly to the lungs (Trowbridge, 1988, 1990). They can be found in the capillaries of the liver, spleen, kidneys and heart and sporadically in septum and pathological exudative fluids from the pleural and peritoneal cavities (Aabo and Hansen, 1978; Koss, 1979; Kumar and Naylor, 1980).

The presence of megakaryocytes in capillary vessels of the human lungs was first found by Aschoff (1893). The studies by other authors reveal that the occurrence of megakaryocytes in the vascular bed of the lungs and in the peripheral blood is a physiological phenomenon (Levine et al., 1990, 1993) which can become considerably intensified in certain pathological conditions (Hume et al., 1964; Aabo and Hansen, 1978). A large increase in the number of megakaryocytes in the lungs is observed in those who die of disseminated intravascular clotting (DIC), acute infections, haemorrhages, certain types of neoplasms, liver failure and shock (Aabo and Hansen, 1978). Breslow et al. (1968) found a considerable increase in the number of megakaryocytes and platelets in the peripheral venous blood in post-operative patients.

The aim of the present study was to analyse the effect of a single large dose of cyclophosphamide (CP), an antineoplastic and immunosuppressive agent, on the change in the number of platelets in arterial and venous blood and to evaluate the occurrence of megakaryocytes in lung tissue according to the time that elapsed from CP administration.

Material and methods

Design of the Study

The experiment used 40 male Wistar rats of 160-180

g body weight (b.w.). The animals were maintained in well a sunlit room, at 18-20 °C on standard granulated diet containing 0.55% of cysteine and methionine. All procedures were in strict accordance with the guide for the care and use of laboratory animals and were approved by the local Animal Care Committee. The animals 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 1 ml PBS. All experimental animals were sacrificed under sodium pentobarbital anaesthesia after 1 (subgroup I, II-1), 3 (subgroup I, II-3), 7 (subgroup I, II-7) and 14 (subgroup I, II-14) days of CP (or PBS) treatment. Just after pentobarbital sodium administration, with the heart action still maintained, blood was collected from the left ventricle (LV), and after two minutes from the right ventricle (RV). Once 0.2ml of blood was collected diluted to 2% EDTA Na₂. The number of platelets was determined using a Cell-Dyna 1500 haematological analyser. The number of platelets was estimated in 7 out of 10 rats of each experimental subgroup; their number was not calculated in the animals subjected to vascular perfusion.

Morphological Study

Histological analysis. The routine paraffin method was used to prepare sections for histological analysis. The sections were stained with HE, according to Fulmer and Lille, impregnated with silver salts according to Gomori and then examined for several parameters. We specifically looked for naked cellular nuclei and/or megakaryocytes, pulmonary congestion or oedema, inflammatory infiltrate and fibrosis. These parameters were graded from (+) to (+++), with (+) indicating the least encountered.

Tissue preparation for TEM. Before opening the thorax, the lungs of 3 animals from each subgroup were perfused with Ringer's solution containing buffered 1.5% glutaraldehyde. The lungs of 3 other animals were not perfused. In the rats perfused, a fine catheter was inserted in the abdominal part of the inferior vena cava to reach the right ventricle of the heart. Then, Ringer's solution with glutaraldehyde was administered at a pressure of 30 cm H₂O. At the same time the abdominal aorta was cut in order to allow easier backflow of perfusive fluids from the lungs. The whole procedure lasted 10 min. Later, small blocks of 1 mm³ vol. were cut out of the lungs and refixed for 3 hours in cold (4 °C) 2.5% glutaraldehyde solution in 0.1 M Na-cacodylic buffer at pH 7.4. Fixed tissue samples were washed with 0.1M cacodylic buffer (pH 7.4), and postfixed in 1% osmium tetroxide in 0.1M cacodylic buffer for 1 hour and washed in buffer again. After dehydration in alcohol-acetone series and embedding in epon, they were cut and contrasted with lead citrate and uranyl acetate and examined in an Opton PC-900 transmission electron microscope.

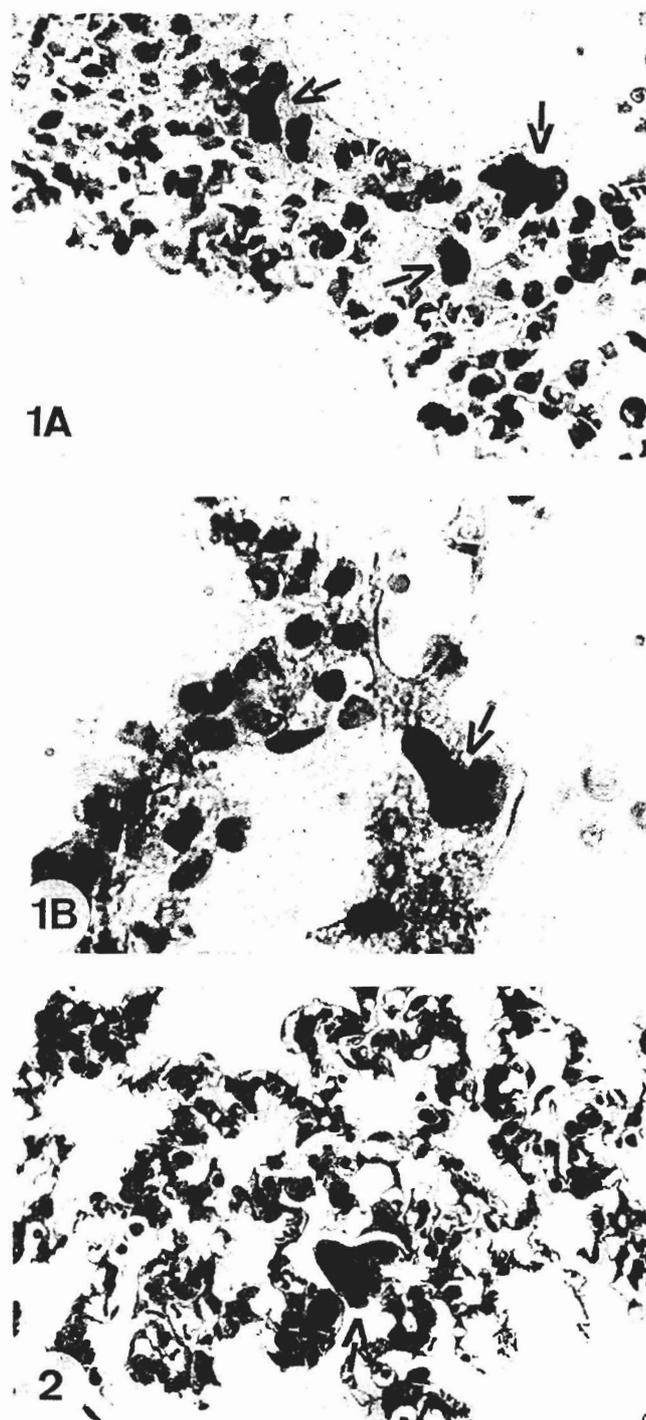


Fig. 1. A and B. Naked nuclei (arrow) of megakaryocytes observed in the lungs of CP-receiving animals. Dilated interalveolar septa are infiltrated with inflammatory cells. Subgroups I-7 and I-14, HE staining. A, x 240; B, x 320

Fig. 2. A megakaryocyte (arrow) seen in the semithin preparation stained with toluidine blue. A fragment of this cell is presented in Fig. 9. Subgroup I-7, x 160

Statistical Analysis

All values are presented as means from seven assays \pm standard deviation (SD). Mann-Whitney U-test was used for the statistical evaluation and the p -value <0.05 was considered significant. Statistical calculations were performed on the Statistica 5.0 programme.

Results

Morphological examination

Light microscope pictures showed foci of slightly intensified congestion and/or oedema of the lungs after 1 day following CP administration (subgroup I-1). After 3 days these changes were much more pronounced. The walls of the alveoli were focally dilated. Within them, monocytes and neutrophilic granulocytes were accumulated. After 7 days following CP administration light microscope pictures revealed more intensified changes of lung parenchyma compared with the previous group. The areas of atelectatic lung parenchyma alternated with the areas of oedema, mostly interstitial.

The latter showed considerable dilation of interalveolar septa with an accumulation of numerous inflammatory cells, mainly monocytes and neutrophilic granulocytes. A new phenomenon (only sporadically observed in subgroup I-3) was the appearance of large, clumpy, naked megakaryocytic nuclei in the vessels of interalveolar septa. The changes described above were particularly well pronounced in subgroup I-14. The vascular lumen showed megakaryocytes and naked cellular nuclei, which may have originated as the result of megakaryocyte disintegration. More intensified features of fibrosis within interalveolar septa and in the

Table 1. Results of histological examinations of the lungs.

GROUPS	NAKED NUCLEI	CONGESTION AND/OR OEDEMA	INFLAMMATORY INFILTRATE	FIBROSIS
I-1	-	+	+	-
I-3	+	++	++	-
I-7	+++	++	+++	+
I-14	++	+	++	++
II-(1-14)	-	-	-	-

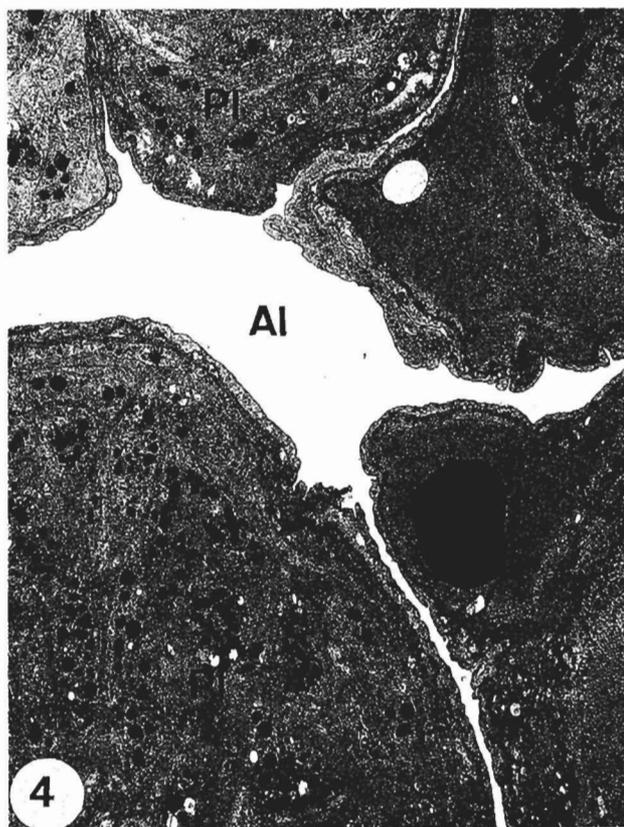
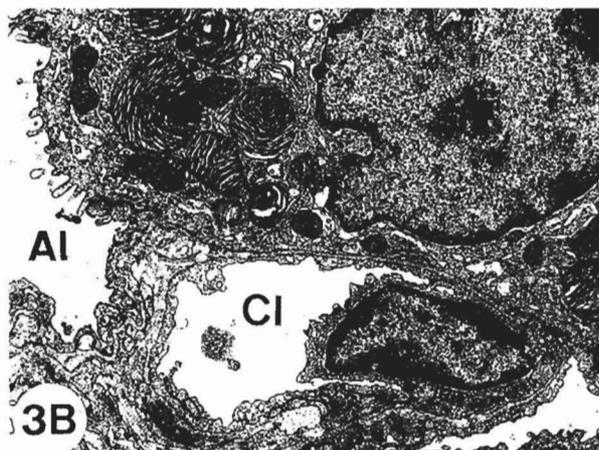
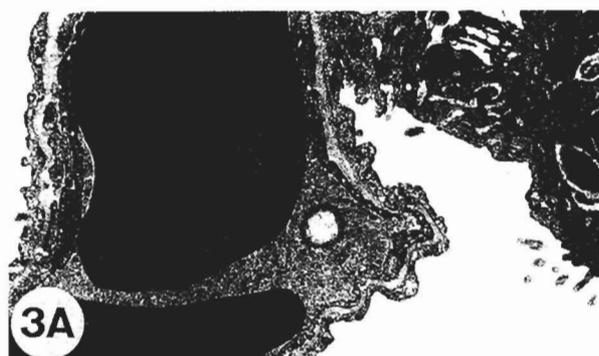


Fig. 3. Fragments of interalveolar septa of control animals. **A.** Without vascular perfusion. **B.** Condition after perfusion. The vascular lumen (CI) is totally devoid of blood cells and plasma. TEM. $\times 4,400$

Fig. 4. Ultrastructural pictures of interalveolar septa 24h after CP administration. Vessels filled with blood platelets (PI) visible in the vicinity of slightly changed blood vessels (on the right). Subgroup I-1. TEM. $\times 3,000$

vicinity of bronchi and blood vessels were observed in subgroup I-14, compared with subgroup I-7. The results of histopathological examinations of the lungs are shown in Table 1, Figs. 1A,B, 2.

Ultrastructural examinations did not reveal any pathological alterations in the structure of the lungs in any time subgroups in control animals. The vascular lumen in the material collected from the non-perfused lungs was filled with plasma and erythrocytes (Fig. 3A); sporadically, neutrophilic granulocytes, lymphocytes, monocytes and blood platelets were seen. The capillaries of the interalveolar septa of the perfused lungs had neither plasma nor cells (Fig. 3B). Only occasionally, single erythrocytes were found in the vascular lumen. After 24 hours from CP administration the lumen of blood vessels showed monocytes and neutrophilic granulocytes (Fig. 5), attached to the endothelium in places and observed in the lumen even after perfusion. Vessels containing platelets were also observed. An interesting finding is that both the vessels devoid of platelets and those rich in platelets occurred in the same neighbourhood (Fig. 4). The vast majority of platelets

were lying, «loosley» in the vascular lumen, not fused with each other nor attached to the endothelium. TEM examinations in subgroup I-3 did not show significant differences in the composition of cells observed in the vascular system of the lungs, compared with subgroup I-1. Focally, however, platelets were joined to the endothelium (Figs. 6, 7). This was seen particularly in the animals subjected to vascular perfusion (Fig. 7). Frequent connections of platelets to one another or to the endothelium were also found in subgroup I-7 (Fig. 8). These changes could be the early stages of blood platelet thrombogenesis and could partially explain the results of quantitative platelet analysis. Focally, in subgroup I-7 as well as in subgroup I-14, the vascular lumen displayed megakaryocytes (Figs. 9, 10) and their fragments (Fig. 8). Megakaryocytes, like platelets, were frequently attached to the endothelium, particularly in subgroup I-7 (Fig. 9).

Quantitative analysis of platelets

Single administration of CP in a dose of 150mg/b.w.

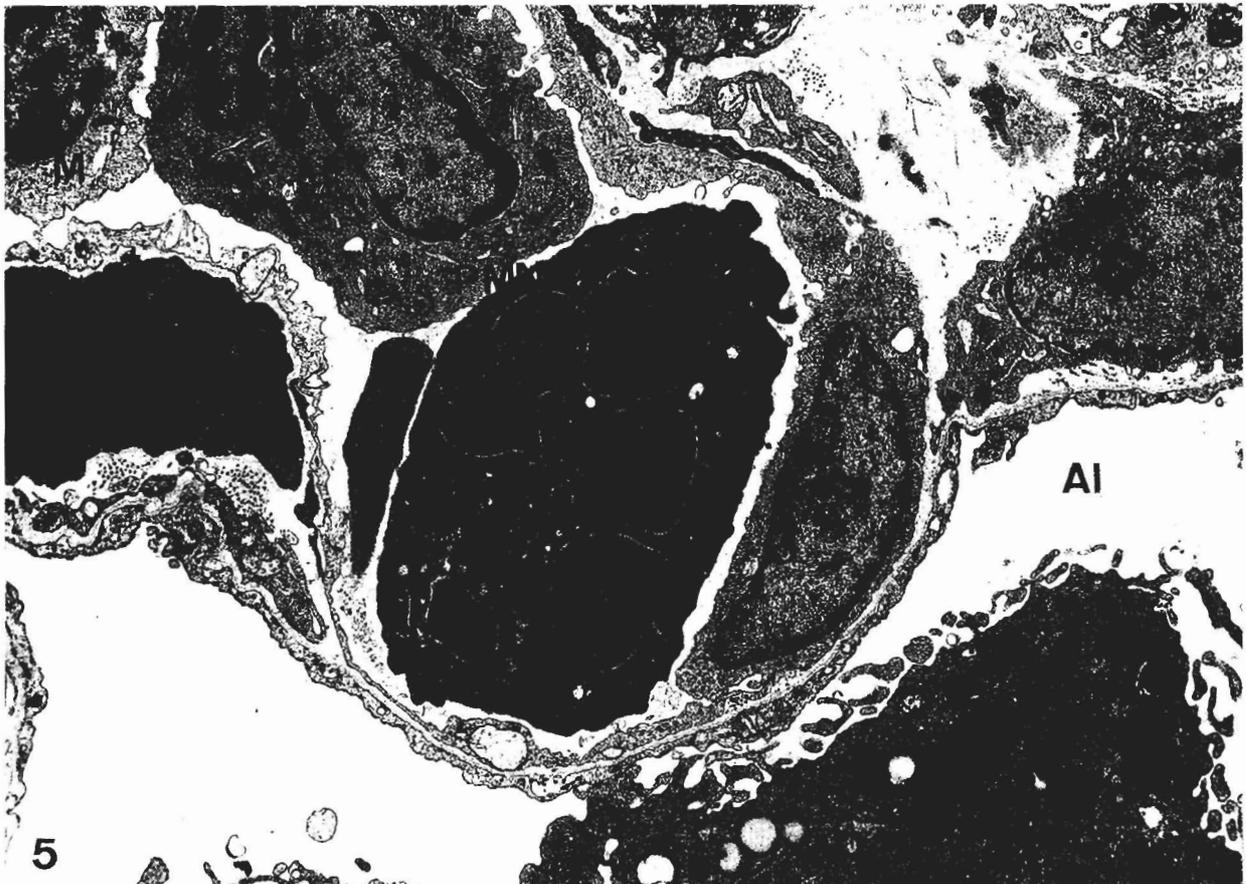


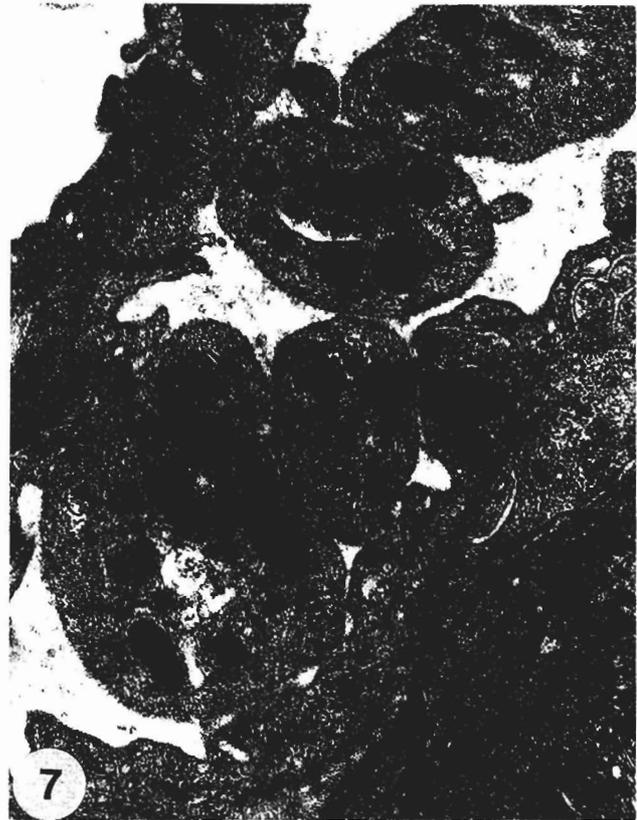
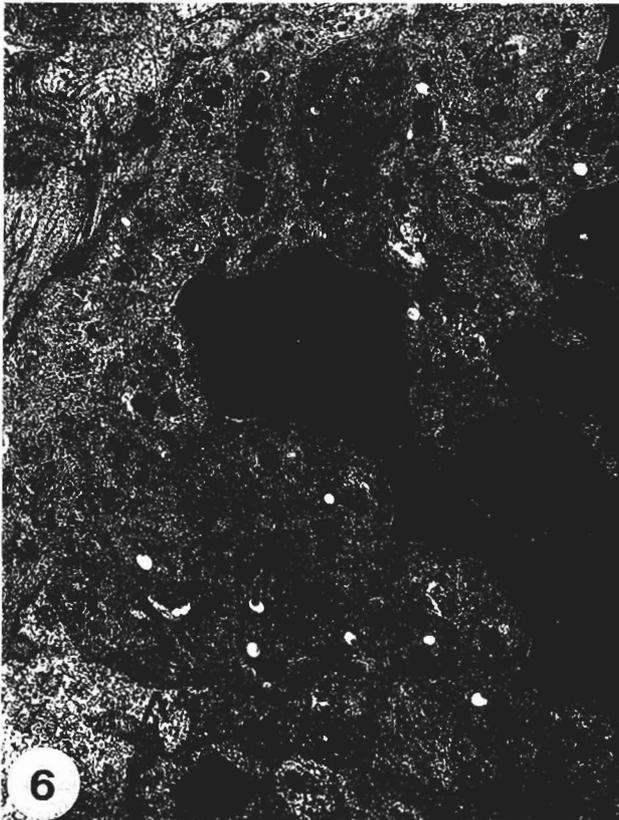
Fig. 5. Ultrastructural pictures of interalveolar septa 24h after CP administration. The vascular lumen, despite perfusion, is filled with neutrophilic granulocytes (PMN), and monocytes (M). The alveolar lumen (Al) shows a fragment of a macrophage (AM). Subgroup I-1. TEM. x 3,000

caused a statistically significant decrease in the number of blood platelets compared with control subgroups. In all control subgroups, blood from the right ventricle had a smaller number of platelets when compared with the left ventricle. Similar dependence was observed in CP-receiving animals (subgroups I-1, 7, 14), excluding subgroup I-3, where the number of right ventricle platelets was higher when compared with the left ventricle ($p=0.007294$). Statistically significant differences were found between the right and left ventricle in CP-treated animals in subgroup I-7 ($p=0.035013$) and in subgroup I-14 ($p=0.001747$).

In blood from the right ventricle, a reduction in the number of blood platelets was observed in all CP-receiving animals when compared with control subgroups (the highest in I-3 and I-7, $p=0.001747$), while in the left ventricle a decrease was found in subgroups I-1 ($p=0.008813$) as well as I-3 ($p=0.001747$) and I-7 ($p=0.001747$). In the left ventricle of subgroup I-14, in CP-treated animals, the number of platelets was higher than in the control subgroup (II-14). However, the differences were not statistically significant. Results of quantitative analysis of platelets in the blood collected from the left ventricle and RV of the heart are shown in Figs. 11-14.

Discussion

Damage to haemopoietic action of the bone marrow with the resulting leucopenia and granulocytopenia and more rarely thrombocytopenia and anaemia, occurs during administration of almost all antineoplastic drugs (Brown and Carbone, 1971; Squires and Lamerton, 1975). CP destroys the granulocytic line. It is generally believed that CP does not significantly damage the thrombopoietic system (Ninkov and Piletic, 1974; Fried and Barone, 1980). The lowest values of blood morphotic parameters are observed between the 7th and 14th day following the administration of CP, while improvement in bone marrow function occurs after 28 days (Pannacciulli et al., 1977). Our present study revealed a statistically significant decrease in the number of platelets in blood from the right ventricle at all time intervals examined. The highest decrease was observed 3 and 7 days after CP administration. A particularly high fall in the number of platelets was found in subgroup I-3 in the blood collected from the left ventricle. We assume that this fall may be associated with platelet arrest in the capillary system, mainly in the interalveolar septa. Morphological observations in TEM seem to support this assumption. In subgroup I-3, platelets frequently



Figs. 6 and 7. The vascular lumen shows blood platelets fused with each other or attached to the endothelium (arrow). **Fig. 6.** Subgroup I-3. TEM. x 4,400. **Fig. 7.** Condition after vascular perfusion. Subgroup I-3. TEM. x 12,000

joined to one another and/or to the vascular endothelium. Platelets were observed in the vascular system even after lung perfusion. The possibility of platelet arrest in lung capillaries was also confirmed by the fact that the number of platelets in the blood that from the left ventricle was lower than that from the right ventricle in subgroup I-3, while in the remaining subgroups this dependence was reversed.

Up to now, there has been no documentation of vessel occlusion by entire megakaryocytes with copious cytoplasm in the pulmonary circulation. There is, however, substantial evidence that megakaryocyte naked nuclei, often with a thin rim of cytoplasm, are seen in these vessels in a variety of mammals (Sharnoff and Kim, 1958; Warheit and Barnhart, 1981; Slater et al., 1983). If the cells deform and squeeze through the pulmonary circulation, megakaryocytes with copious cytoplasm should be found in the pulmonary veins and central arterial blood (Levine et al., 1993). The MK found at these sites are predominantly naked nuclei (Tinggaard Pedersen, 1978; Trowbridge, 1988). The origins and fates of the megakaryocyte enucleate cytoplasmic bodies and naked nuclei remain uncertain (Levine et al., 1993). The fragments could be produced

by detachment of megakaryocyte pseudopods in the marrow sinusoids as they extend through very small endothelial pores (Scurfield and Radley, 1981); however, relatively few naked nuclei have been found in marrow examined by electron microscopy (Radley and Haller, 1983). Our observations in TEM seem to confirm the previous supposition that enucleate cytoplasmic bodies as well as naked nuclei could originate in the vascular bed of the lungs.

The results of quantitative analysis in subgroup I-14 are in our opinion of particular interest. A statistically significant increase ($p=0.001747$) was observed in this group in the number of platelets from the left ventricle when compared with the right ventricle. The increase was also found in subgroup I-7 ($p=0.035013$) and in subgroup I-1 ($p=0.063929$); however, the differences observed in subgroup I-1 were not statistically significant. The results of quantitative analysis in subgroup I-14, together with TEM examinations provide good evidence that the vascular system of the lungs is where platelets are produced after CP administration. Of particular importance is the presence of megakaryocytes in the vascular system of the lungs (subgroups I-7 and I-14), known as the direct source of blood platelets. We

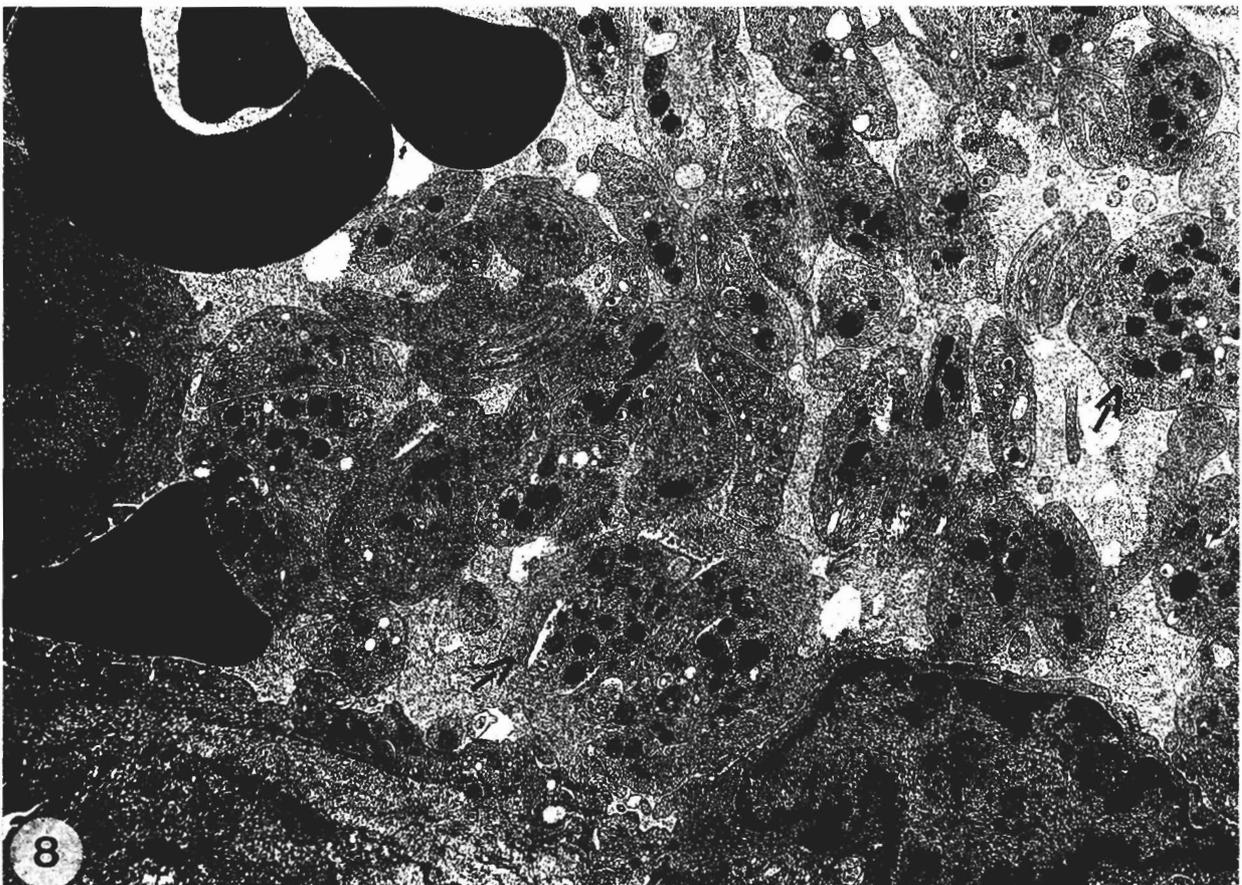


Fig. 8. The vascular lumen is filled with numerous blood platelets and fragments of megakaryocytes (arrow). Subgroup I-7. TEM. $\times 3,000$

Cyclophosphamide and lung thrombopoiesis

have not found, however, any reports in the literature that would consider the lungs a major place of thrombopoiesis during CP administration. The presence of megakaryocytes in the lungs of CP-treated animals, revealed in the present study, may suggest the purposefulness of similar studies in CP-treated people. Considering that CP is a common drug applied to the treatment of neoplastic diseases, the finding of an increased number of extramarrow megakaryocytes during therapy with CP may have a diagnostic importance. Megakaryocytes, particularly those found in the peripheral blood, sputum or cytological material aspirated during thin-needle biopsy of the lung can cause diagnostic errors. Because of the polypoid character of their nucleus, megakaryocytes can be mistaken for cells of a malignant neoplasm (Koss, 1979; Kumar and Naylor, 1980).

Enhanced pulmonary thrombopoiesis is undoubtedly important for the development of pulmonary complications following CP administration, including fibrosing alveolitis (Sulkowska and Sulkowski, 1997b). It cannot be excluded that increased accumulation of platelets in lung tissue is one of the causes of pulmonary

fibrosis after CP as well as after lung injury induced by other factors (Sulkowski et al., 1994; Sulkowska et al., 1996; Sulkowska and Sulkowski, 1997). The hypothesis is supported by the studies of Martin et al. (1983), who, based on the studies of Risk et al. (1982) and Sultan et al. (1981) ascribe stimulation of fibrosis to the platelet growth factor stored in platelet alpha granules. The study by Vignaud et al. (1991) confirms and extends work by Antoniadis et al. (1990) identifying platelet-derived growth factor (PDGF-B)-related gene products and peptides in the lungs of patients with idiopathic pulmonary fibrosis (IPF). Vignaud et al. found that the percentage of interstitial macrophages exhibiting B-chain expression was 3-fold higher in IPF patients than in control subjects. Of note is that positive macrophages were observed both in fibrotic as well as anatomically normal areas of lung from patients with IPF. This observation suggests that PDGF-related peptides are involved during all stages of the fibrotic process, from initiation through evolution.

Platelet accumulation in lung capillaries provides favourable conditions for the formation of platelet thrombi observed in a number of lung tissue injuries,

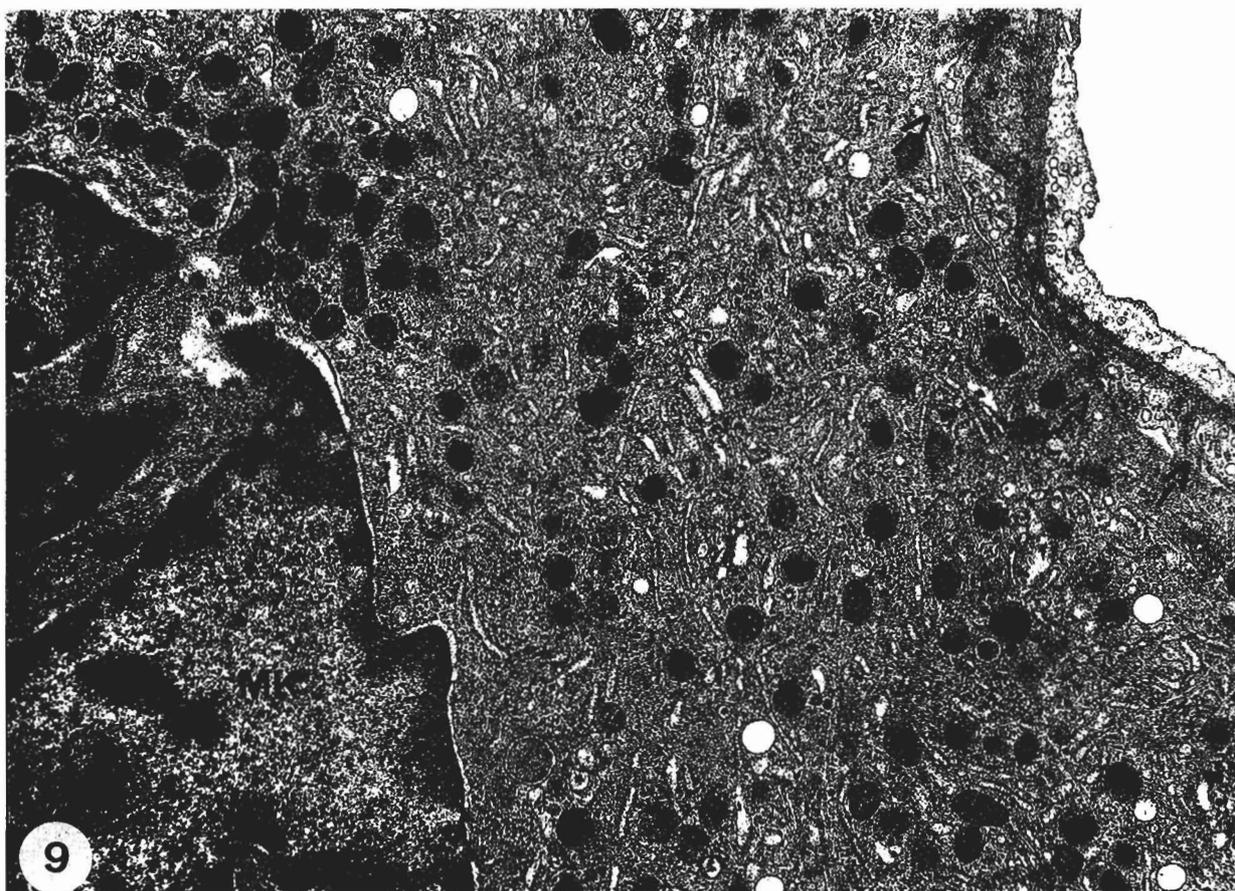


Fig. 9. Fragment of megakaryocyte (MK) from Fig. 2. Typical nucleus and the cytoplasm divided into platelet territories can be seen. The cell is tightly joined to the endothelium in places (arrow). Subgroup 1-7 (condition after vascular perfusion). TEM, x 3,000

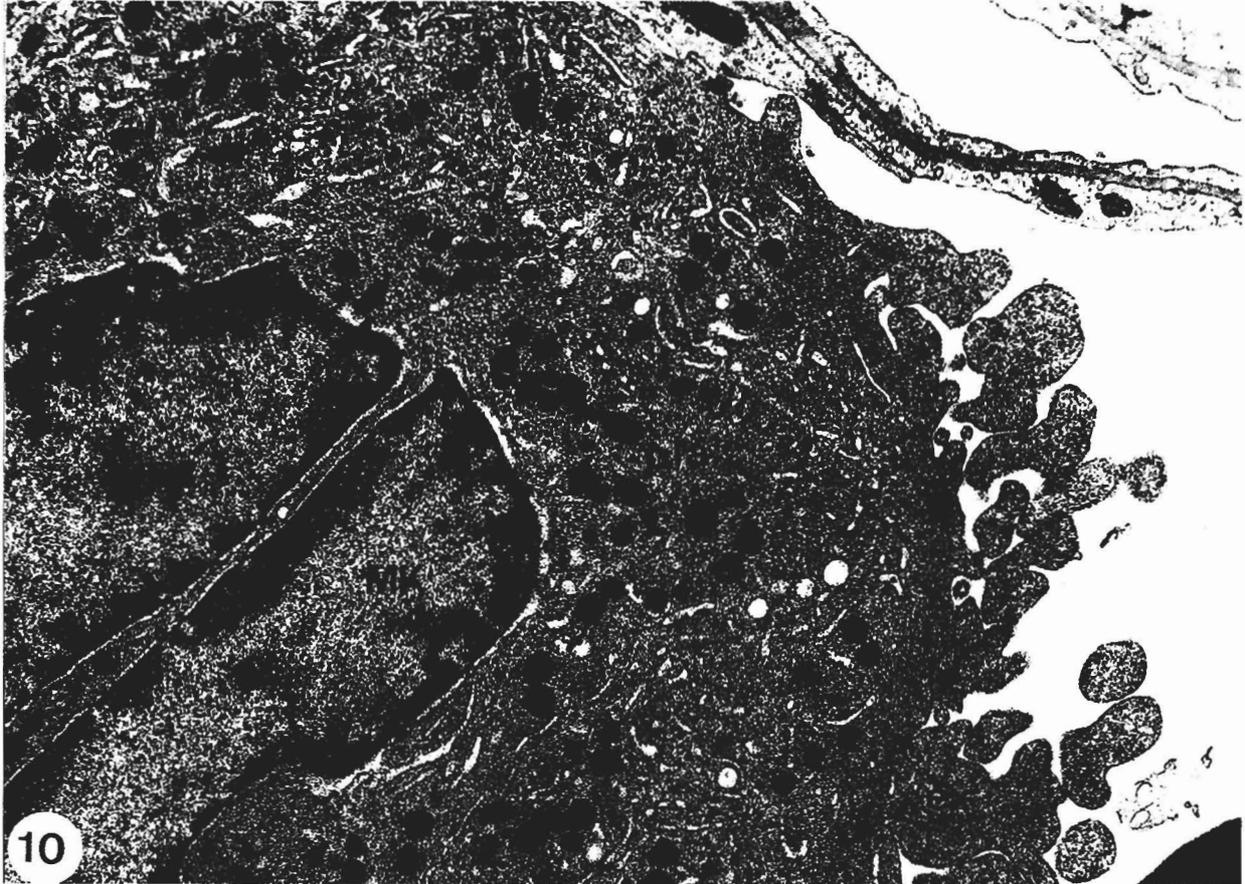


Fig. 10. A megakaryocyte (MK) with preserved division of the cytoplasm in the external layer (ex) forming amoeboid processes devoid of cellular organelles and platelet territory (pla) situated close to the cellular nucleus. Subgroup I-14 (condition after vascular perfusion). TEM. x 4,400

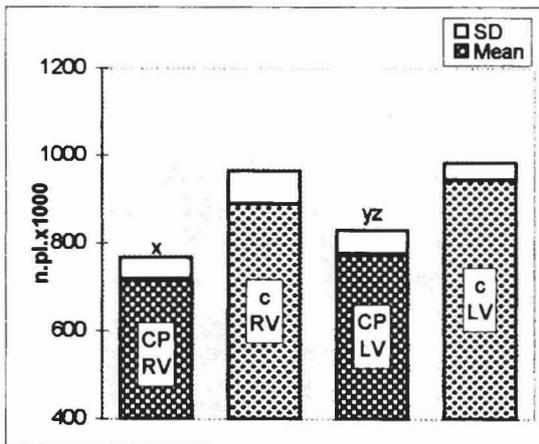


Fig. 11. Mean numbers of platelets (n.pl) ± standard deviation (SD) collected from the right (RV) and the left (LV) ventricle of the heart in control (c) and CP-treated animals. Statistically significant difference: x: $p=0.002678$ (comparison between CP-RV and c-RV); y: $p=0.008813$ (comparison between CP-LV and c-LV); z: $p=0.063929$ (comparison between CP-RV and CP-LV).

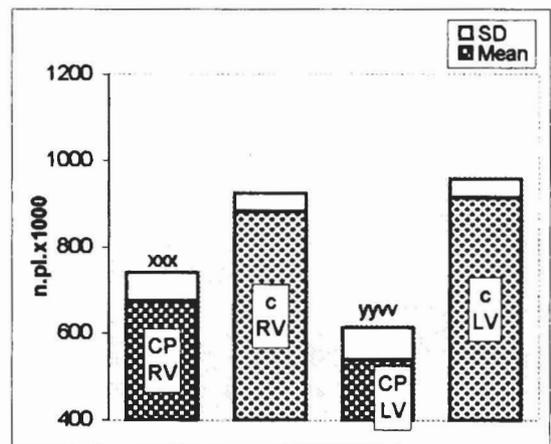


Fig. 12. Mean numbers of platelets (n.pl) ± standard deviation (SD) collected from the right (RV) and the left (LV) ventricle of the heart in control (c) and CP-treated animals. Statistically significant difference: xxx: $p=0.001747$ (comparison between CP-RV and c-RV); yy: $p=0.001747$ (comparison between CP-LV and c-LV); vw: $p=0.007294$ (comparison between CP-RV and CP-LV).

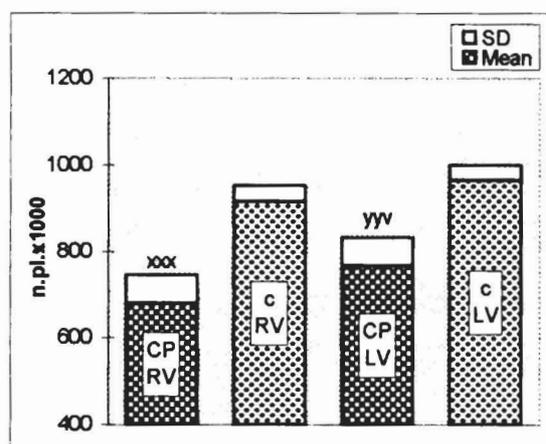


Fig. 13. Mean numbers of platelets (n.pl) \pm standard deviation (SD) collected from the right (RV) and the left (LV) ventricle of the heart in control (c) and CP-treated animals. Statistically significant difference: xxx: $p=0.001747$ (comparison between CP-RV and c-RV); yy: $p=0.001747$ (comparison between CP-LV and c-LV); v: $p=0.035013$ (comparison between CP-RV and CP-LV).

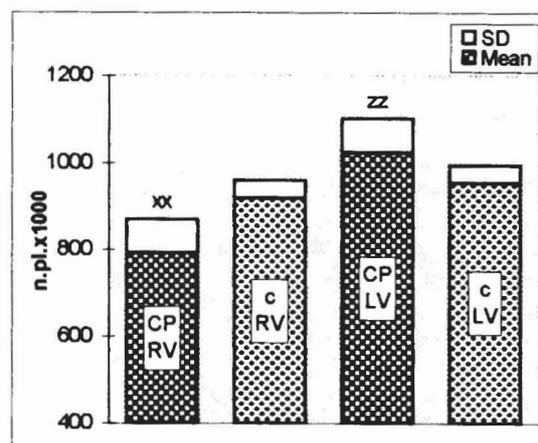


Fig. 14. Mean numbers of platelets (n.pl) \pm standard deviation (SD) collected from the right (RV) and the left (LV) ventricle of the heart in control (c) and CP-treated animals. Statistically significant difference: xx: $p=0.008813$ (comparison between CP-RV and c-RV); zz: $p=0.001747$ (comparison between CP-RV and CP-LV).

particularly in Adult Respiratory Distress Syndrome (ARDS) of septic etiology. Platelets, like granulocytes and macrophages, contain a variety of inflammatory reaction mediators. Mobilized in the lower part of the respiratory system, they can play a paradoxical role and become a direct source of lung destruction (Herscovitz, 1985). Products of platelet secretion (histamine, serotonin, kinin) can stimulate shrinkage of the endothelium, causing dilation of intracellular junctions and increased vascular permeability. All these factors can intensify damage to lung tissue, this being a major complication and a cause of death in CP-treated patients (Venkatesan and Chandrakasan, 1995; Malik et al., 1996).

Present studies, being a continuation of earlier investigations (Sulkowska and Sulkowski, 1998) seem to indicate a significant role of blood platelets in damage-repair processes taking place in the lungs following cyclophosphamide administration, including platelet contribution to the processes that lead to the development of fibrosing alveolitis. Although a single administration of a large dose of cyclophosphamide caused a decrease in the number of platelets in the blood from both ventricles of the heart in the early phase of lung damage, the decrease in the left ventricular blood may have been associated with platelet arrest in the lung vascular bed, as indicated by morphological findings. On the other hand, in the later period, the number of platelets increased, particularly in the left ventricular blood, which supported by ultrastructural analysis points to the lungs as a potential place of blood platelet production following therapy with cyclophosphamide.

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Cyclophosphamide and lung thrombopoiesis

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