Thrombin-Stimulated Effects on Megakaryocytopoiesis and Pulmonary-Platelet Interactions

D. B. WARHEIT[1], S. O. SALLEY[2, 3] and M. I. BARNHART[3, *]

1 *E. I. du Pont de Nemours and Company, Inc., Haskell Laboratory for Toxicology and Industrial Medicine, P. O. Box 50, Elkton Rd., Newark, DE 19714, USA*
2 *Dept. of Chemical and Metallurgical Engineering, College of Engineering, Wayne State University*
3 *Dept. of Physiology, Wayne State University, School of Medicine Detroit, MI*
* *Deceased*

Abstract. The effects of thrombin stimulation on megakaryocytopoiesis and pulmonary-platelet interactions were investigated before and after administration of the compound to 15 mongrel dogs. Each dog served as its own control. Thrombin was given to encourage the traffic of megakaryocytes into the lung and to study the thrombin-stimulated effects on megakaryocytopoiesis in the bone marrow. Our results showed that thrombin increased the numbers of bone marrow cells in general and megakaryocytes (MK) in particular. In addition, the maturation cycle of megakaryocytes was accelerated and the number of MK migrating into the central venous circulation was nearly doubled. Most of the circulating MK ultimately became sequestered in pulmonary capillaries, where platelets were shed into the arterial circulation. We conclude that thrombin has a major stimulatory effect on megakaryocytopoiesis in the bone marrow and that the lung plays an important role as a vascular filter and regulator of circulating platelet count.

Key words: Pulmonary megakaryocytes, megakaryocytes, platelets — Circulating platelet count — Bone marrow megakaryocytes

Introduction

The function of the lung as a storage site for megakaryocytes (MK) and circulating platelets has been in question ever since 1893, when Aschoff first observed megakaryocytes in human lung capillaries (Aschoff 1893). While the precise mechanism of MK shedding of platelets remains controversial, it is clear that platelet release by MK has traditionally been considered a bone marrow event. A number of investigators have quantified or described MK in both the

central and peripheral blood circulations as well as in the pulmonary vasculature under physiological and pathological conditions (Seebach and Kernohan 1952; Scheinin and Koivuniemi 1963; Kaufman et al. 1965a, 1965b; Crosby 1976; Rešl et al. 1987).

In earlier reports, we suggested that the lung plays an important accessory role as a vascular filter and regulator of circulating platelet count, based on morphological evidence of megakaryocytes in circulating blood and lung microvasculature (Warheit and Barnhart 1979,1980,1981). In these studies, scanning and transmission electron microscopic correlations were made on the same individual cells to identify platelet-containing megakaryocytes within pulmonary capillaries. Several previous but indirect studies had presented interpretations regarding the existence and fate of pulmonary MK based on counts and light microscopic evaluations of the cells in pulmonary arterial vs. venous blood, or anterograde vs. retrograde perfusions. Our electron microscopic studies on *in situ* lung megakaryocytes provided useful and direct information regarding the ultrastructural morphology and potential physiology of these cells. Since in the normal individual only small numbers of MK can be found either in the venous blood or within the lung, we elected to stimulate megakaryocytopoiesis and megakaryocyte migration from the bone marrow via thrombin-induced microthrombosis. In this manner we were better able to evaluate megakaryocyte and platelet dynamics within the lung prior to and following intravascular stress.

In the present study we have utilized a thrombin-stimulated microthrombosis model to study quantitative aspects of bone marrow megakaryocytopoiesis as well as megakaryocyte transport and platelet sequestration within the lungs following intravascular stress. Our results showed that thrombin accelerates the maturation cycle of megakaryocytes and augments the numbers of MK migrating into the venous circulation. The majority of these platelet-containing cells subsequently lodge in pulmonary capillaries. The results of these studies suggest that the lung serves as a reservoir for platelets under normal conditions and as a repository for the cells following intravascular stress.

Materials and Methods

Fifteen normal healthy mongrel dogs were anesthetized with 50 mg/kg of sodium pentobarbital, intubated, and ventilated on a Harvard animal respirator. Catheters were placed in the right atrium and aorta via the right jugular vein and right carotid artery, respectively. Arterial pressure and the electrocardiogram were monitored continuously via a Hewlett Packard physiologic recording system. The thorax was opened along the midline and the right apical and cardiac lobes were isolated by placing sutures around the corresponding bronchus, pulmonary artery and pulmonary vein of each lobe. Purified bovine thrombin (courtesy of Drs. Seegers, McCoy, Walz and Teng) was

then infused at a dose of either 100 or 500 Iowa U/kg body weight via either the jugular or femoral veins, over a period of 50 minutes. The thrombin activity was assessed immediately prior to the surgery. Arterial and venous blood samples were collected at the following intervals (1) control, (2) post-thoracotomy, (3) immediately following termination of thrombin infusion, (4) 40—45 minutes post-thrombin, and (5) 2 hours post-thrombin. The control apical lobe was removed prior to the thrombin infusion. The second and experimental cardiac lobe was then ligated and excised during the greatest level of thrombocytopenia (as determined by blood platelet counts from previous experiments).

Immediately after removal, each isolated lung lobe was perfused via the pulmonary artery with phosphate-buffered saline, followed by 0.6 mol/l cacodylate-buffered 1% glutaraldehyde (GAH) (300 mOsm, pH = 7.3). A Harvard infusion/withdrawal pump was used at physiologic pressure. The glutaraldehyde was prepared using a distillation process of the stock solution.

The airway and epithelial lining of the lung lobe was similarly exposed to buffered glutaraldehyde by instilling the fixative fluid at constant pressure of 20 cm H_2O above the lobe's hilum. The fixative-filled lobe was kept immersed in fixative for several hours. Then each lobe was cut into 1 cm^3 pieces and immersed in buffered 1% GAH for 24 hours. Correlative transmission electron microscopic specimens were cut into 1 mm^3 pieces and similarly immersed in buffered GAH for 24 hours. At the end of the experiment, dogs were euthanized with an overdose of sodium pentobarbital followed by an intravenous injection of potassium chloride.

Bone Marrow Biopsy and Smear Preparation

The biopsy procedure was carried out prior to the initiation of as well as 45 minutes following the termination of thrombin infusion. An incision was made on the lateral aspect of the thigh. The vastus lateralis and adductor muscles were separated by blunt dissection to expose the femur. The periosteum was scraped from the bone and a small indentation was made with a scalpel. An electric drill equipped with a 1/16th inch bit was used to drill a hole into the marrow cavity. An 18 gauge needle attached to a 3 or 5 ml plastic disposable syringe was inserted into the marrow cavity, and a sample was withdrawn. The hole was then filled with a small piece of bone wax (Ethicon, Somerville, NJ). Speed was essential to avoid clotting of the sample. The marrow sample was smeared on clean glass slides, allowed to dry, and then stained with Leishman's stain (Allied Chemical, Morristown, NJ).

Technique for Preparation of In Situ Bone Marrow Specimens

A modification of a technique reported by Becker and De Bruyn (Becker and DeBruyn 1976) was used to prepare bone marrow specimens. Phosphate-buffered saline (PBS) followed by 1% GAH was infused through a catheter secured in the deep femoral artery, leading to the nutrient artery of the femur. The femur was isolated as described earlier. Following removal of the bone, a rotating dental saw of high speed steel was utilized to make a cross cut at each end of the shaft. This was followed by longitudinal cuts about 1 mm apart and making contact with the cross cuts. With a fine forceps, the bony splinter thus separated was removed and chilled fixative was dropped onto the exposed bone marrow. The whole marrow was then immersed for 24 h in chilled 1% GAH.

Technique for Obtaining Femur Cell Counts

In order to quantititate the concentration of megakaryocytes per femur, it was necessary to count all the cells in the contralateral femur. While the percentage of megakaryocytes was obtained from bone marrow biopsies in the right femur, the total number of cells were counted in the left femur,

using a dilution technique. Following isolation of the femur, the bone was removed. A sterile syringe containing 50 ml of heparinized PBS was used to aspirate the entire marrow contents into a beaker. Serial dilutions were made, and the cells were counted in a hemocytometer.

Platelet Count Procedure

Whole blood was drawn from catheters placed in the right atrium and aorta for the corresponding venous/arterial platelet counts. These counts were performed by the method of Brecher and Cronkite (1950). Corresponding hematocrit values were obtained simultaneously with the platelet counts.

Counting of Bone Marrow Smears

Megakaryocyte differentials were assessed on Leishman stained bone marrow smears, using a Leitz Lux SM light microscope. The megakaryocytes were classified according to the following four stages (Barnhart and Noonan 1978):

Stage I — Megakaryoblast
Stage II — Promegakaryocyte
Stage III — Adult Megakaryocyte (Reserve + Platelet Forming MK)
Stage IV — Naked Nucleus

Prethrombin MK differentials were compared with post-thrombin MK differentials. In addition, 10,000 bone marrow cells were counted from the smears and the number of MKs/10,000 cells in both prethrombin and post-thrombin slides were assessed and compared.

Circulating Blood MK

The technique of Kaufman et al. (1965a; 1965b), as modified by Tinggaard Pedersen (1971, 1974) was used to quantitate circulating blood MK. Briefly, 2 ml of whole blood was obtained via catheters placed in the right atrium and aorta. The syringe was then rapidly inverted 20 times, and the blood transferred to a centrifuge tube containing 13.3 ml of a polyvinyl pyrrolidone-formalin solution (Sigma Chemical Co., St. Louis, MO). A 4% formalin solution was then added to obtain a better fixation of the cells. The cells were centrifuged at 170x*g* for 20 minutes; the supernatant was decanted and the sediment resuspended in PBS and filtered through Millipore filters (pore size = 4.5 μm). The filters containing the cells were stained with either a Papanicolaou or May-Grünwald-Giemsa stain. Subsequently, the numbers of MK were quantified using light microscopy.

Scanning Electron Microscopy (SEM)

After completion of fixation by immersion in 1% GAH, SEM specimens were cleared in a cacodylate-buffered sucrose solution and then placed through a series of graded dehydration steps in ethanol. Samples were critical-point-dried using Freon® 13. Most specimens were subjected to an ethanol-infiltrated cryofracture technique using liquid nitrogen (Humphreys et al. 1974), and were similarly critical-point-dried. All samples were then placed on stubs, gold sputtered, silver plated, and placed in an ETEC-Autoscan electron microscope for study and photography.

Transmission Electron Microscopy

Following glutaraldehyde fixation and washing, specimens were post-fixed in 1% OsO_4 for 2 hours.

Table 1. Megakaryocyte Series of Cells in Bone Marrow

	Differential Percentages (± S.D.)			
	Thrombin (100 U/kg) $n = 8$			
	Stage I	**Stage II**	**Stage III**	**Stage IV**
Prethrombin %	32 ± 14	20 ± 10	36 ± 9	12 ± 7
Post-thrombin %	29 ± 8	22 ± 9	26 ± 10	23 ± 7
	Thrombin (500 U/kg) $n = 2$			
Prethrombin %	26 ± 18	20 ± 21	44 ± 3	11 ± 1
Post-thrombin %	32 ± 1	17 ± 18	17 ± 1	34 ± 17

After dehydration, infiltration and embedding in Maraglas, ultrathin sections were cut with a diamond knife. These were doubly stained with uranyl acetate and lead citrate and examined in an RCA-EMU-4 microscope operated at 50 kV.

Statistical Analyses

All statistical analyses were carried out using Student's *t*-test analysis of the data.

Results

Bone Marrow Concentrations

The mean megakaryocyte differential percentages for pre- and post-thrombin specimens are given for all experiments in Table 1. The percentages of stage IV MK in the bone marrow were significantly enhanced following thrombin administration. In addition, thrombin stimulated a significant increase in the concentration of bone marrow megakaryocytes (Fig. 1). MK comprised approximately 0.2% of all bone marrow cells in control specimens (21.4 MK/10,000 cells). Following thrombin infusion, the incidence of megakaryocytes was increased by 65% to 37 MK/1000 cells. Analysis of the data indicated that evaluation of the two factors independently, (i.e., MK differential percentages and concentration) did not accurately reflect the thrombin-initiated effects upon bone marrow MK. As a result, we combined these factors to analyze the mean values of MK compartments before and after the thrombin stimulus (Fig. 2). Accordingly, the finding of significant quantititative differences in 3 of 4 compartments implies that there was an acceleration of the maturation rate of megakaryocytopoiesis, combined with an enlargement in the total megakaryocyte pool.

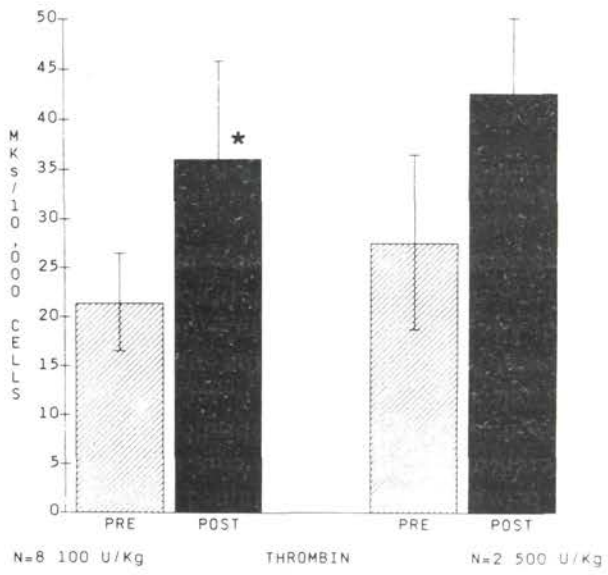


Fig. 1. Bone marrow MK concentrations (values ± S. D.) prior to and immediately following thrombin administration. The numbers of MK were significantly increased ($*p < 0.05$) in dogs infused with 100 U/kg of thrombin.

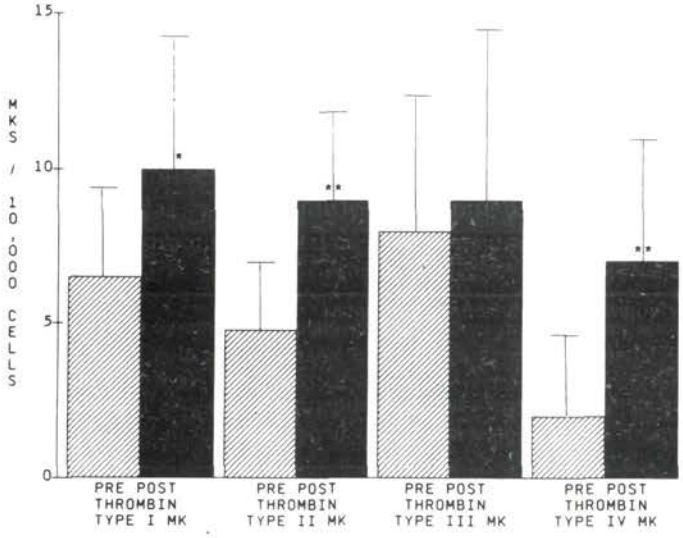


Fig. 2. Bone marrow MK differential concentrations (values ± S. D.) before and after thrombin administration (100 U/kg). Note the significant increases in Types I, II, and IV MK following thrombin stimulation. ($*p < 0.05$; $**p < 0.01$; N = 8 dogs).

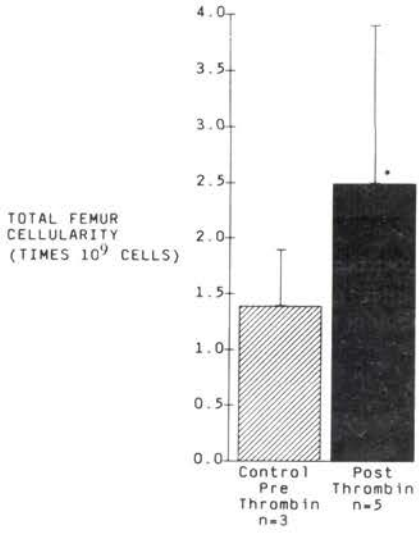


Fig. 3. Changes in total femur cellularity (values ± S. D.) prior to and following thrombin (100 U/kg). The absolute numbers of cells in the marrow were significantly increased following thrombin stimulation. (*$p < 0.01$).

In order to determine whether the thrombin stimulus had a direct effect upon the MK compartment of the bone marrow, or perhaps might have augmented MK numbers as a by-product of an enhanced bone marrow cellularity effect, total femur cellularity values were quantified in dogs prior to and following thrombin administration. Our results demonstrated that total femur cellularity values nearly doubled within 45 minutes post-thrombin (Fig. 3). These data suggested that thrombin stimulation may effect several influences upon the bone marrow. In addition to a direct action on MK compartments, thrombin may have produced direct stimulatory effects upon undifferentiated cells, as well as on committed cells in the precursor compartments of the granulocytic and erythrocytic series.

As a consequence of the femur cellularity data, it was necessary to modify our conclusions based upon MK changes per 10,000 cells and assess changes in total femur megakaryocyte content (Table 2). In this light, our results indicated that substantial increases in MK numbers occurred in all MK compartments following thombin infusion. The most significant changes occurred in the Type IV compartment, signaling that many MK were shedding their platelets prematurely. The increases seen in the other compartments suggested that the void created by the premature liberation of platelets from adult MK was rapidly

Table 2. Changes in Total Femur Megakaryocyte Content

	Pre-Thrombin	Post-Thrombin
Total Marrow Cellularity	$1.4 \pm 0.4 \times 10^9$	$2.6 \pm 1.1 \times 10^9$
Total Marrow MK	$3.0 \pm 0.7 \times 10^6$	$9.1 \pm 2.2 \times 10^6$
Megakaryocyte Morphologic **Classification**		
Type I	$1.0 \pm 0.5 \times 10^6$	$2.7 \pm 0.8 \times 10^{6**}$
Type II	$06 \pm 0.3 \times 10^6$	$2.0 \pm 0.8 \times 10^{6*}$
Type III	$1.1 \pm 0.3 \times 10^6$	$2.4 \pm 0.9 \times 10^{6*}$
Type IV	$0.3 \pm 0.2 \times 10^6$	$2.1 \pm 0.6 \times 10^{6**}$

Values given are mean no. of cells $\pm$ S.D. for one femur ($n = 7$)
Thrombin dose was 100 U/kg
$*p < 0.01$ (Student's t-test)
$**p < 0.005$

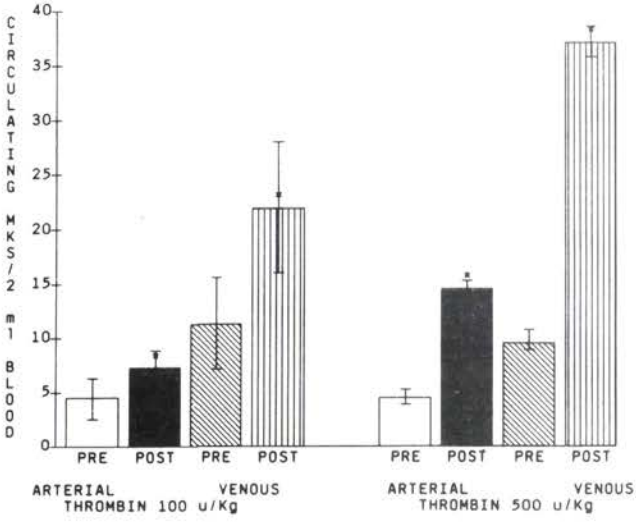
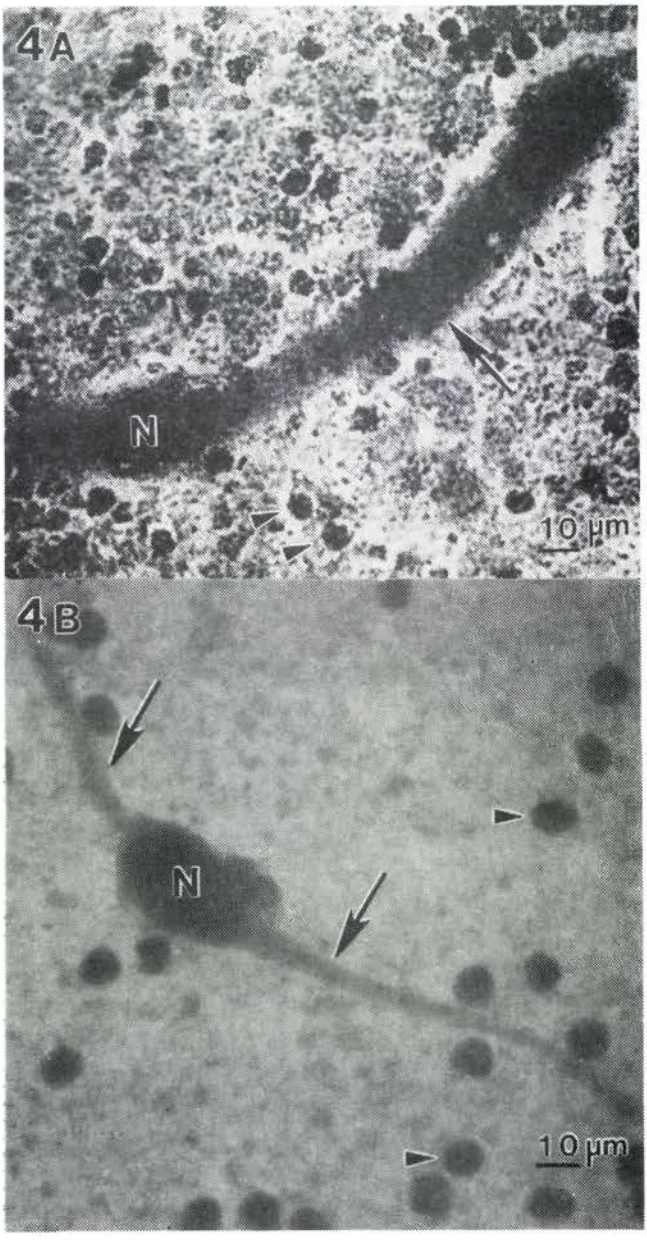


Fig. 4. Changes in circulating arterial and venous MK prior to and following thrombin infusion ($\pm$S. D.). Significant increases in circulating MK were observed following thrombin stimulation. These data suggest that thrombin induced MK migration from the bone marrow. ($*p < 0.01$). $N = 8$ dogs.

filled by the acceleration of maturation times from immature MK compartments (stages I and II). Furthermore, there was a corresponding movement of unrecognized precursor cells into the stage I compartment.



Figs. 4*A* and *B*. Light micrographs of circulating blood megakaryocytes stained on Millipore filters. 4*A*. Note the large nucleus (N) and copious cytoplasm observed in this central venous MK (arrow). *4B*. Another MK has thin bipolar fragments of cytoplasm (arrows) extending from the nucleus. Neutrophils (arrowheads) can also be observed and are included for size reference.

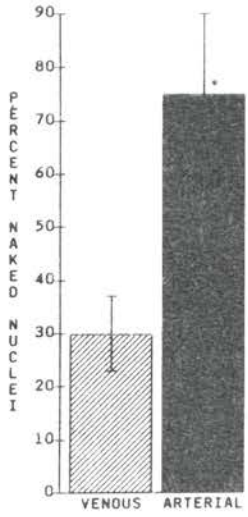


Fig. 5. Percent circulating MK naked nuclei immediately following thrombin infusion (100 U/Kg). The presence of increased numbers of naked nuclei in the arterial circulation suggests that the lung serves as a filter for circulating, platelet-containing MK. ($*p < 0.01$; $N = 5$).

Circulating Megakaryocyte Data

The mean values of arterial and venous circulating MKs/2 ml of blood were gained from counts on Millipore filters. These data revealed a significant increase in circulating arterial and venous MK following thrombin infusion (Fig. 4). Light microscopic examination of MK on Millipore filters revealed that central venous MK often contained copious amounts of cytoplasm. Most of these cells were elongated, suggesting that a return to original form was unlikely following passage through narrow channels made in the endothelial cells during entry into bone marrow sinusoids (Becker and DeBruyn 1976; Lichtman et al. 1978). We observed a great variation in size and shape of central venous MK. Some megakaryocytes measured 100–140 μm in length and contained abundant cytoplasm (Fig. 4*A*), while some MK had elongated but thin bipolar fragments of cytoplasm extending from the nucleus (Fig. 4*B*). In many instances the nucleus was centrally located, while in a few MK there was a unidirectional extension of the cytoplasm. Occasionally, a nucleus was separated from its cytoplasm. This may have been an artifact of the preparation technique. Thirty

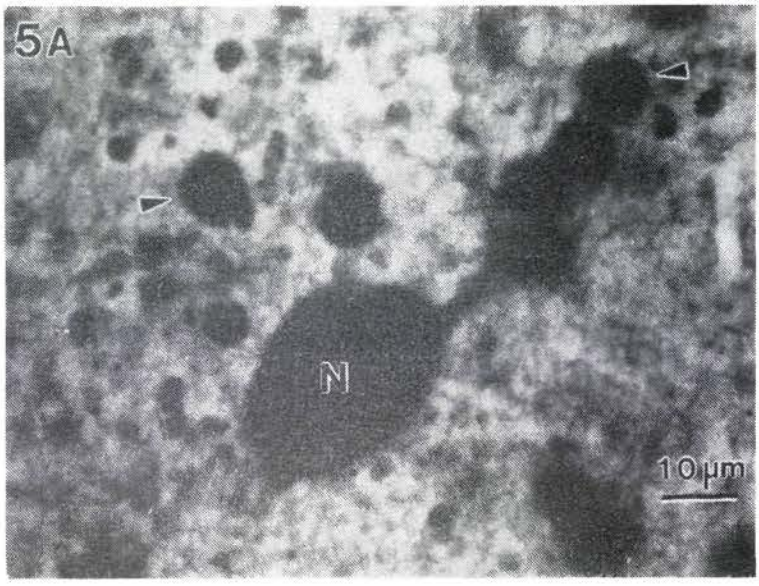


Fig. 5A. Light micrograph of a circulating MK naked nucleus (N) observed in the arterial circulation of a dog exposed to thrombin. Neutrophils (arrowheads) are included for size reference.

Table 3. Mean Platelet Counts/μl Blood

Expt. Parameter	Thrombin 100 U/kg $n = 9$	Thrombin 500 U/kg $n = 2$
Control Arterial	3.1 ± 0.3	2.5 ± 0.2
Control Venous	2.8 ± 0.4	2.3 ± 0.2
Post-thoracotomy Arterial	2.6 ± 0.3	———
Post-thoracotomy Venous	2.2 ± 0.3	———
Post-thrombin Arterial	1.7 ± 0.4	1.2 ± 0.4
Post-thrombin Venous	1.4 ± 0.3	1.1 ± 0.4
45 minutes Post-thrombin Arterial	1.8 ± 0.4	1.4 ± 0.3
45 minutes Post-thrombin Venous	1.6 ± 0.3	1.3 ± 0.3
2 h Post-thrombin Arterial	2.2 ± 0.4	1.9 ± 0.3
2 h Post-thrombin Venous	1.6 ± 0.3	1.3 ± 0.4

Values given are mean no. of cells (± s.d.) $\times 10^5$

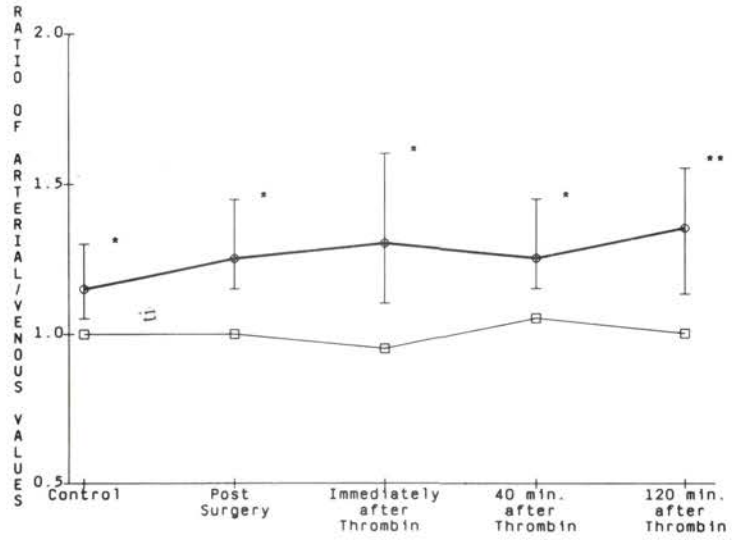


Fig. 6. Arterial/venous ratios of platelet counts and hematocrits before and after thrombin administration. Note that the maintenance of a hematocrit ratio of 1 strongly suggests that the insertion of platelets into the circulation occurs following passage through the pulmonary vasculature. (*$p < 0.05$; **$p < 0.01$; $N = 8$)

percent of the MK found in the venous circulation consisted of effete naked nuclei, indicating that these cells may have been small enough to avoid being trapped in the pulmonary vasculature and thus recirculate. Seventy-five percent of the circulating arterial MK were devoid of cytoplasm (i.e., naked nuclei) (Fig. 5, 5*A*).

Blood Platelet Counts

Arterial platelet counts were consistently higher than venous levels (Table 3). Both arterial and venous platelet counts were significantly diminished following thrombin administration and had not recovered to control values after 2 hours. Corresponding arterial and venous hematocrit values were not significantly different. Hence, the discrepancy between arterial and venous platelet counts could not be attributed to any dilution of venous blood prior to passage into the lungs. The arterial/venous platelet count ratios were significantly greater than unity at all time periods (Fig. 6).

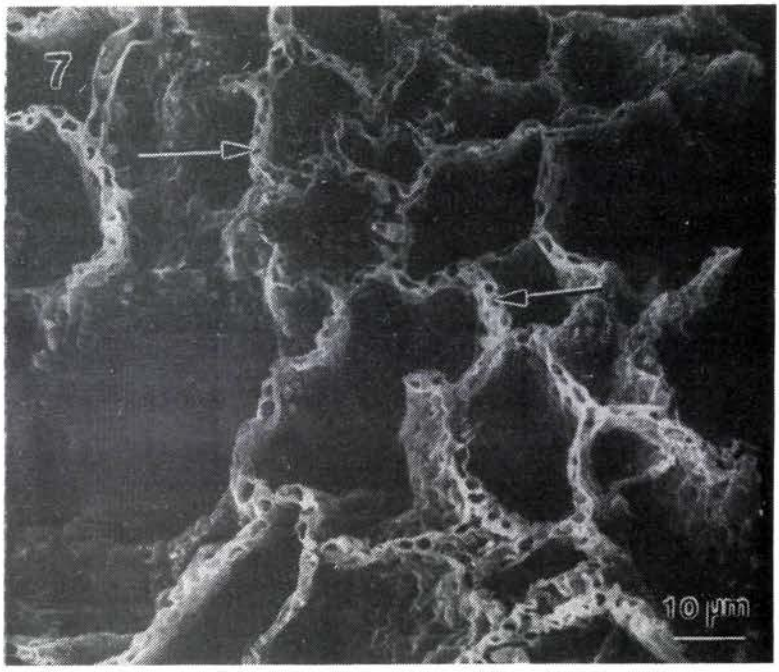


Fig. 7. Scanning electron micrograph of a cryofractured lung preparation reveals empty capillaries (arrowheads) resulting from perfusion of an unobstructed circulation in a control lobe.

Ultrastructural Results

Following intravascular perfusion of thrombin, numerous pulmonary capillaries became clogged with blood cells and platelets due to the degree of stasis produced when the experimentally induced disseminated microthrombi were sieved into the lungs (Figs. 7 and 8). Close examination of the capillaries after thrombin stress revealed that platelets were often present as platelet aggregates. Megakaryocytes were easily identified in ultrathin sections by transmission electron microscopy (TEM) (Figs. 9 and 10). Note the elaborate membranes of the demarcation membrane system in this platelet-forming MK; platelet granules were also observed. Most MK observed in lung capillaries were either adult forms or naked nuclei.

Discussion

Thrombin is known to have a variety of pathophysiologic effects in producing

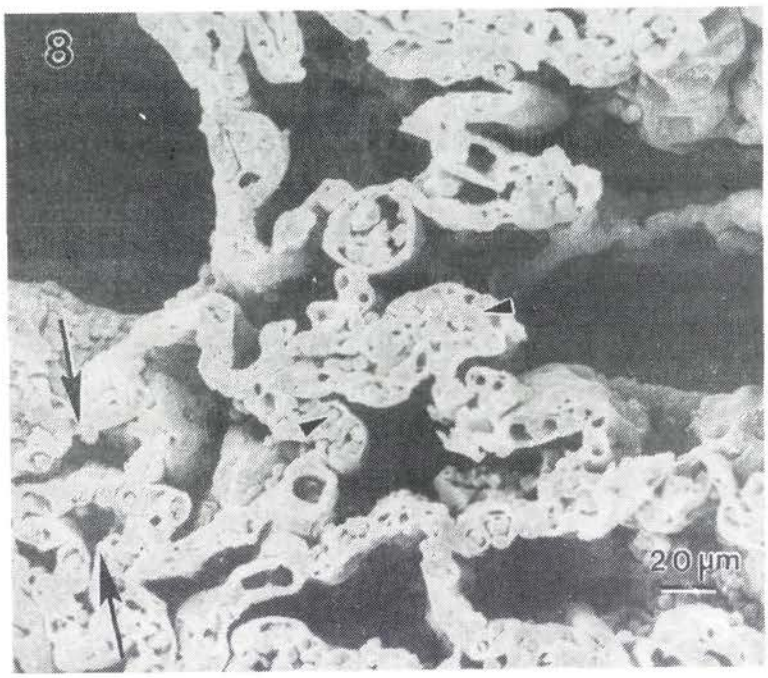


Fig. 8. Scanning electron micrograph of a cryofractured lung preparation reveals vascular congestion (arrowheads) 40 minutes after thrombin was infused. The lung is poorly expanded (arrows) although it was fixed under pressure.

lung vascular injury (Johnson et al. 1983). In this regard, alterations in pulmonary vascular pressures, permeability and the development of pulmonary edema are known to be consequences of thrombin-induced microthrombosis (Johnson et al. 1983; 1982; Lo et al. 1985). Whether perturbations of pulmonary vascular pressures have any effect upon pulmonary-platelet interactions remains to be determined. In the current study, thrombin was utilized to facilitate thrombocytopenia and augment the numbers of both medullary and circulating megakaryocytes. Our data showed that the concentrations of marrow MK increased following thrombin infusion. Earlier reports indicated that marrow MK numbers increased only after 2—3 days of marked thrombocytopenia (Kimura et al. 1985). Alternatively, numerous investigators have demonstrated direct stimulatory effects of thrombin on guinea pig (Fedorko 1977; Leven and Nachmias 1982; Leven 1988; Miller 1983) and mouse MK (Radley et al. 1987). In the latter study, Radley and colleagues investigated MK development *in vitro* and reported that mature MK undergo thrombin-induced microtubular depolymerization prior to platelet formation. Therefore, on the basis of these studies

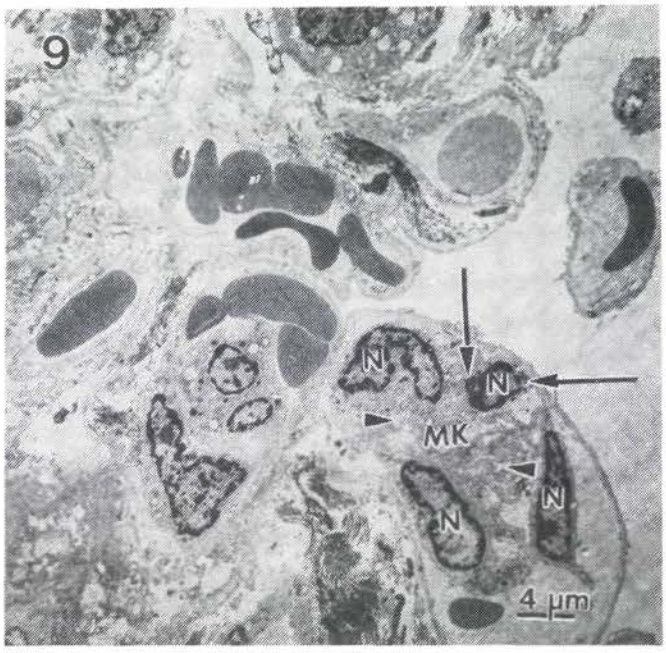


Fig. 9. Transmission electron micrograph which reveals a platelet-forming MK sequestered in a pulmonary capillary. Note the MK multilobular nuclei (N) and attached platelets (arrows). In addition, demarcation membrane systems (DMS) can be discerned (arrowheads). The pulmonary MK is enlarged in Fig. 10.

and the data presented here, it seems likely that thrombin directly stimulates the MK pool, since the effects could not be accounted for by the development of thrombocytopenic conditions.

Intravascular infusion of thrombin stimulated the migration of megakaryocytes from the bone marrow into the central venous circulation, which accounted for a substantial increase in the numbers of circulating MK. Most of the circulating venous MK became lodged in pulmonary capillaries, although we also observed a two-fold increase in the numbers of arterial megakaryocytes (primarily naked nuclei) following thrombin-stimulated microthrombosis, suggesting that a greater proportion of platelet-containing megakaryocytes were shedding platelets in the lung prematurely. It was interesting to discover that 75% of the circulating arterial MK were devoid of cytoplasm, whereas only 30% of circulating venous MK consisted of effete naked nuclei. These results suggested that the lung serves as an effective vascular filter for platelet-containing megakaryocytes.

Arterial platelet counts were significantly higher than corresponding venous

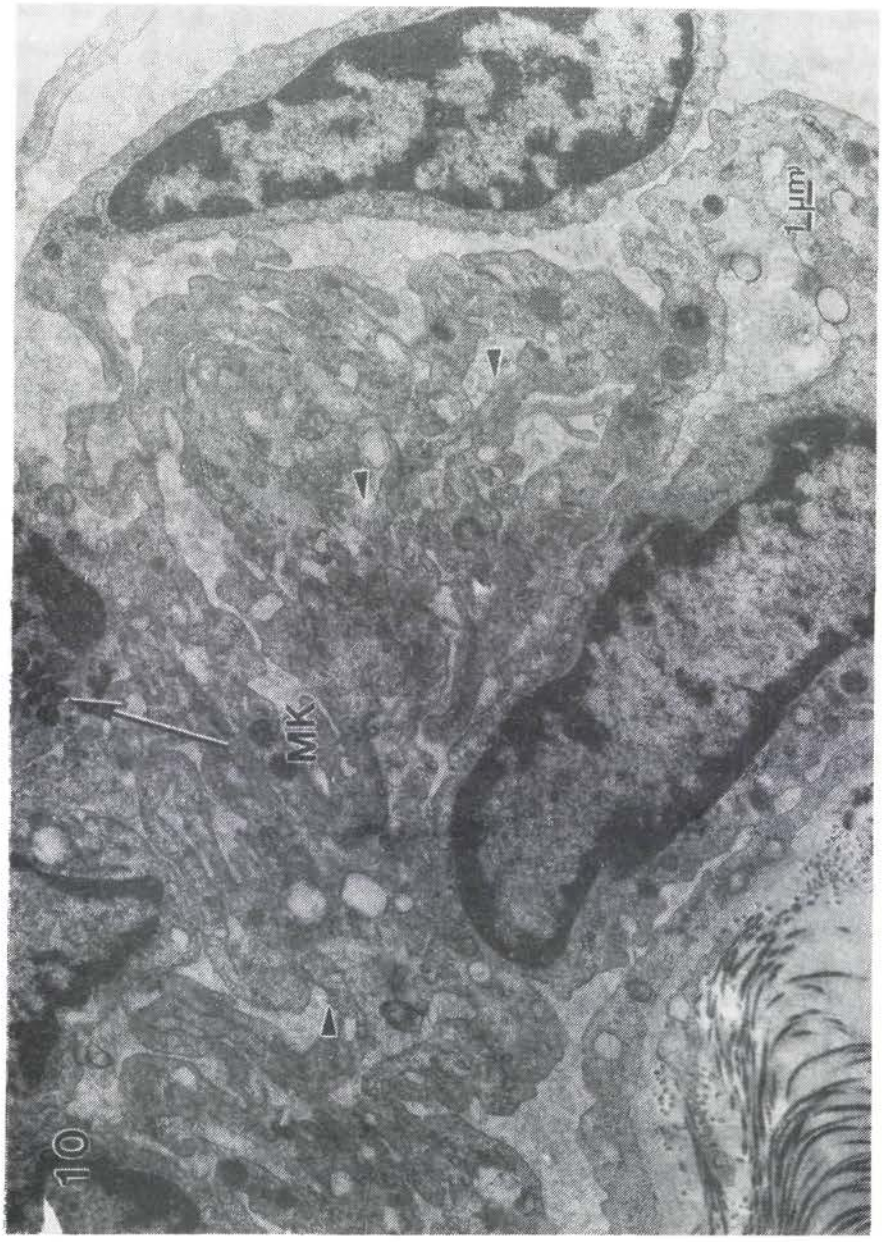


Fig. 10. An enlargement of Fig. 9. A platelet-containing (arrows) MK is lodged in a pulmonary capillary. The characteristic demarcation membrane system can be observed as well as platelet granules (arrowheads).

levels under normal conditions, confirming the work of previous investigations (Kaufman et al. 1965b; Tinggaard Pedersen 1971; 1978). Our data indicate that platelets are inserted into the circulation during passage through the lung vasculature. Infusion of intravascular thrombin stimulated a marked thrombocytopenia, which facilitated a significant increase in arterial/venous platelet ratios over hematocrit values. Accordingly, it would appear that either a greater number of venous platelets were either being retained in the lung, and/or an increased proportion of platelets were inserted into the arterial circulation following passage through the lungs.

Transmission electron microscopic studies demonstrated the presence of platelet-containing megakaryocytes in the lung following thrombin administration. A large number of platelet aggregates were observed during the period of thrombocytopenia. Several of the observed pulmonary capillaries were clogged with blood cells and platelet aggregates.

Several investigators have recently challenged the widely regarded concept that the majority of circulating platelets are shed via proplatelet processes (Lichtman et al. 1978; Handagama et al. 1986; 1987) from the bone marrow sinusoids. Trowbridge, Martin and colleagues, using a mathematical model of platelet formation have proposed that physical fragmentation of MK cytoplasm in the pulmonary capillaries accounts for the circulating platelet pool and thus, the pulmonary circulation is the main source of platelet production (Martin et al. 1982; Slater et al. 1983; Trowbridge et al. 1982). In support of this theory, 1) MK are known to migrate from the bone marrow sinusoids into the general circulation and are considered to be normal consitutents of venous and arterial blood; 2) the presence of MK in pulmonary vessels is a normal finding at autopsy, and the frequency of intrapulmonary MK is increased during cardiovascular and pulmonary disease states (Aabo and Bendix-Hansen 1978; Sharma and Talbot 1986; Wells et al. 1984); 3) the presence of naked MK nuclei and increased platelet numbers in the arterial circulation strongly suggests that the lung serves as a reservoir for platelets. The evidence presented supports the notion that the lung plays an important role as a vascular filter and regulator of circulating platelet count, however, the extent to which pulmonary MK contribute to the maintenance of the *normal* circulating platelet population remains to be determined.

The development of platelet aggregates in lung capillaries may influence subsequent cellular events producing pathologic lesions in the lung. Forrest (1977) has shown that increased numbers of vasoactive platelet granules are stored and secreted following platelet aggregation. The release of these phlogistic constituents produces alterations which mirror anaphylactic reactions, i.e., spasmogenic effects and increases in vascular permeability. Lonky and Wohl

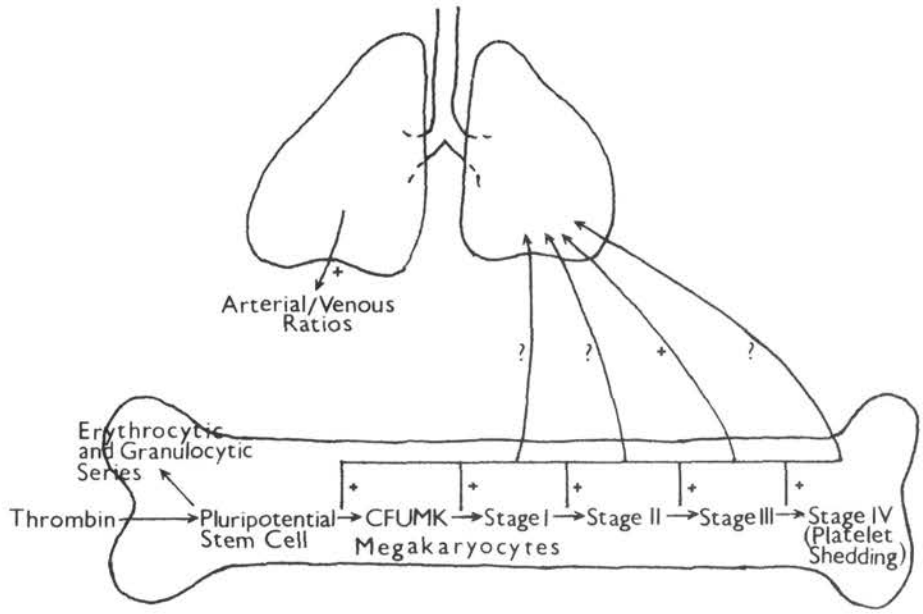


Fig. 11. A schematic detailing a proposed mechanism for the effects of thrombin on megakaryocytopoiesis and migration of bone marrow MK into the central venous blood. The data presented suggest that thrombin infusion has stimulatory effects upon the pluripotential stem cell, the megakaryocytic colony-forming cells (CFU-MK) and accelerates the maturation cycle of MK stages I—IV. In addition, increased numbers of platelet-containing MK migrate into the central venous circulation and become trapped in the lung. Shedding of pulmonary MK into the arterial circulation would account for the increased arterial/venous platelet ratios.

(1981) have shown that platelet factor 4 stimulates neutrophils to release human leukocyte elastase against lung elastin. Combinations of platelet factor 4 and human leukocyte elastase were instilled into the lungs of animals and produced emphysematous effects. Kaplan and colleagues (1982) have demonstrated *in vitro* that thrombin stimulates the secretion of platelet factor 4 during the release reaction of platelet aggregation. Similarly, Stimler et al. (1981) and Benveniste (1974) have described a phospholipid mediator of anaphylaxis (i.e., platelet activating factor) released from basophils and leukocytes which aggregates platelets and releases their vasoactive amines. It is conceivable that thrombin-induced microthrombosis as well as any other intravascular stress might produce similar effects.

Alternatively, it is now well established that a prostaglandin counterbalance to platelet aggregation is produced in the lung due to endothelial cell release of prostacyclin. Prostacyclin (PGI_2) prevents platelet aggregation *in vitro* (Mon-

cada et al. 1977) and *in vivo*, in several experimental models (Higgs et al. 1978; Ubatuba et al. 1979). Furthermore, it has been reported that PGI_2 can disaggregate platelets that have already aggregated *in vivo* (Martin et al. 1980). Therefore thrombin-stimulated microthrombosis may be a temporary event, and the subsequent return to normal platelet levels may be a function of prostacyclin-mediated disaggregation of platelets as well as megakaryocyte shedding of platelets.

We have shown that a subpopulation of megakaryocytes migrates out of the bone marrow and becomes trapped in the lung. Subsequently, under normal conditions, platelets are inserted into the arterial circulation via shedding pulmonary megakaryocytes. Both of these events, megakaryocyte migration and platelet insertion, are exacerbated following intravascular stress, and may account for the increased arterial/venous platelet ratios observed after thrombin administration (Fig. 11). These results suggest that the lung serves as a vascular filter and reservoir for platelets under normal conditions and as a repository for the cells following intravascular stress.

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References

Aabo K., Bendix-Hansen K. (1978): Megakaryocytes in pulmonary blood vessels. Incidence at necropsy, clinicopathological relations, especially to disseminated intravascular coagulation. Acta Pathol. Microbiol. Scand. (Sect. A). **86,** 285—291

Aschoff L. (1893): Ueber capillare Embolie von riesenkernhaltigen Zellen. Arch. Path. Anat. Physiol. **134,** 11—26

Barnhart M. I., Noonan S. M. (1978): Fine structure and surface features of platelets and megakaryocytes. Thromb. Haemost. **63,** (suppl.) 3—36

Becker R. P., DeBruyn P. P. H. (1976): The transmural passage of blood cells into myeloid sinusoids and the entry of platelets into the sinusoidal circulation: a scanning electron microscopic investigation. Amer. J. Anat. **145,** 183—205

Benveniste J. (1974): Platelet-activating factor, a new mediator of anaphylaxis and immune complex deposition from rabbit and human basophils. Nature (London) **249,** 581—582

Brecher G., Cronkite E. P. (1950): Morphology and enumeration of blood platelets. J. Appl. Physiol. **3,** 365—377

Crosby W. H. (1976): Normal platelet numbers- pulmonary-platelet interactions. Ser. Haematol. **8,** 89—97

Fedorko M. E. (1977): The functional capacity of guinea-pig megakaryocytes. Lab. Invest. **36,** 310—320

Forrest J. B. (1977): A quantitative morphological study of early platelet aggregation in rabbit lungs. Microvasc. Res. **14,** 111—129

Handagama P., Jain N. C., Kono C. S., Feldman B. F. (1986): Scanning electron microscopic studies of megakaryocytes and platelet formation in the dog and rat. Amer. J. Vet. Res. **47,** 2454—2460

Handagama P. J., Jain N. C., Feldman B. F., Kono C. S. (1987): Scanning electron microscope study of platelet release by canine megakaryocytes in vitro. Amer. J. Vet. Res. **48,** 1003—1006

Higgs E. A., Higgs G. A., Moncada S., Vane J. R. (1978): Prostacyclin (PGI_2) inhibits the formation of platelet thrombi in arterioles and venules of the hamster cheek pouch. Brit. J. Pharmacol. **63,** 535—539

Humphreys W. J., Spurlock B. O., Johnson J. S. (1974): Critical point drying of ethanol-infiltrated cryofractured biological specimens for scanning electron microscopy. Scannning Electron Microsc. **I,** 275—282

Johnson A., Tahamont M. V., Kaplan J. E., Malik A. B. (1982): Lung fluid balance after pulmonary embolization: effects of thrombin vs. fibrin aggregates. J. Appl. Physiol. **52,** 1565—1570

Johnson A., Tahamont M. V., Malik A. B. (1983): Thrombin-induced lung vascular injury. Role of fibrinogen and fibrinolysis. Amer. Rev. Respir. Dis. **128,** 38—44

Kaplan A. P., Silverberg M., Dunn J. T., Ghebrehiwet B. (1982): Interaction of the clotting, kinin-forming, complement, and fibrinolytic pathways in inflammation. Ann. NY. Acad. Sci. **389,** 25—38

Kaufman R. M., Airo R., Pollack S., Crosby W. H., Doberneck R. (1965a): Origin of pulmonary megakaryocytes. Blood **25,** 767—775

Kaufman R. M., Airo R., Pollack S., Crosby W. H. (1965b): Circulating megakaryocytes and platelet release in the lung. Blood **26,** 720—731

Kimura H., Segal G. M., Lee M. Y., Adamson J. W. (1985): Megakaryocytopoiesis in the rat: response to thrombocytopenia induced by exchange transfusion. Exp. Hematol. **13,** 1048—1054

Leven R. M., Nachmias V. T. (1982): Cultured megakaryocytes: changes in the cytoskeleton after ADP-induced spreading. J. Cell. Biol. **92,** 313—323

Leven R. M. (1987): Megakaryocyte motility and platelet formation. Scanning Microscopy **1,** 1701—1709

Lichtman M. A., Chamberlain J. K., Simon W., Santillo P. A. (1978): Parasinusoidal location of megakaryocytes in marrow: a determinant of platelet release. Amer. J. Hematol. **4,** 303—312

Lo S. K., Pearlman G. D., Niehaus G. D., Malik A. B. (1985): Thrombin-induced alterations in lung fluid balance in awake sheep. J. Appl. Physiol. **58,** 1421—1427

Lonky S. A., Wohl H. (1981): Stimulation of human leukocyte elastase by platelet factor 4. J. Clin. Invest. **67,** 817—826

Martin J. F., Suggett A. J., Leach E., Moncada S. (1980): The effect of prostacyclin on platelet aggregation and disaggregation *in vivo*. Thromb. Res. **18,** 749—751

Martin J. F., Trowbridge E. A., Salmon G. L., Slater D. N. (1982): The relationship between platelet and megakaryocyte volumes. Thromb. Res. **28,** 447—459

Miller J. L. (1983): Characterization of the megakaryocyte secretory response: studies of continuously monitored release of endogenous ATP. Blood **61,** 967—972

Moncada S., Higgs E. A., Vane J. R. (1977): Human arterial and venous tissues generate prostacyclin (Prostaglandin X) a potent inhibitor of platelet aggregation. Lancet **1,** 18—20

Radley J. M., Hartshorn M. A., Green S. L. (1987): The response of megakaryocytes with processes to thrombin. Thromb. Haemost. **58,** 732—736

Rešl M., Kuna P., Petýrek P. (1987): Megakaryocytes in rabbit pulmonary blood vessels. Gen. Physiol. Biophys. **6,** 383—386

Scheinin T. M., Koivuniemi A. P. (1963): Megakaryocytes in the pulmonary circulation. Blood **22,** 82—87

Seebach L. M., Kernohan J. W. (1952): Megakaryocytes in the pulmonary capillaries. Amer. J. Clin. Path. **22,** 646—651

Sharma G. K., Talbot I. C. (1986): Pulmonary megakaryocytes: "missing link" between cardiovascular and respiratory disease? J. Clin. Pathol. **39,** 969—976

Slater D. N., Trowbridge E. A., Martin J. F. (1983): The megakaryocyte in thrombocytopenia: a microscopic study which supports the theory that platelets are produced in the pulmonary circulation. Thromb. Res. **31,** 163

Stimler N. P., Brocklehurst W. E., Bloor C. M., Hugli T. E. (1981): Anaphylatoxin-mediated contraction of guinea pig lung strips: a nonhistamine tissue response. J. Immunol. **126,** 2258—2261

Tinggaard Pedersen N. (1971): Circulating megakaryocytes in blood from the inferior vena cava in adult rats. Scand. J. Haemat. **8,** 223—230

Tinggaard Pedersen N. (1974): The pulmonary vessels as a filter for circulating megakaryocytes in rats. Scand. J. Haemat. **13,** 225—231

Trowbridge E. A., Martin J. F., Slater D. N. (1982): Evidence for a theory of physical fragmentation of megakaryocytes, implying that all platelets are produced in the pulmonary circulation. Thromb. Res. **28,** 461—475

Ubatuba F. B., Moncada S., Vane J. R. (1979): The effect of prostacyclin (PGI_2) on platelet behaviour, thrombus formation in vivo and bleeding time. Thromb. Haemost., **41,** 425—434

Warheit D. B., Barnhart M. I. (1979): Megakaryocytes in the lung after thrombin initiated diffuse microthrombosis: a preliminary report on ultrastructure. Scanning Electron Microsc. **1979/III,** 801—808

Warheit D. B., Barnhart M. I. (1980): Circulating megakaryocytes and the microvasculature of the lung. Scanning Electron Microsc. **1980/III,** 255—262

Warheit D. B., Barnhart M. I. (1981): Ultrastructure of circulating and platelet-forming megakaryocytes: a combined correlative SEM-TEM and SEM histochemical study. Ann. NY. Acad. Sci. **370,** 30—41

Wells S., Sissons M., Hasleton P. S. (1984): Quantitation of pulmonary megakaryocytes and fibrin thrombi in patients dying from burns. Histopathology. **8,** 517—527

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