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THE KINETICS OF REVERSIBLE-SEQUESTRATION OF LEUKOCYTES BY THE ISOLATED PERFUSED RAT LUNG

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by

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Abstract

The kinetics and morphology of sequestration and margination of rat leukocytes were studied using an isolated perfused and ventilated rat lung preparation. Whole rat blood, bone marrow suspension, or suspensions of peritoneal neutrophils, referred to as leukocyte suspensions, were used to perfuse the isolated rat lung either in the single-pass or continuous-circulation mode. Samples were collected from the lung affluent and effluent suspensions at various intervals and their leukocyte concentrations were determined. The lung was also perfused with latex particle suspensions and the passage of particles through the lung capillaries was studied. Lungs prefused with leukocyte or latex particle suspensions were fixed by the tracheal infusion technique, ethanol-cryofractured, and processed for scanning electron microscopy.

When a leukocyte suspension was perfused through the lung in the single-pass mode, the rate of sequestration decreased as more cells were perfused. In contrast, latex particles of a size comparable to that of leukocytes were totally stopped by the lung. With the scanning electron microscope, latex particles were seen plugging the capillaries and small arterioles. When the leukocyte suspension was recirculated through the lung, cells were rapidly removed from circulation until a steady state was reached, after which no net removal of cells by the

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lung occurred. For the range of concentrations studied, the fraction of cells remaining in circulation at the steady state was independent of the starting concentration. These results indicate that leukocytes are reversibly sequestered from circulation, i.e., sequestered cells are in rapid exchange with circulating cells. Scanning electron microscopy of the fixed lungs perfused with leukocyte suspension showed that the sequestered cells marginated and attached to the luminal surface of the endothelium of post-capillary venules and veins.

A mathematical model was developed based on the assumption that, at any time, the attachment and detachment of leukocytes to blood vessel walls follows first-order kinetics; the rate of attachment is equal to a constant, k_a, times the circulating cell concentration and the rate of detachment is equal to a constant, k_b, times the number of cells attached to the vascular endothelium of the lung. The model correctly predicts the following characteristics of the system: a) The kinetics of the sequestration of leukocytes by the lung. b) The existence of a steady state when a suspension of leukocytes is recirculated through the lung. c) The independence of the fraction of cells remaining in circulation from the starting concentration for all values of starting concentration. The model provides an explanation for the existence and distribution of the circulating and the marginated granulocyte pools in-vivo, under normal conditions, and the failure of leukocyte infusion therapy to raise the circulating leukocyte concentration of patients. The model also provides parameters with which individual leukocytes can be differentiated with respect to their affinity toward sequestration and margination.

J C Schooley Edward L Lelgon

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I. INTRODUCTION

A. Statement of the Problem

Ever since the discovery of the essential role of leukocytes in the body defense mechanism against foreign invasion, a great deal of attention and investigation has been focused on identifying and better understanding various steps and factors involved in this defense system.

Among the various components of this system, granulocytes are responsible for providing a fast initial counter attack against invading microorganisms outside the body's vascular network. This involves a series of steps, including margination and adherence to the vascular endothelium, diapedesis through vascular walls, directional movement of cells along chemotactic gradients and phagocytosis of the invading microorganisms. Among these steps, margination and adherence to the vascular endothelium have been poorly understood and characterized; of special interest are the following aspects:

1. Under normal conditions, blood granulocytes are distributed among two pools, a circulating pool and a marginated pool. The two pools are about equal in size and they are in rapid equilibrium. The kinetics and mechanisms responsible for such a distribution are not known.

2. In patients with defective leukocyte systems, attempts to substantially raise their blood leukocyte concentrations by transfusion of leukocyte suspensions have been unsuccessful. The reason has been traced to sequestration of infused leukocytes by the vasculature of

peripheral organs, especially the lung. The mechanisms and the kinetics of this sequestration phenomenon are not understood.

3. The relation between the sequestration phenomenon described in aspect 2 and the physiological (normal) marginability of leukocytes described in aspect 1 is not known.

4. Understanding of the adhesion of leukocytes to vascular endothelium is still in its infancy. Although there are some clinical correlations between the in-vivo therapeutic treatments and the in-vitro adherence to glass beads or nylon fibers, these in-vitro test systems obviously cannot explain the nature and mechanism of adhesion of leukocytes to endothelial cells. Tissue cultures of endothelial cells, although more physiologic than artificial surfaces, still suffer from the fact that the surfaces provided by the cell in culture may not be identical with the in-vivo luminal surface of endothelial cells.

Therefore, in order to approach the questions described in 1, 2, and 3 above and to provide an alternative to test systems described in 4, I developed an isolated perfused rat lung and, under different conditions, perfused it with various suspensions of leukocytes. This system enjoys the following advantages over previous systems described above:

1. It measures the interaction of leukocytes with the luminal surfaces of the endothelium developed and formed in-vivo.

2. It contains the three dimensional structure of the blood vessels in the lung.

3. It is a proper system for kinetic studies of sequestration and margination phenomena.

4. It allows morphological studies of sequestration and margination.

B. Historical Background

1. Leukocytes

Leukocytes can be categorized in different ways depending on the type of characteristic under study. They can be classified functionally, with monocytes and granulocytes included in the phagocytic cell group and lymphocytes included in the non-phagocytic group, or they can be classified morphologically. Here, I will use a criterion based on the morphology of the cell nucleus under the light microscope. Accordingly, the cells with a single round or kidney shaped nucleus (including lymphocytes and monocytes) are called mononuclear cells, and the cells with a lobulated nucleus are called polymorphonuclear cells or granulocytes.

a. Lymphocytes

Lymphocytes have been classified as T-cells for thymus-derived (that is, thymus-processed, thymus-dependent) lymphocytes and B-cells for "bursa-equivalent"-derived (that is, nonthymus-processed, thymus-independent) lymphcytes (1).

T-cells. Current evidence indicates that in mouse embryos (2), as in the chick embryo (3), thymic lymphocytes are derived from stem cells circulating in the blood stream which migrate into the thymus around day 12 of the embryo's development. In the thymus, the stem

cells differentiate into small lymphocytes bearing the O and TL antigenic markers (4). Further differentiation of these thymus lymphocytes, accompanied by reorganization of surface markers and emigration out of the thymus, results in peripheral T-lymphocytes basically involved in "cell-mediated" immunity.

B-cells. A considerable amount of information is available on the B-lymphocytes of birds. The bursa of birds has been considered the main site of differentiation and maturation of stem cells to B-cells. Chromosome marker studies in parabiosed chick embryos have demonstrated that lymphocytes within the follicles of the bursa are derived from migrant blood-born stem cells (5).

Although no mammalian bursal-equivalent organ has been defined, all mammalian lymphocytes of thymic-independent origin are referred to as B-lymphocytes. Both foetal liver (6) and bone marrow (7) are known to be sources of stem cells which can mature into B-lymphocytes, and the two main candidates for a bursal-like role in their maturation have been intestinal lymphoid tissue and the marrow itself (8).

Cooper, et al (9,10) have proposed a scheme of B-cell maturation based on their experimental findings. They suggest that antibody variability in terms of both specificity and class is generated following the replication of multipotential stem cells within the bursa. Since immunoglobulin within the embryonic bursa is seen mainly at cell surfaces rather than within cell cytoplasm, and because cytoplasmic fluorescence following labelling with fluorochromed antibodies of chicken immunoglobulin is weak and easily quenched relative to

mature plasma cells, they postulate that immunoglobulins produced by bursal lymphocytes are not secreted but are incorporated into cell membranes as "receptors" or "antigen-recognition antibodies." Thus, the basis of this scheme is that the bursa is the site of maturation of stem cells to B-axis lymphocytes which possess surface immunolgobulin "receptors." These lymphocytes are committed to specific classes and class divisions of antibodies which they make and secrete once they have migrated to peripheral tissues and have been stimulated by antigen. Hence, the differentiation step from B-lymphocyte to antibody-secreting cell (plasma cell) takes place as a separate event in the peripheral lymphoid system.

Life-span. By injecting ^{3}H-thymidine and monitoring the accumulation of labelled lymphocytes, Everett, et al (11) were able to distinguish two populations of lymphocytes, a short-lived population having an intermitotic interval of less than two weeks, the majority of which label within 4-5 days, and a long-lived population with an intermitotic interval of several months, the majority of which do not label during a short course of ^{3}H-thymidine administration.

Buckton, et al (12) used an independent approach to measure human lymphocyte longevity. They collected blood lymphocytes of a human subject who had received large doses of X-irradiation several years previously and stimulated them to divide in culture. They could then observe unstable chromosomal abnormalities not compatible with post mitotic survival, suggesting that these cells were dividing for the first time since irradiation, which had been ten years earlier.

Although, generally, long-lived lymphocytes have been equated with T-cells and short-lived lymphocytes with B-cells (13-15), recent evidence indicates that there are significant numbers of short- and long-lived lymphocytes in both T- and B-cell populations (16-20).

Recirculation. It was Gowans (21) who first demonstrated that, in rats, a large proportion of lymphocytes recirculate between blood and lymph, while others do not. He showed that the main route from blood to lymph was through specialized endothelial cells lining the post-capillary venules in lymph nodes and Peyer's patches (22). After migrating through the substance of the node, recirculating lymphocytes enter efferent lymphatics and pass via the thoracic duct back into the blood. Lymphocytes also leave the blood in the spleen, possibly by passing between the endothelial cells of the marginal sinus (23) and, after some time in the white pulp, return to the blood in the spleen by an unknown route.

Post-capillary venules in the rat and other mammalian species, including man, normally are lined by endothelial cells which have abundant cytoplasm (22). Post-capillary venules in rats thymectomized at birth, and in lymphocyte-depleted rats, were dilated and empty; endothelial cells lining the venules had relatively little cytoplasm (23). In rodents, the majority of recirculating lymphocytes are T-cells (22,24,25). Although B-cells recirculate between lymph and blood (26), their recirculation differs from that of T-cells in that the tempo is slower, without a clear model time, and it takes place through the follicular (thymus-independent) areas of the spleen, Peyer's patches and lymph nodes (27,28).

The interaction of lymphocytes with the high endothelial cells of post-capillary venules is very specific. When murine lymphocyte suspensions were layed over fixed sections of syngenic lymph nodes at 7°C, the cells adhered specifically to high endothelial venules (HEV) but not to other vascular structures (29). If the incubation is performed at 24°C, binding is severely reduced and at 37°C, there are few, if any, HEV that exhibit adherent thoracic duct lymphocytes compared to optimal attachment occurring at 7°C, suggesting that lymphocytes that adhere to HEV are capable of rapid dissociation under physiologic conditions (30).

Besides the lymph nodes and the spleen, physiological migration of lymphocytes across the endothelium also occurs to some extent in other organs such as the kidney (31). Lymphocyte migration from the blood stream to the tissues shows several types of specificity; organ specificity, T- and B-cell specificity, and immunological specificity. When rats were challenged against infection by Listeria monocytogenes, some lymphocytes were found to be able to be able to migrate across the vessel and enter the site of infection in the peritoneal cavity (32,33).

In summary, it is clear the lymphocytes can leave the blood stream at various organs for different reasons, and to do so they have to cross over the endothelial barrier of blood vessels. So far, post-capillary venules are the only known sites of lymphocyte migration. Migration takes place by the lymphocyte first attaching to the endothelium, and then by its intercellular passage through the endothelial cells (34).

b. Monocytes

Morphologically, monocytes can be differentiated from lymphocytes by their large size, kidney shaped nucleus and abundant, pale blue cytoplasm in Romanovsky-stained smears. Blood monocytes are phagocytes which eventually migrate out of the blood stream to form tissue macrophages. In rats, monocytes make up about six percent of the total blood lymphocytes.

The earliest recognizable precursors of monocytes in bone-marrow are promonocytes (35-37). The promonocyte is a dividing cell capable of self renewal (37). After a developmental period of 1-3 days in bone marrow (38,39), monocytes enter the blood stream within a few hours after the last mitosis (37).

The egress of monocytes from the blood is a random process; the mean half-time for the disappearance of labelled cells is 71 hours in pulse-labelled human subjects, 12-14 hours in pulse-labelled rats (39), and 3.1 days in continuously-labelled mice (38). Based on the transfusion of labelled monocytes into rats, Whitelaw (40) concluded that labelled, transfused monocytes initially leave circulation with a half-time of 0.2 hour. They first collect on the walls of small vessels and then migrate outward until equilibrium is established between the circulating pool and a large extra-vascular pool which contains about twenty-five times as many monocytes as does the circulating blood. From this pool, they leave randomly and irreversably with a half-time of 14 hours. The spleen and the lungs make up a large part of the pool, and splenectomy reduces the size of the extravascular compartment and slows the rate of sequestration (40).

c. Polymorphonuclear Cells - Granulocytes

Neutrophilic Granulocytes. In Karnowsky-stained blood smears, these cells are identified by their nucleus, which is segmented into two to five, most frequently three, lobes connected by thin chromatin strands; and by their neutral staining cytoplasm. They tend to be of a uniform size of about 7 μm in diameter and make up about 25 percent of circulating blood leukocytes in rats.

Maturation. Under normal conditions, bone marrow is the maturation site of neutrophils. In-vitro, a class of stem cells committed to the granulocytic-monocytic line can be identified (41) that, under suitable conditons, will give rise to granulocyte and macrophage colonies. In-vivo, the earliest precursor is the myeloblast. This is a relatively undifferentiated, small cell which gives rise to the more differentiated precursor, the promyelocyte. The promyelocyte is the earliest cell that can be identified with certainty as a neutrophil precursor. The diagnostic feature is the formation and presence of azurophil granules, which can be recognized histochemically by their positive staining for peroxidase (42-45).

The next in line is the myelocyte. The myelocyte stage is characterized by the formation of a second class of granules, i.e., specific granules, which can be distinguished from azurophil granules by their lack of peroxidase (42-45). The myelocyte gives rise to the metamyelocyte, which has lost the capacity for cell division. The metamyelocyte matures into the stab (band) form which eventually gives rise to the fully mature neutrophil.

The kinetics of granulopoiesis in the bone marrow has been extensively studied by several investigators using different techniques and approaches. Based on mitotic indices of human bone marrow cells, Cronkite's estimate of compartment transit time, i.e., the average time from the entrance of a cell into a compartment until it or its progeny leaves the compartment, was 23 hours for myeloblasts 78.3 hours for promyelocytes, 26 hours for myelocytes and 84 hours for the non-dividing metamyelocyte series. By adding the transit times of different compartments from the myeloblast to the last myelocyte division, the elapsed time could vary from 3.5 to 9.5 days (46).

Studies on granulopoiesis in the dog using ^{3}H.Tdr and labelled mitosis techniques by Maloney and Patt (47) revealed that the ratio of flash-labelled granulocytes to erythrocyte progenitors was unity (1.02 ± 0.05) in the eight dogs studied, which means that gross production rates were essentially the same. Since the net production, as revealed by the peripheral blood turnover, is greater for erythrocytes than for granulocytes, they suggested that there must be some ineffective granulopoiesis at the myelocyte level. However, the same data have been alternatively interpreted by Craddock (48) to mean that certain cells along the pathway of myeloid maturation are self-sustaining, and are, in effect, differentiated stem cells.

Normal Distribution. The granulocytes of the peripheral blood are distributed between freely circulating granulocyte pools (CGP) and the marginal granulocyte pool (MGP). Looking at the intact mesentry of guinea-pigs, rabbits, and cats, under the light microscope, Vejlan (49)

was the first to reveal that, under ordinary conditions, some of the white blood cells were axially flowing while others were marginal and wall adherent. The marginal cells were almost all polymorphonuclear leukocytes with occasional lymphocytes. Marginated cells were observed exclusively in blood vessels described by Vejlans as veins and post-capillary venules.

Ever since Vejlans published his results, numerous studies on the distribution of granulocytes have become available, confirming Vejlan's findings (50). A useful technique employed in studying the kinetics of granulocytes in the peripheral blood was the use of radioactive diisopropyl fluorophosphate (DFP^{32}), a potent, irreversble inhibitor of a number of proteases and esterases such as trypsin, chymotrypsin, and various cholinesterases. When DFP^{32} is incubated with blood in-vitro or is injected intravenously, only neutrophils, among other blood leukocytes will pick up the label, i.e., DFP^{32} is an exclusive marker of neutrophils among other leukocytes (51,52). Labelled granulocytes, when returned to the circulation of the donor, distribute into a pool of cells approximately two times larger than that calculated from the blood volume and the total number of labelled granulocytes injected (53,54). This pool has been referred to as the "total blood granulocyte pool" (TBGP) and consists of two subcompartments or pools; the circulating granulocyte pool, and the marginated granulocyte pool. The equilibrium between these two pools is sufficiently rapid and complete to allow them to be considered as one kinetically, and the size of the TBGP is the sum of CGP and MGP.

In a group of 109 normal subjects studied (52), the ratio of the CGP to the TBGP was 0.44 for the entire group, with a wide variation in individuals. The labelled granulocytes left the circulation in a random fashion (exponential function) with a half-time disappearance of 6.7 hours.

Epinephrine and physical exercise shift cells from the MGP to the CGP without increasing the size of the TBGP (55). Granulocytes are phagocytic cells which perform their principal function in the extra vascular space. To do this, each granulocyte establishes close contact with the endothelium before any attempt is made for emigration. The marginated granulocyte pool, under normal conditons, serves as a reservoir of cells ready to function throughout the body organs and tissues. The occurrence of inflammation and local injuries focuses the attention of the system on the site of the injury, which results in a rapid increase of margination and migration of cells from the post-capillary venules of the injured tissues to the extravascular space (56,57). Availability and the proper concentration of circulating granulocytes is a key factor in the body's prompt response to and defense against various forms of infection. The common failure to raise the granulocyte level to the expected degree by transfusing large volumes of normal or leukemic granulocytes to patients with various forms of agranulocytosis and granulocytopenia (58) has been attributed to the sequestration of the transfused cells by the pulmonary circulation (59,60), and well as other organ and tissue circulation (61,62).

Test of Stickiness of Granulocytes. To better understand the phenomenon of granulocyte margination, sequestration by the vascular beds of various organs, and the sequence of events involved in inflammation, a great deal of attention has been focused on the stickiness of granulocytes. Several techniques have been developed and used in studying granulocyte stickiness in-vitro.

Garvin initiated the investigation of granulocyte adherence in-vitro, using a column packed with glass beads for his assay system (63). He demonstrated that the presence of divalent cations was required for full adherence; the adhesiveness of granulocytes was independent of cyanide and dinitrophenol, but was almost completely eliminated by iodoacetemide. Under all conditoins mentioned above, the loss of adhesiveness was concurrent with the loss of the usual ability of granulocytes to migrate. Lymphocytes were retained on the columns to a much lesser extent than granulocytes under all conditions. Other investigators have modified Garvins' assay system, but their studies have essentially confirmed Garvin's original findings (64,65). Recently, it was demonstrated that the retention of leukocytes in glass bead columns used as a measure of leukocyte adhesiveness is affected by the presence and the condition of erythrocytes and platelets (66,67).

Further investigation of granulocyte stickiness in-vitro was made possible by McGregor's (68) introduction of nylon fiber columns as a test system for granulocyte stickiness. He demonstrated that in-vitro exposure of whole blood to varying concentrations of ethanol caused a

dose-dependent significant inhibition of adherence, while sodium salicylate or hydrocortisone sodium succinate had no effect. However, in all volunteer subjects receiving prednisone or aspirin, impaired adherence developed.

The use of nylon fiber columns by other investigators has made available more information about the adhesiveness of granulocytes. Boxer, et al have shown that the state of microtubule assembly may directly affect the properties of the granulocyte plasma membrane without requiring alteration of cyclic nucleotides as an intermediary. Adherence of granulocytes to nylon fibers was inhibited in a dose-dependent fashion by exposure of these cells in-vitro to inhibitors of microtubule assembly (69).

Aspirin profoundly inhibited the in-vitro augmentation of human and mouse granulocyte adherence to nylon fiber induced by bacterial endotoxin. Granulocytes obtained from normal volunteers during the 48 hours following ingestion of aspirin did not respond normally to endotoxin stimulation, and pretreatment of mice with sodium salicylate prior to intraperitoneal infection with streptococcus pneumoniae impaired granulocyte exudation and resulted in uncontrolled bacteremia and greater lethality of infection (70).

The adherence of granulocytes to nylon fibers is inhibited by in-vivo administration of anti-inflammatory agents (71). In contrast, in patients with acute inflammation, the mean adherence of granulocytes was twice normal; their plasma contained a factor that augmented adherence of normal cells (72). Further investigation on the role of

plasma factors resulted in the finding of a close correlation between the activation of complement (C) and the stickiness of granulocytes. Whether C was preactivated in-vitro and then infused intravenously, activated in-vivo by administration of the cobra venom factor, or activated during extracorporeal circulation in human hemodialysis, shortly after C activation, circulating granulocytes disappeared, while their stickiness (measured by nylon fiber filtration) increased strikingly. Thereafter, when circulating granulocytes return and actually rebound to above baseline levels, their stickiness declines in parallel (73,74).

Prostaglandins are found to be inhibitors of granulocyte adherence. The in-vitro treatment of cells with PGI_2 inhibited their adherence to nylon fibers in correlation with a rise in the intracellular level of camp (75).

Another approach to study granulocyte adhesiveness was the use of tissue cultures of endothelial cells. Leukocytes showed increased adhesiveness to tissue culture monolayers of endothelial cells compared to epithelial cells, fibroblasts, kidney cells and plastic petri dishes without monolayers on them (76,77). Under proper conditions, granulocytes were able to migrate through the intercellular junctions of the endothelial cell monolayer in tissue cultures (77,78). A number of similarities (75,76) and differences (78,79) between the adhesiveness of leukocytes to nylon fibers or glass beads and endothelial cells in culture have been described. It is obvious that the surface of the endothelial cells in culture is more similar than are nylon fibers or glass beads to the kind of surfaces which a leukocyte encounters in-vivo.

2. The Lung

The lung is the organ of exchange where the respiratory gases, carbon dioxide and oxygen, are equilibrated between the blood and the atmosphere across the thin alveolar epithelium. For this, the lung has a large surface area and a rich blood supply. The lung is normally supplied by two arterial systems, the pulmonary artery which supplies the lungs with deoxygenated blood and arises from the right ventircle, and the bronchial arteries which convey oxygenated blood from the thoracic aorta. The adult pulmonary arterial system, to use an electrical analogy, lies in series with respect to the systemic circulation and receives the whole blood volume of the body.

Although built for gas exchange, the lung also serves numerous nonrespiratory functions, mainly as a consequence of its location in the circulatory sense between the right and left sides of the heart (80-82). Filtering capability of the lung is perhaps the earliest of such functions discovered, yet the least understood one (for a review of this subject, see reference 80).

The lung acts as a filter that rids the blood of particles larger than normal red blood cells. After injection of glass microspheres into the dog pulmonary artery, approximately 50 percent of spheres 2.8 - 4.0 μm in diameter could be found in the systemic circuit during the first circulation, but only 6 percent of those spheres eight μm or larger got through the pulmonary circuit when compared with simultaneously injected tagged erythrocytes (83).

The sequestration of granulocytes (60) and lymphocytes (84) from blood traversing the lungs has suggested a role for the lung in maintaining a stable level of circulating leukocytes (59). In their early, extensive studies, Ambrus, et al (60) showed that until a certain level was reached, white blood cells were rapidly removed from heparinized dog blood circulating in the heart-lung preparations from dogs. This level was then maintained and appeared to be independent of the leukocyte count of the infused blood. They observed a similar disappearance of leukocytes in the cross transfusion of intact donor dogs with heart lung preparations. Differences were also found between leukocyte levels of blood before and after passage through the lungs in long and short range in-vivo cardiac catheterization experiments. The fall of the white blood cell counts was due mainly to the disappearnace of granulocytes.

The endothelium of the pulmonary vascular bed is of the continuous type (85). However, the cells at the level of the alveolar-capillary unit differ from those of the pulmonary arteries and veins. The endothelium of the main-stem pulmonary artery contains fibrillar structures and abundant surface projections (82,86). The fibrillar material also occurs in endothelial cells of bronchial arterioles (87), but is not prominent in pulmonary capillary endothelial cells. Endothelial projections occur at the capillary level, but are most prominent in veins and arteries. Endothelial cells have abundant caveolae intracellulares (pinocytotic vesicles) (88).

Among the available techniques for studying the fine structure of the lung, the light and transmission electron microscope, although widely used and established, have the disadvantage of having a limited field of observation. On the other hand, the scanning electron microscope (SEM), used in this study, although lacking many capabilities of the light and transmission electron microscope, has the advantage of providing a larger field of observation.

The SEM has recently become an important tool in studying soft biological tissues. Among such tissues, the lung, because of its delicacy and complexity, requires a more careful tissue preparation. Several studies utilizing scanning electron microscopy of both normal and pathological lungs have been published (89-95). However, the information provided on the lung vasculature, especially on smaller blood vessels is still minimal, mostly due to difficulties involved in preparation of lung tissue for SEM. To obtain specimens small enough for observation with the SEM, fixed lung is usually cut or torn by a sharp razor blade. The mechanical deformation suffered by the exposed surfaces is so severe that structures such as capillaries or small venules and arteries become unsuitable for observation. To minimize such mechanical distortions, several alternative techniques such as ethanol-cryofracturing (96), and resin-cracking (97) have been developed. Ethanol-cryofracturing is a straight forward technique which causes minimal damage to exposed, fractured surfaces. When applied to the lung, ethanol-cryofracturing, in combination with vascular perfusion-fixation, yields a suitable specimen for examining the vascular network of the lung along with other morphological features.

II. MATERIAL AND METHOD

A. Animals

Male CD rats (Charles River Co.) of about 350 grams in weight were used in all experiments.

B. Isolated Perfused Lung Preparation

The animals were anesthetized by intraperitoneal injections of Sodium Pentobarbital (Diamond Labs, Inc.), 3.9 mg per 100 g of body weight. Following anesthesia, a tracheotomy was performed by inserting a silicon rubber (silastic tubing) cannula, 0.16 mm^{ID} x 0.24 mm^{OD} (Dow Corning Co.), into the trachea and securing it in place by a ligature. Blood coagulation was prevented by either injecting Heparin Sodium, 1,000 U.S.P. units (Upjohn Co.), through the jugular vein, or by mixing 2,000 U.S.P. units of heparin with Sodium Pentobarbital, used for anethesia, and injecting them intraperitonealy. The diaphragm and rib cage were then incised, exposing the heart and lungs. Respiration was restored immediately after opening the chest cavity by connecting the tracheal cannula to a Harvard respirator, model 680 (Harvard Apparatus Co.), under maximum inflation pressure of 18 cm H_2O and 45 strokes per minute. Stroke volume was adjusted to 3 ml per stroke. Pressure was measured by a Statham pressure transducer, model 5E (Gould Statham Inst., Inc.).

The pulmonary artery was cannulated through an incision in the right ventricle, and the cannula (same material and size as used for the tracheal cannula) was secured in place by a ligature with the heart and lungs still intact in the chest cavity. The left atrium was then incised to allow the perfusate to drip out.

Perfusate was pumped through the lung by a Harvard peristaltic pump, model 1203 (Harvard Apparatus Co.), at a flow rate of about 9 ml per minute, with a peak-to-peak pressure of 3-20 cm H_2O. Flow rate was measured by a magnetic flow probe, model 300A (North Carolina Medical Electric Co.) and a square-wave flow meter, model 321 (North Carolina Med. Elec. Co.). Perfusion pressure was measured by a Statham tranducer, model P23db (Gould Statham Co.). Both the perfusion and respiration pressures were recorded by a HP recorder, model 7402A (Hewlett Packard Co.).

The heart was totally excised as soon as the pulmonary artery cannula was secured in place; immediately thereafter the lungs were removed from the chest cavity and suspended from the tracheal cannula in ambient air at room temperature. The whole surgical operation, from the moment that the chest cavity was opened to the moment when the lungs were removed, took about eight minutes.

Perfusates were maintained in two types of water-jacketed glass reservoirs. A funnel shaped reservoir was used for maintenance of cell or particle free buffers, and a flat bottom reservoir was used for cell or particle suspensions. The flat bottom is especially useful for placing the reservoir on a magnetic stirrer to constantly and gently stir the cell or particle suspension. Figure 1 illustrates these two kinds of reservoirs.

The floor of the flat-bottomed reservoir was made flush with the perfusate outlet arm so that cells or particles would not have any barrier on their way out, and a uniform transfer of particles from the reservoir, through the perfusion tubing to the lung, was assured.

The temperature of the perfusates was kept constant by circulating water from a variable temperature bath and circulator, model 2095 Masterline (Forma Scientific Co.) through the water jackets around the reservoirs.

C. Perfusion

To wash as much blood as possible out of the lung, it was initially perfused and washed for ten minutes with one of the following buffers: Dulbecco's Medium supplemented with 20 percent fetal calf serum (GIBCO), Dulbecco's Phosphate buffered saline solution (GIBCO), Hank's Balanced Salt Solution (GIBCO). Only lungs which could be washed clean and white were used for the experiments. Immediately following this initial cleansing, whatever perfusate to be used in the experiment was perfused through the lung.

1. Single-Pass Perfusion

In this mode, the perfusate was passed only once through the lung. The pulmonary venous effluent was then collected and treated as desired. The experimental set-up was a modification of Pickett's method (98). Figure 2 provides a graphic representation of the single-pass perfusion set-up.

2. Continuous-circulation Perfusion

In this mode, the perfusate (cell or particle suspension) was circulated through the lung for the desired period. Samples were taken either from the pulmonary venous effluent drops or from the reservoir at various intervals. The experimental set-up was a modification of Bassett's method (99). Figure 3 is a schematic representation of the continuous-circulation perfusion set-up.

D. Cell and Particle Suspensions

Three kinds of cell suspensions and a suspension of latex particles were used in these experiments. Cell suspensions were whole blood, bone marrow cells, and peritoneal neutrophils.

1. Whole Blood

Whole blood was collected by bleeding rats through the vena cava with a heparinized 20 ml plastic syringe. An average of about 13 ml of blood could be obtained from each rat.

2. Bone Marrow

Bone marrow from two femurs and tibias of a rat was suspended in Dulbecco's Medium supplemented with 20 percent fetal calf serum (GIBCO). The marrow was gently passed through successively smaller needles (gauge 19 to 27) to produce a single cell suspension. The suspension volume was kept constant and equal to 70 ml in all experiments. These suspensions were used for continuous-circulation experiments. Cell concentration in the suspension was adjusted to the desired values according to the experimental scheme.

3. Neutrophils

Neutrophils were prepared from the peritoneal cavity by injecting 30 ml of 0.1 percent glycogen (GIBCO) in physiological saline into the peritoneal cavity of a rat. Four to 16 hours later, the cavity was opened. The cells were pipetted out, centrifuged, and resuspended in Hank's Balanced Salt Solution (HBSS). These cells were washed once more with HBSS and then resuspended in HBSS or other buffers to make the neutrophil suspension with the desired concentration and volume.

These suspensions were used in both single-pass and continuous-circulation experiments; in the latter case, the suspension was supplemented with 20 percent fetal calf serum.

4. Latex Particles

Latex particles (Dow Chemical Co.) with a diameter of 7.6 ± 2.3 μ were suspended in Dulbecco phosphate buffered saline (GIBCO) to the desired concentration. This suspension was used for single-pass experiments. In all suspensions using artificial medium, the pH was adjusted to 7.3.

E. Scanning Electron Microscopy

Isolated perfused rat lungs were fixed either by tracheal infusion or vascular perfusion of the fixative. In both cases, the fixative was 1.5 percent Glutaraldehyde in .1 M sodium cacodylate buffer with osmolarity of 310 mosmol/kg and pH of 7.4. For tracheal infusion fixation, the airways were washed three times by infusing 5 ml of the fixative through the trachea. The airways were then filled with the fixative at a maximum pressure of 20 cm H_2O, the trachea was clamped, and the whole lung was immersed in fresh fixative for ten minutes. For vascular perfusion fixation, the lung was initially perfused for ten minutes with Dulbecco's PBS, pH = 7.4, followed by fixative perfusion for another ten minutes. The whole lung was then immersed in fresh fixative for an additional ten minutes.

In either case, the lung was subsequently cut by a sharp razor blade into several pieces about one cm long and a few mm thick. The pieces were returned to the fixative and were degassed under vacuum to

remove any air trapped in the airways and to allow the tissue to sink completely into the fixative. Fixation was then continued for 24 hours at 4°C.

Fixed samples were rinsed with several washes of 0.2 M sodium cacodylate buffer, pH = 7.4, for a total of four hours. Samples were then post-fixed in 0.1 percent osmium tetroxide in 0.1 M sodium cacodylate buffer, pH = 7.4, at 4°C for ten hours. Excess osmium tetroxide was removed by several washes in 0.2 M sodium cacodylate buffer for four hours. Finally, samples were washed twice in distilled water before dehydration. Samples were dehyrated in a graded series of ethanols.

Ethanol-cryofracturing was performed as described by Humphreys (96). Samples in the final 100 percent ethanol rinse were encapsulated in parafilm sleeves, frozen in liquid nitrogen and fractured by a pre-cooled razor blade. Fractured specimens were then thawed in a large volume of fresh 100 percent ethanol.

Specimens were dried by CO_2 in a critical point dryer (Polaron Co.) as described by Cohen (100). Dried specimens were mounted on studs and coated with gold palladium in a sputter-coater, model Hummer (Technics Co.). A field emission scanning electron microscope, model 50 (Coates Welter), with accelerating voltage of about 15-20 KV, was used for observation. The same angle of tilt was used in taking all the photographs, and no attempt was made to take stereo-pair photographs for critical size determination.

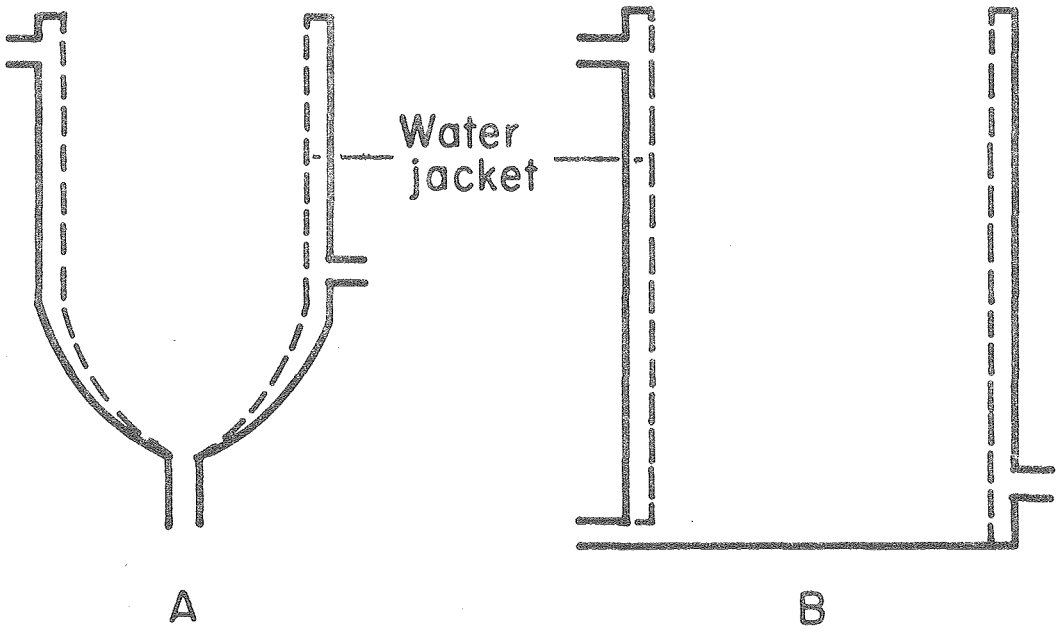

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Figure 1. A. The funnel shaped reservoir. B. The flat-bottom reservoir.

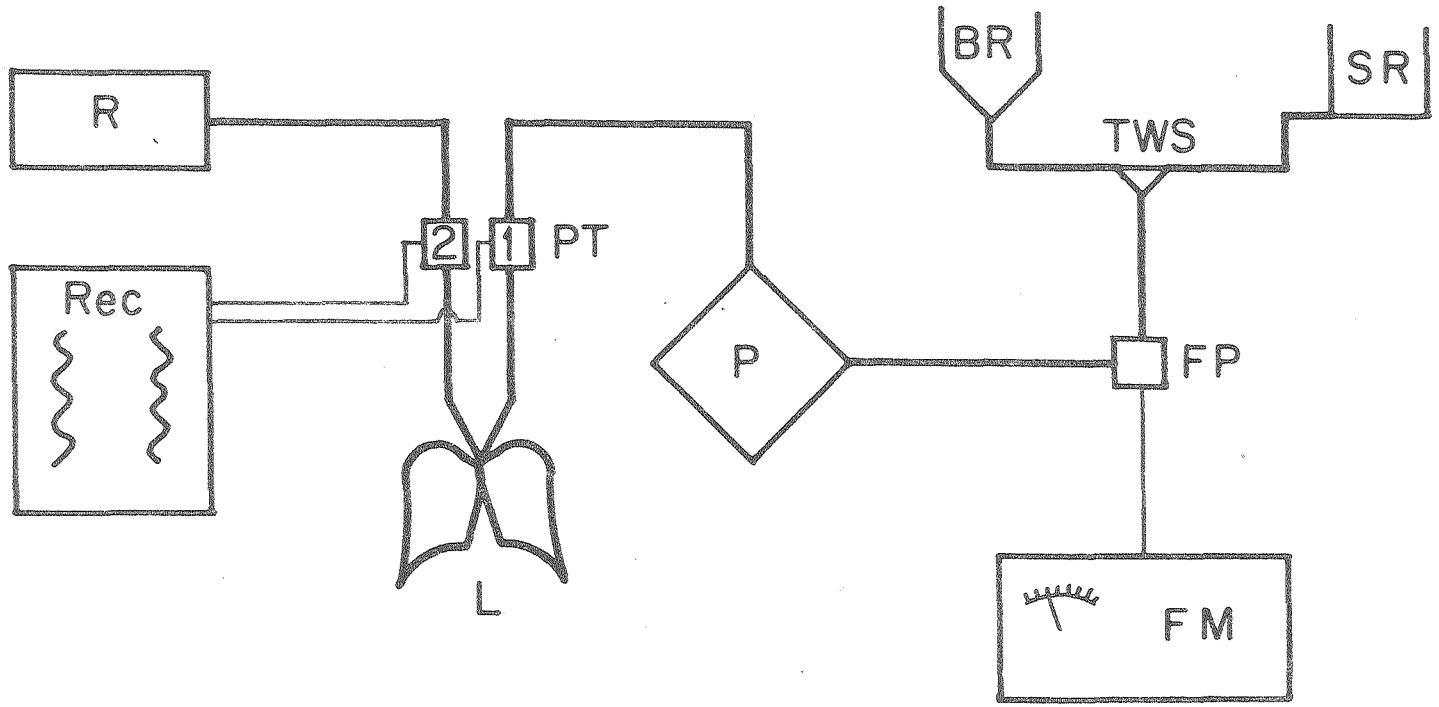

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Figure 2. Single-pass perfusion set-up SR: particle or cell suspension reservoir; BR: Buffer reservoir; TWS: three-way stopcock; FP: Flow probe; MW: Flow meter; P: pump; PT-1: Pressure transducer, liquid; PT2: Pressure transducer, air; L: Lung; R: Respirator; Rec: Recorder.

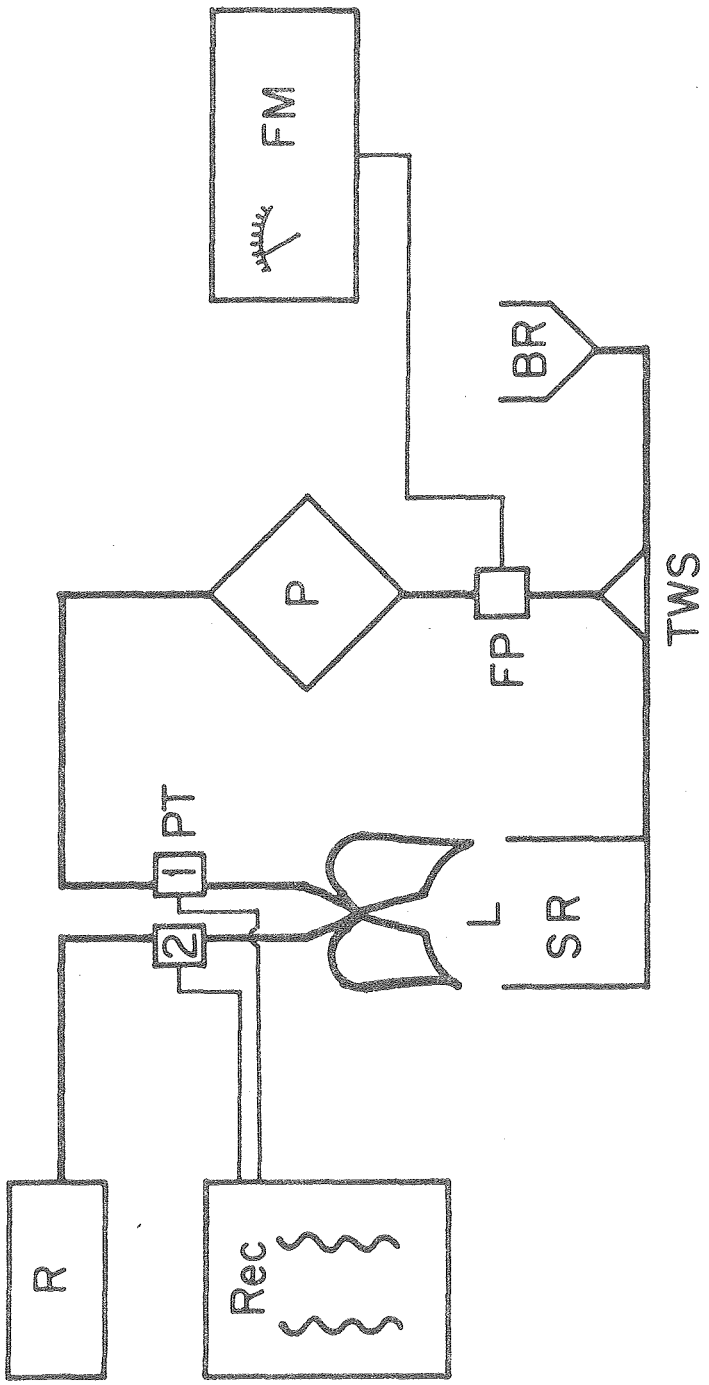

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Figure 3. Continuous-circulation perfusion set-up. See Fig. 2 for abbreviations.

III. RESULTS

A. Kinetics of Sequestration

Before attempting to determine the mechanism of sequestration of leukocytes by the blood vascular bed, the fundamental question of whether or not such a phenomenon exists at all must be addressed. One approach to this question consists of passing a suspension of leukocytes, with as little manipulation as possible, through a network of blood vessels and monitoring the leukocyte concentration at various times and locations in the system. In the absence of sequestration, one would expect that the same number of cells entering the network of blood vessels would leave the system. The most physiological leukocyte suspension with the least amount of manipulation is freshly prepared whole blood.

As a general control experiment, blood or other leukocyte suspensions were circulated through the system without the lung in place. In either the single-pass or the continuous-circulation modes, there was no detectable retention of cells by the system.

Ninety ml of freshly collected rat blood was used to perfuse, in the single-pass mode, an isolated rat lung as described in the method section. The flow rate of perfusion in this series of experiments, and in all other experiments to be described, was kept constant and equal to nine ml/min. The blood leukocyte concentration was determined, using a hemocytometer, before the initiation of perfusion and in samples of lung effluent suspension collected at various intervals after the initiation of perfusion. The results of such an experiment

are illustrated in Fig. 4, in which the log of the fraction of leukocytes recovered in the lung effluent is plotted as a function of time. Each point is the average of three experiments. It is evident from these results that not all cells that enter the lung leave it; i.e., the isolated perfused lung sequesters blood leukocytes. It is also obvious that the sequestration rate is not constant, it is high in the early moments of perfusion, but decreases with the continuation of perfusion.

There are at least three possible explanations for such a variable sequestration rate; a) the opening of lateral shunts, b) the gradual death of the lung, c) the saturation of the lung with leukocytes.

If the opening of lateral shunts is responsible for the decrease in the sequestration rate, then the replacement of leukocytes with other particles of the same size should not change the outcome of the experiment. To test this, the lung was perfused with suspensions of latex particles of about the same size as leukocytes. Figure 5 illustrates results of such an experiment. Two suspensions of latex particles, 7.6 ± 2.3 μm in diameter, with concentrations of 3.69×10^6 and 9.3×10^6 particles/ml were prepared and used to perfuse two isolated rat lungs in the single-pass mode. Experimental conditions were similar to those for Fig. 4. Each point on Fig. 5 is the average of two experiments and the Y axis represents the fraction of particles which were recovered from the lung. It is evident from these results that the latex particles are also sequestered by the lung. However, the rate and pattern of sequestration are totally different from those

of leukocytes. Very few, or no, particles can pass through the lung. Results of this experiment, therefore, contradict the lateral shunt hypothesis as a possible explanation for the variable rate of leukocyte sequestration observed in Fig. 4.

The claim that latex particles have actually plugged the capillaries and small blood vessels is also supported by morphological evidence. In this case, at the end of the latex particles perfusion experiment, the lung was fixed by the tracheal infusion technique and processed for scanning electron microscopy. Figure 18 shows the fractured surface of such a lung. Several small blood vessels plugged by latex particles are visible in the picture.

The second possible explanation for the variable rate of sequestration of leukocytes is the gradual death and malfunction of the lung. The following experiment was designed to test this possibility: Eight rats were bled to collect enough blood for at least eight minutes of single-pass perfusion. Half of this blood was used to perfuse a rat lung for three minutes. Samples for the determination of leukocyte concentration were collected from the reservoir and the lung effluent suspension at 30 second intervals. At the end of this phase, a cell free buffer, Dulbecco's PBS (GIBCO), was perfused through the lung for four minutes to wash away the blood from the earlier perfusion. Finally, at the end of the buffer perfusion, the rest of the blood perpared for phase 1 was perfused through the lung in the single-pass mode. Samples were taken from the reservoir and from the lung effluent suspension, and the fraction of cells recovered was

determined. If the dying lung hypothesis is correct, one would expect that in the second blood perfusion, all the cells going into the lung would pass through it without any sequestration. The results of the experiment are illustrated in Fig. 6. It is evident from these results that not all of the cells going into the lung in the second blood perfusion could pass through it and that the same pattern of variable sequestration observed in the first blood perfusion was repeated in the second blood perfusion. These results, therefore, exclude the dying lung hypothesis as a possible explanation for variable-rate sequestration of leukocytes by the lung.

The results of these experiments leave the hypothesis of lung saturation with leukocytes as the only possible explanation. In the experiments described above, a constant concentration of blood leukocytes is continuously passing over the vascular bed of the lung, and the lung effluent leukocyte concentration, from a low level, gradually approaches that of the input suspension. There are two possible explanations for this: a) Leukocytes irreversibly attach to the luminal surface of endothelium of blood vessels and, as the perfusion continues, the lung saturates with cells and removes fewer and fewer cells from circulation, or b) Cells attach reversibly to the vascular endothelium and, as the perfusion continues, the system reaches a steady state at which the number of cells removed by the lung from circulation equals the number of cells released back into circulation by it.

Results described in Fig. 4 provide no information as to the reversibility of attachment. Therefore, in order to clarify the nature of attachment of leukocytes to blood vessel walls and further test the soundness of the lung saturation hypothesis, the following experiments were performed. Leukocyte suspensions of known volume and concentration were repeatedly circulated through isolated rat lungs (see Fig. 3). At various intervals after the initiation of perfusion, samples were collected from the suspension reservoir and from the lung effluent suspension and their leukocyte concentration was determined. If the irreversible-attachment hypothesis is correct, one would expect that the lung would remove and deplete all the cells in the suspension; i.e., that the cell concentration in the suspension reservoir would eventually drop to zero exponentially.

Results from such experiments are illustrated in Fig. 7. In these experiments, the cell suspension was prepared from bone marrow. The reason for using bone marrow suspension was the desire to have a rather high and yet variable concentration of white cells in a large volume of suspension. Cell concentration was adjusted to 9×10^6 nucleated cells/ml and the volume of the perfusate suspension was 70 ml. Each point in Fig. 7 is the average of two experiments.

Contrary to the irreversible attachment hypothesis prediction, the cell concentration in the reservoir did not drop to zero; actually it reached a steady state and maintained this value for the rest of the perfusion period. Lung effluent cell concentration was very low (usually 5 to 10 percent of initial concentration) at the beginning,

but then it gradually increased until it reached a steady state where the same number of cells going into the lung could pass through and come out of it.

Results of this experiment do not exclusively support the reversible attachment hypothesis, for there is still the possibility that the irreversible attachment of cells exists, but that this attachment is selective for a specific group of cells and, once this group of cells is removed from circulation, the remaining cells in the suspension circulate through the lung without being sequestered and removed from circulation.

If the above explanation is correct, then one would expect that in the plateau region in Fig. 7, replacing the lung in the circuit with a new lung should not distrub the steady state, because all the specific cells to be removed should have already been removed by the first lung. This hypothesis was tested and the results are illustrated in Fig. 8. For this purpose, a bone marrow suspension similar to the one used in the previous experiment was prepared and used to perfuse a rat lung in the continuous-circulation mode. Samples were collected from the reservoir and the lung effluent suspension and the nucleated cell concentration in each was determined. When the steady state was reached, the lung was replaced by a new lung from another rat the perfusion was continued. Samples from the reservoir and the lung effluent suspension were collected and the nucleated cell concentration in each was determined. The results indicate that, in contrast to the selective cell removal hypothesis prediction, introduction of the second lung

disturbed the steady state, and the whole pattern of cell removal observed in the first lung was repeated in the second lung.

To examine the effect of the input cell concentration on sequestration and on the steady state of the system, three experiments were performed. In the first experiment the circulating suspension was replaced at the steady state with a fresh suspension having a concentration equal to the steady state concentration. To do this, a stock suspension of bone marrow cells was prepared and part of this stock suspension was used to make 70 ml of 8×10^6 nucleated cell/ml. This suspension was then used to perfuse a rat lung in the continuous-circulation mode. Samples were taken from the reservoir and from the lung effluent suspension to determine the steady state concentration. Then, from the stock suspension, a new suspension with a volume of 70 ml and a concentration of 2.1×10^6, which was approxmately equal to the steady state concentration of 2.7×10^6, was prepared. This new suspension was used to replace the circulating suspension at the plateau region. Samples from the reservoir and the lung effluent were collected and their leukocyte concentration was determined. The results are illustrated in Figs. 9 and 10. Replacement of the circulating suspension at the plateau region with a new suspension having a concentration equal to that of the steady state concentration did not distrub the steady state situation.

In the second experiment, the circulating suspension was replaced with a fresh suspension of a higher concentration than that of the steady state concentration. Here, two bone marrow suspensions, one

with a concentration of 5.3×10^6 nucleated cell/ml and one with 10×10^6 nucleated cell/ml were prepared. The first suspension (5.3×10^6 cell/ml) was used to perfuse a rat lung in the continuous-circulation mode. Samples were collected from the reservoir and the lung effluent suspension and their nucleated cell concentration was determined. Once the steady state was established, the circulating suspension was replaced with the other suspension (10×10^6 cell/ml) and the nucleated cell concentration in the reservoir and the lung effluent suspension was determined at various intervals. The results are illustrated in Fig. 11. The increase in circulating cell concentration disturbed the steady state and the whole pattern of sequestration was repeated. There was an increase in the rate of sequestration and the establishment of a new plateau level.

For the third experiment, the circulating suspension was replaced at the plateau level with a fresh suspension with a concentration lower than the steady state concentration. For this experiment, a bone marrow suspension with a concentration of 15.8×10^6 nucleated cell/ml was prepared. Part of this suspension was used to perfuse a rat lung in the continuous circulation mode and the remainder of the suspension was kept as stock suspension for future use. A steady state concentration of 3.35×10^6 nucleated cell/ml was determined, and from the stock suspension described above, a fresh suspension was prepared with a concentration of 1.195×10^6 nucleated cell/ml, approximately half of the steady state concentration. The circulating suspension was replaced with this new suspension and the nucleated

cell concentration in samples from the reservoir and the lung effluent suspension was determined at various intervals. Figures 12 and 13 illustrate the results of this experiment. When the circulating cell concentration was decreased, the lung released cells into circulation and raised the circulating cell concentration above the initial concentration of the second suspension.

To study the effect of the initial (starting) leukocyte concentration on the steady state concentration, the following experiment was performed: Three different suspensions of bone marrow cells, with concentrations of 5.38×10^6, 9.3×10^6 and 15.2×10^6 nucleated cell/ml were prepared. These suspensions were used to perfuse three isolated rat lungs in the continuous-circulation mode. The steady state concentration was determined, and the results are plotted in Fig. 14. For the range of concentrations examined, the fraction of cells remaining in circulation was a fairly constant number, 31.2 ± 3.9 percent of the initial concentration.

Both the blood and the bone marrow suspension used in previous experiments were a heterogeneous mixture of several cell types; erythrocytes, platelets, monocytes, lymphocytes and granulocytes. Whether or not the individual cell type in the mixture behave according to the reversible attachment pattern observed for the mixture of cells is the next question to be answered. Also, the active attachment and detachment of cells to blood vessel walls would require a different rate constant for cells which are morphologically and functionally different. Therefore, the following experiments were designed to find

out whether; a) the individual cell type in the blood would follow the general pattern of reversible-attachment to blood vessel walls, and b) the rate constants or the concentration at the plateau level varies for different cell types.

A suspension of peritoneal neutrophils was prepared. Such a suspension, if prepared carefully, would contain virtually no erythrocytes or platelets. About 90 percent of cells are neutrophils, and the rest of them are mostly macrophages and monocytes with some lymphocytes. The suspension volume and concentration were 70 ml and 5.12×10^6 nucleated cell/ml, respectively. The flow rate was 9 ml/min. This suspension was used to perfuse an isolated rat lung in the continuous-circulation mode. Samples were collected from the reservoir and from the lung effluent suspension at various intervals and the total nucleated cell concentration of each was determined. Also, a differential count was performed on these samples. Although with the differential counts, four cell types, granulocytes, monocytes, macrophages and lymphocytes, could be detected, the distinction between the last three types was not always clear and, therefore, in the differential counts all mononuclear cells were considered as one group and neutrophilic granulocytes as another group. Occasionally some eosinophilic granulocytes were present in these suspensions, but since they make up a very small fraction, about 1.5 percent of the total cells, they were ignored in the calculations. The results are illustrated in Fig. 15. The following conclusions are evident from these results; a) the cell suspension used here, although different

from bone marrow suspension and the whole blood used in previous experiments, follows the same pattern predicted by reversible-attachment hypothesis, b) individual cell types in the suspension, neutrophils and mononuclears, follow the same pattern of reversible-attachment as does the whole suspension, c) the rate constants differ for different cell types and compositions. For the crude peritoneal suspension, the plateau level is approximately 14 percent of initial concentration, while for bone marrow suspensions this value is 31.2 ± 3.9 percent of initial concentration. For neutrophils in the peritoneal suspension, the plateau level is 5.5 ± 2 percent of initial concentration, and for mononuclear cells in the peritoneal suspension, it is 72 ± 7 percent of initial concentration.

The results also indicate that, under similar conditions, the lung sequesters a larger proportion of neutrophils than mononuclear cells. Further evidence for this increased affinity of neutrophils to blood vessel walls was obtained from experiments with whole blood perfusions in the single-pass mode. If a differential count is done on samples from the experiments described in Fig. 4 to separate neutrophils from mononuclear cells (in this case lymphocytes and monocytes), neutrophils will show a higher degree of retention and sequestration by the lung than mononuclear cells. Figure 6 is the illustration of differential counts in such experiments. After two and a half minutes of perfusion, about 50 percent of neutrophils are still being retained by the lung, while at the same time less than 20 percent of mononuclear cells are being retained.

Active reversible attachment of blood cells, especially neutrophils, to blood vessel walls, suggests that the chemical composition of the perfusion medium, the medium in which cell interaction with blood vessel walls occurs, might affect the rate of attachment and detachment. To test this, the effect of divalent cations on the equilibrium state for attachment of neutrophils to lung blood vessel walls was examined. For this purpose, a suspension of peritoneal neutrophils was prepared. The concentration and the total number of neutrophils in the suspension was 0.9×10^6 cells/ml and 36×10^6 cells, respectively. This suspension was used to perfuse an isolated rat lung in the single-pass mode. All of the lung effluent suspension was collected and its total number of neutrophils was determined by a whole cell count and a differential count. The ratio of the total number of neutrophils in the lung effluent suspension to that of the original suspension is the non-sequestrable fraction of neutrophils. As indicated in Table 1, in the absence of CA^{++} and MG^{++}, this fraction of non-sequestrable neutrophils increased by about 92 percent.

Table 1. The effect of Ca^{++} and Mg^{++} on the sequestration of neutrophils by the isolated perfused rat lung

Treatment	Non-sequestrable Fraction (%)	% increase in non-sequestrable fraction over control	P value	(n)
Control	6.42	-----	--	(5)
No Ca^{++} and Mg^{++} medium	12.33	92.34	0.05	(6)

Figure 4. Sequestration of blood leukocytes by isolated perfused rat lung. Rat whole blood was perfused, in the single-pass mode, through an isolated rat lung. On the X axis, zero represents the time when blood perfusion begins. Points on the Y axis show the ratio of the concentration of leukocytes recovered to perfused leukocytes on a log scale. The sequestration rate, the fraction of cells which are removed from circulation by the lung, gradually decreases as the perfusion continues, finally leveling off after five minutes at about five percent or less. The input leukocyte concentration was $10.18 \pm 3.37 \times 10^6$ cells/ml. Bars are 1 standard error of the mean.

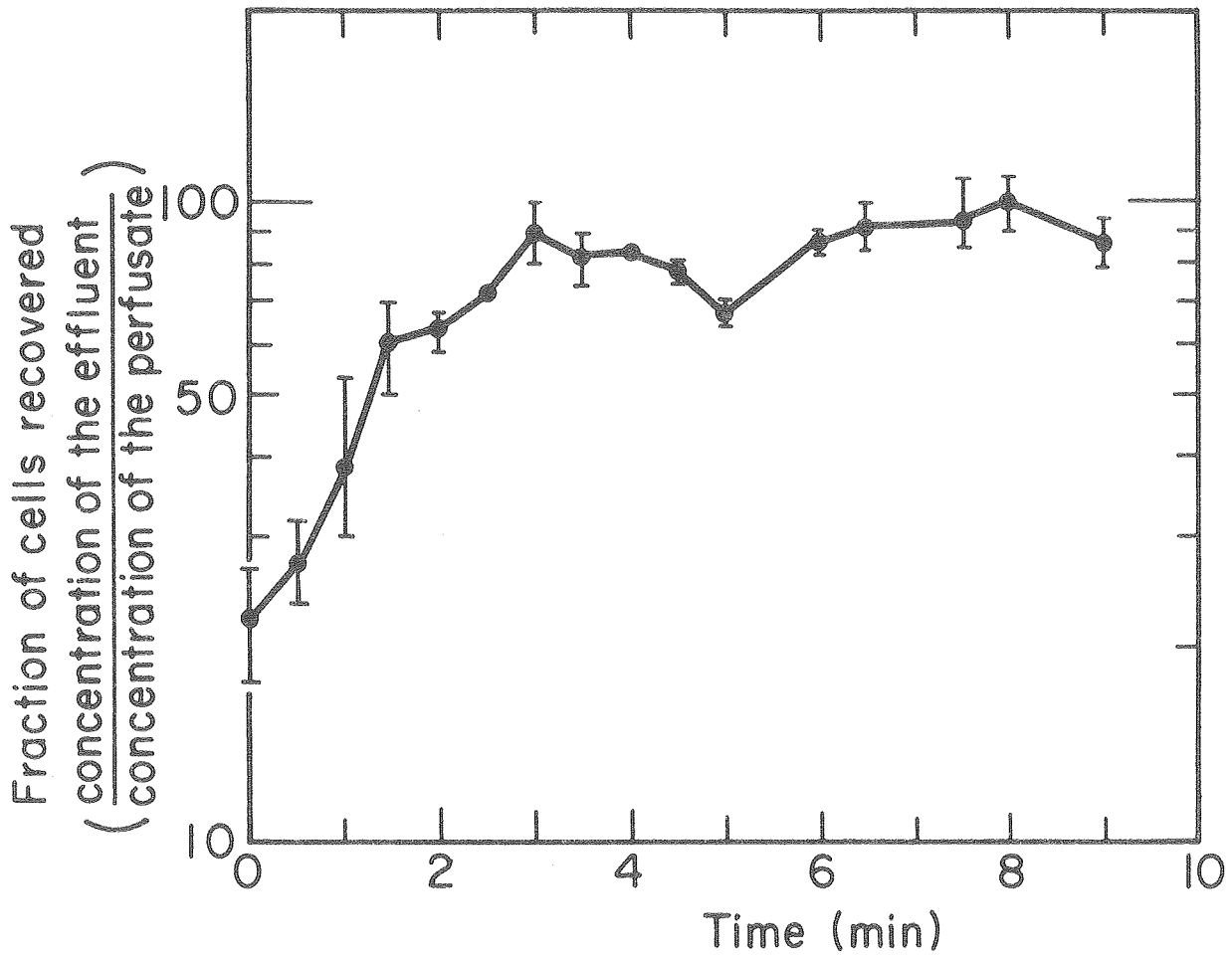

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Figure 4

Figure 5. Sequestration of latex particles by isolated perfused rat lung. Latex particles of 7.6 ± 2.3 μ in diameter were perfused, in the single-pass mode, through an isolated rat lung. The Y axis is the ratio of particle concentration in the lung effluent suspension to the initial particle concentration. Only the first two samples from the lung effluent suspension contained a very small number of particles; the rest of the samples did not contain any particles at all. The experimental conditions are similar to those of Fig. 4.

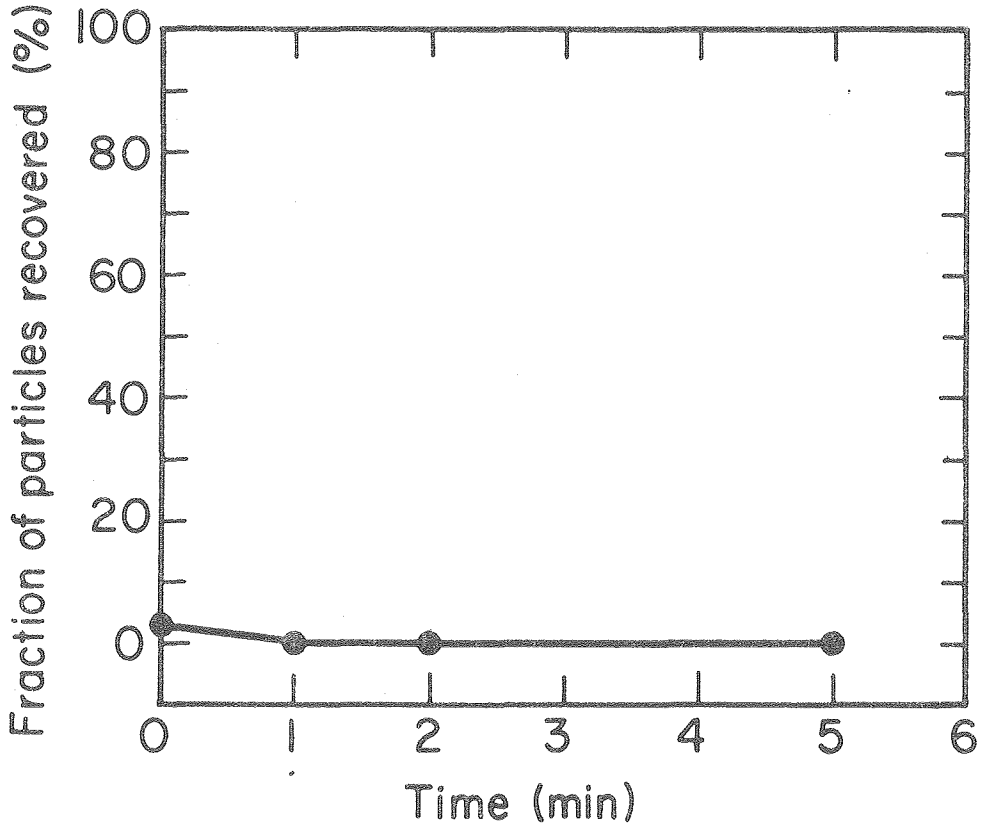

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Figure 5

Figure 6. Sequestration of blood leukocytes by isolated perfused rat lung. A stock of blood was prepared from eight rats. The lung was perfused for three minutes with half of this blood. Then the lung was perfused with a cell free buffer for four minutes to wash the blood away. Finally, the lung was perfused with the rest of the blood stock prepared earlier. The leukocyte concentration in the stock blood was $8.52 \pm 0.3 \times 10^6$ cells/ml. The Y axis is similar to that of Fig. 4.

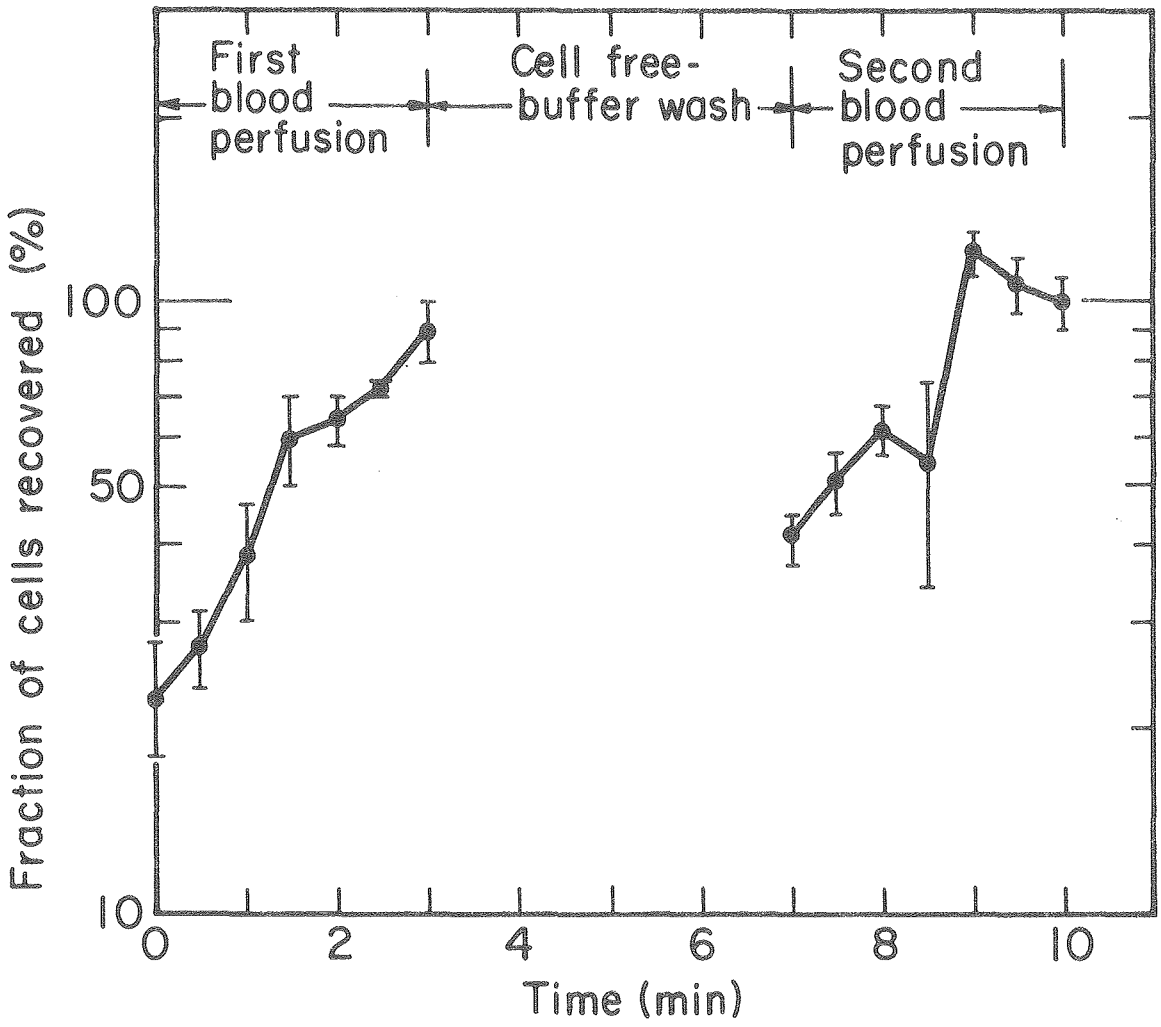

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Figure 6

Figure 7. Sequestration of bone marrow cells by isolated perfused rat lung in the continuous-circulation mode. Bone marrow suspension with a concentration of $9.25 \pm .55 \times 10^6$ nucleated cells/ml was perfused through the lung in the continuous-circulation mode (see Fig. 3), and at various intervals, samples were collected from the reservoir and the lung effluent suspension and their nucleated cell concentration was determined. The Y axis is the ratio of leukocyte concentration in samples to the initial concentration of cells in the suspension on a log scale. The lung did not deplete the cells in the suspension; sequestration levels off very quickly and the system maintains a steady state at which approximately 30 percent of the cells remain in circulation. Net cell sequestration and removal is less than five percent at the plateau region.

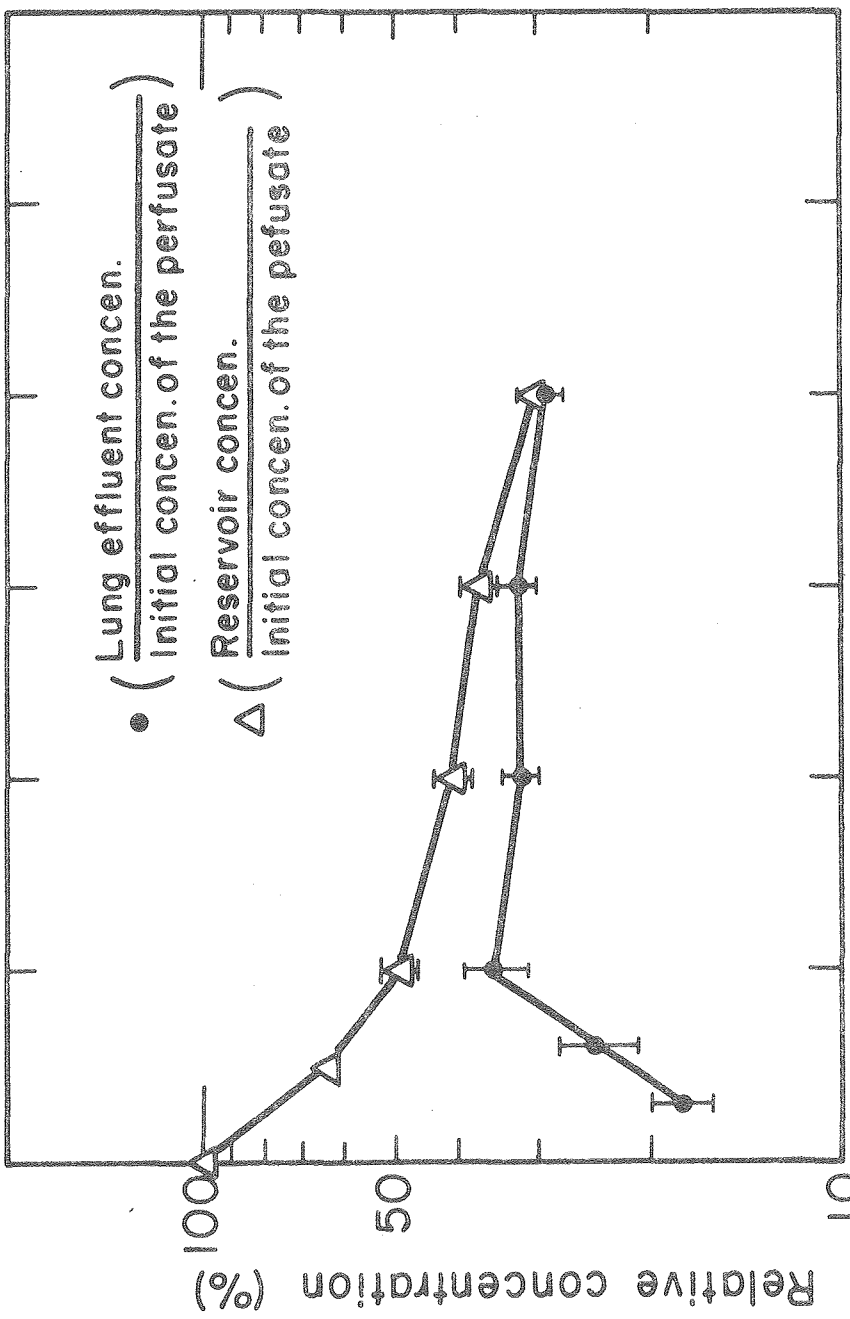

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Figure 7

Figure 8. Sequestration of bone marrow cells by two successive lungs. Bone marrow suspension with a concentration of $8.86 \pm 0.1 \times 10^6$ cells/ml was perfused through an isolated rat lung in the continuous-circulation mode. After reaching the steady state, the lung in the circuit was replaced with a new lung and the perfusion in the continuous-circulation mode was continued. The Y axis is the ratio of leukocyte concentration in the samples from the reservoir and the lung effluent suspension to the initial perfusate concentration on a log scale. Introduction of the new lung disturbs the steady state of the system. The sequestration pattern observed with the second lung is similar to that of the first lung, indicating that sequestration is not totally selective and specific for one cell type. The second plateau has a different concentration from the first one.

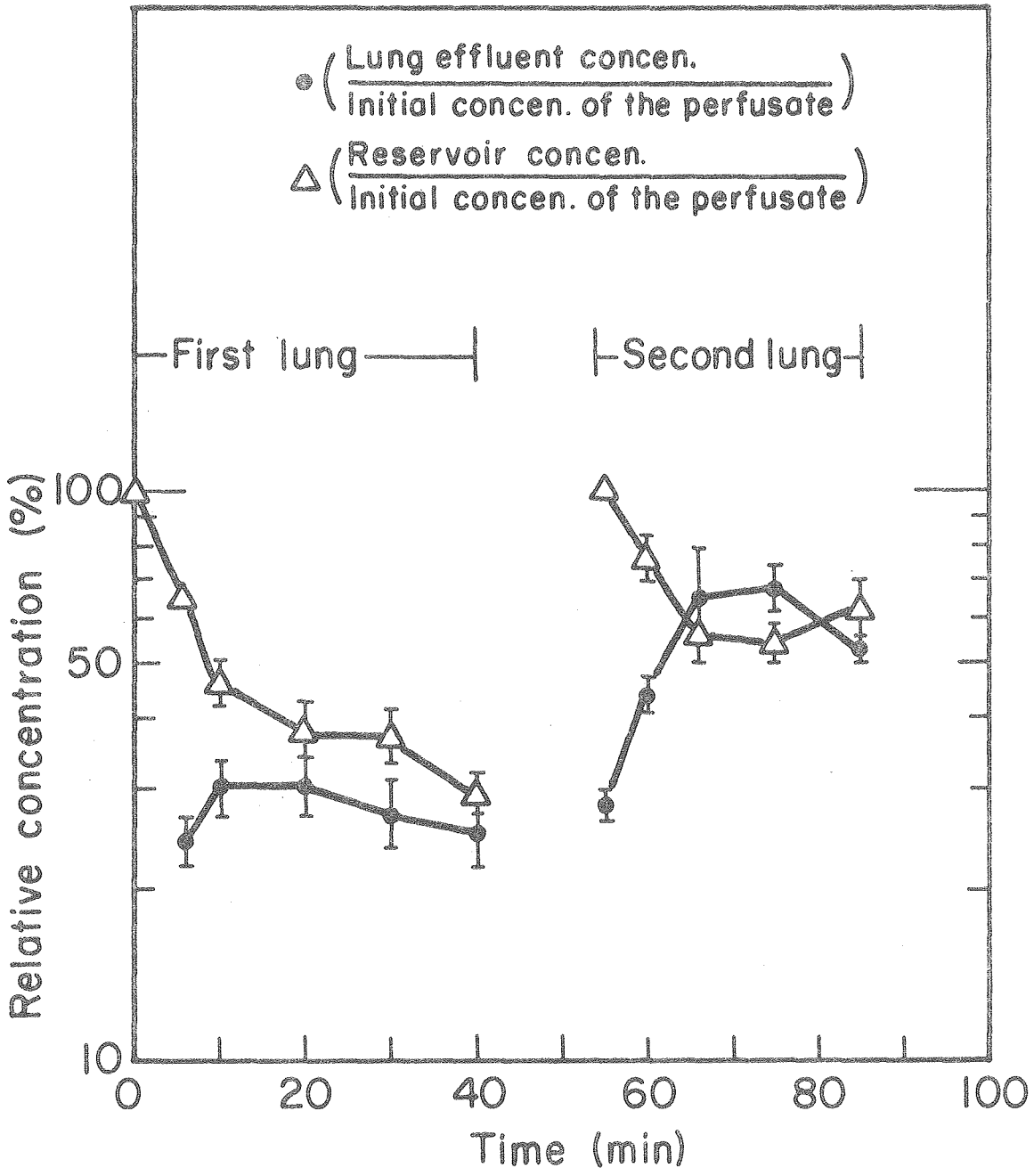

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Figure 8

Figure 9. Sequestration of bone marrow cells by isolated perfused rat lung. A bone marrow suspension was perfused through the lung in the continuous-circulation mode. At the plateau, the circulating suspension was replaced with a new suspension with a concentration approximately equal to that of the steady state concentration. The Y axis represents the absolute value of the nucleated cell concentration in the lung effluent samples on a log scale. Replacement of the circulating suspension with a new suspension with a concentration approximately equal to that of the steady state concentration does not distrub the steady state.

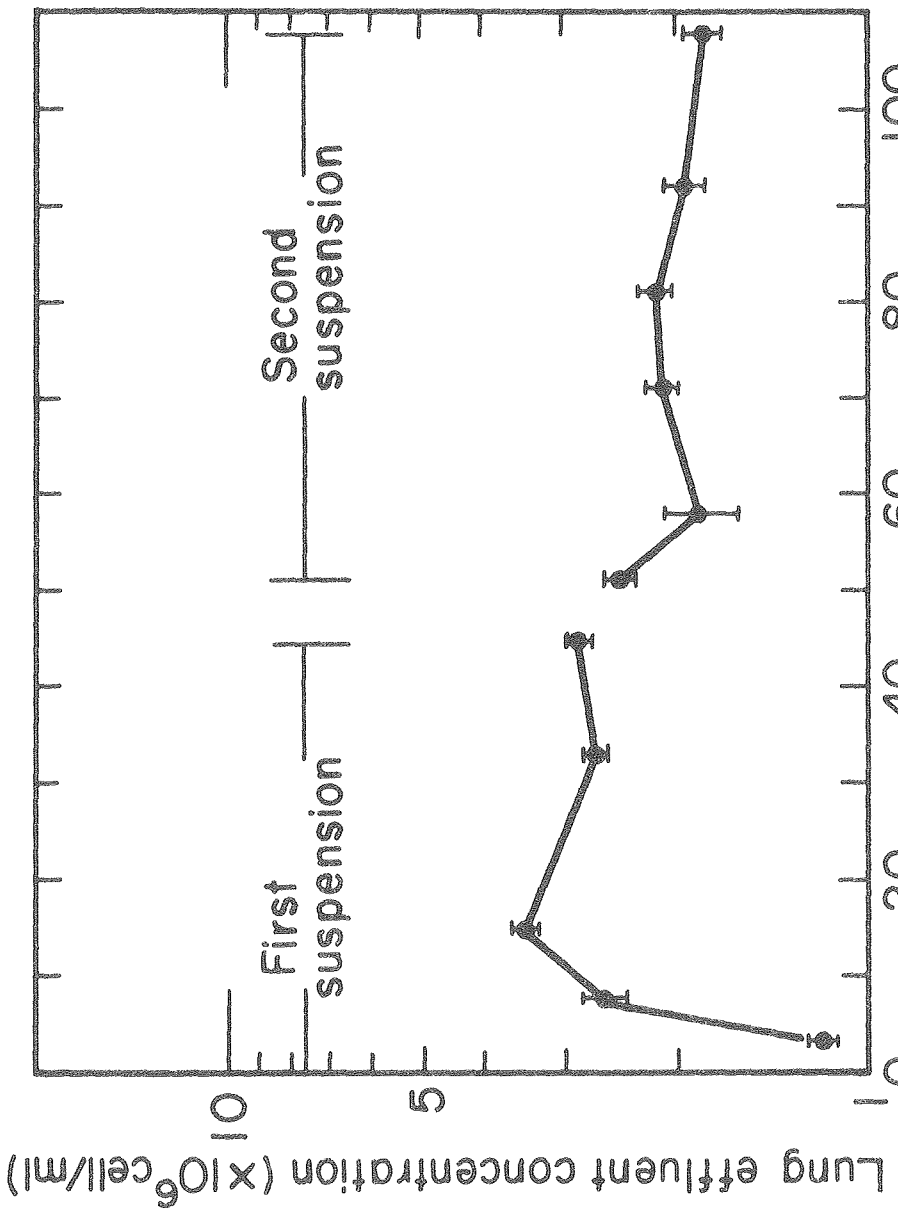

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Figure 9

Figure 10. Same as Fig. 9, except that the Y axis in this figure represents the log of the ratio of nucleated cell concentration in the lung effluent samples to the initial concentration in the reservoir. For the second suspension, the steady state circulating concentration is 97.8 percent of the initial concentration of the second perfusate, while for the first suspension, this number is 33 percent.

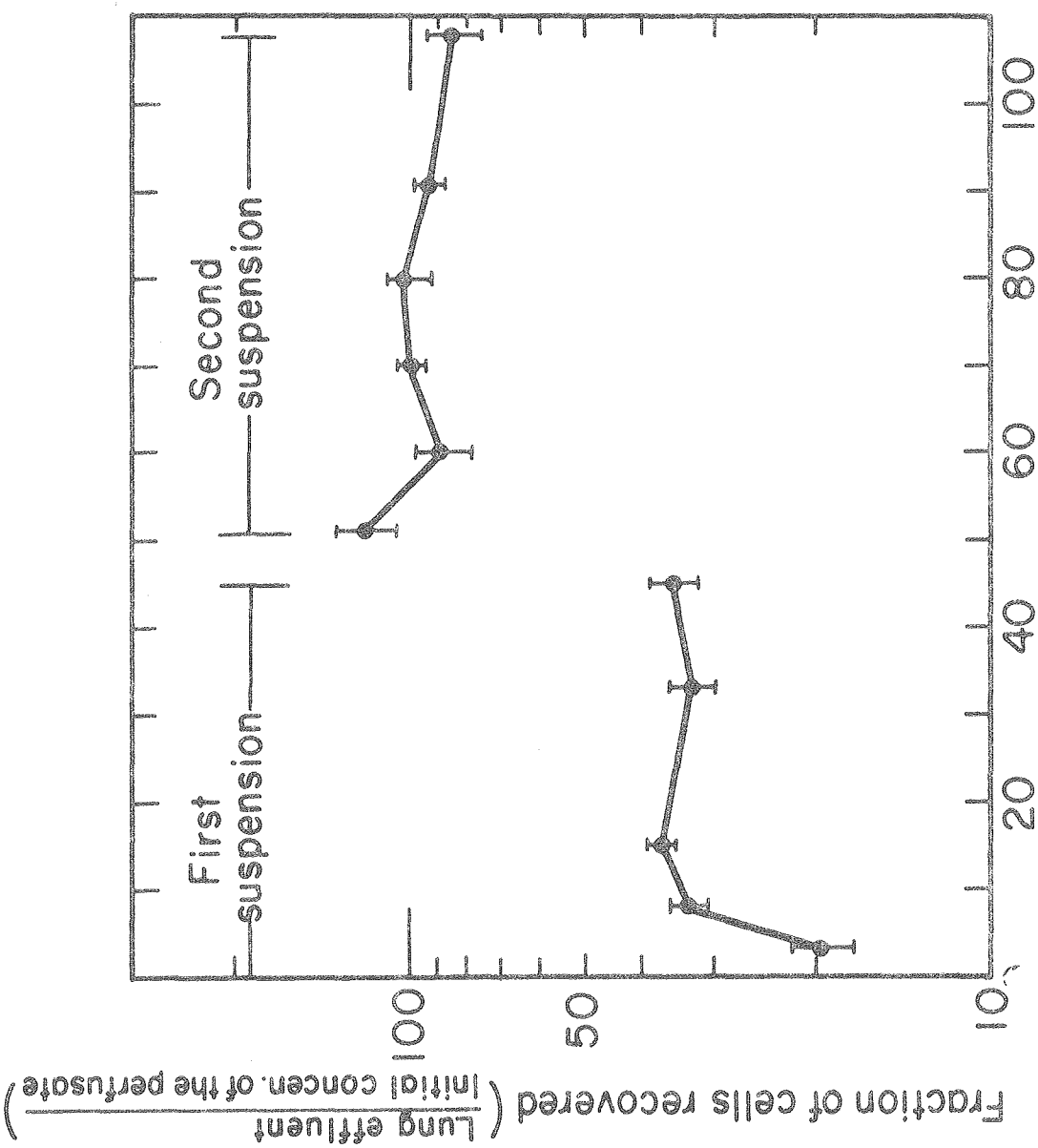

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Figure 10

Figure 11. Same as Fig. 9, but the second suspension had a higher concentration than the steady state concentration of the first suspension. The Y axis represents the absolute value of the nucleated cell concentration in the reservoirs or lung effluent samples on a log scale. Introduction of the second suspension with a higher concentration than the steady state concentration disturbed the steady state and resulted in a new steady state with a higher plateau value.

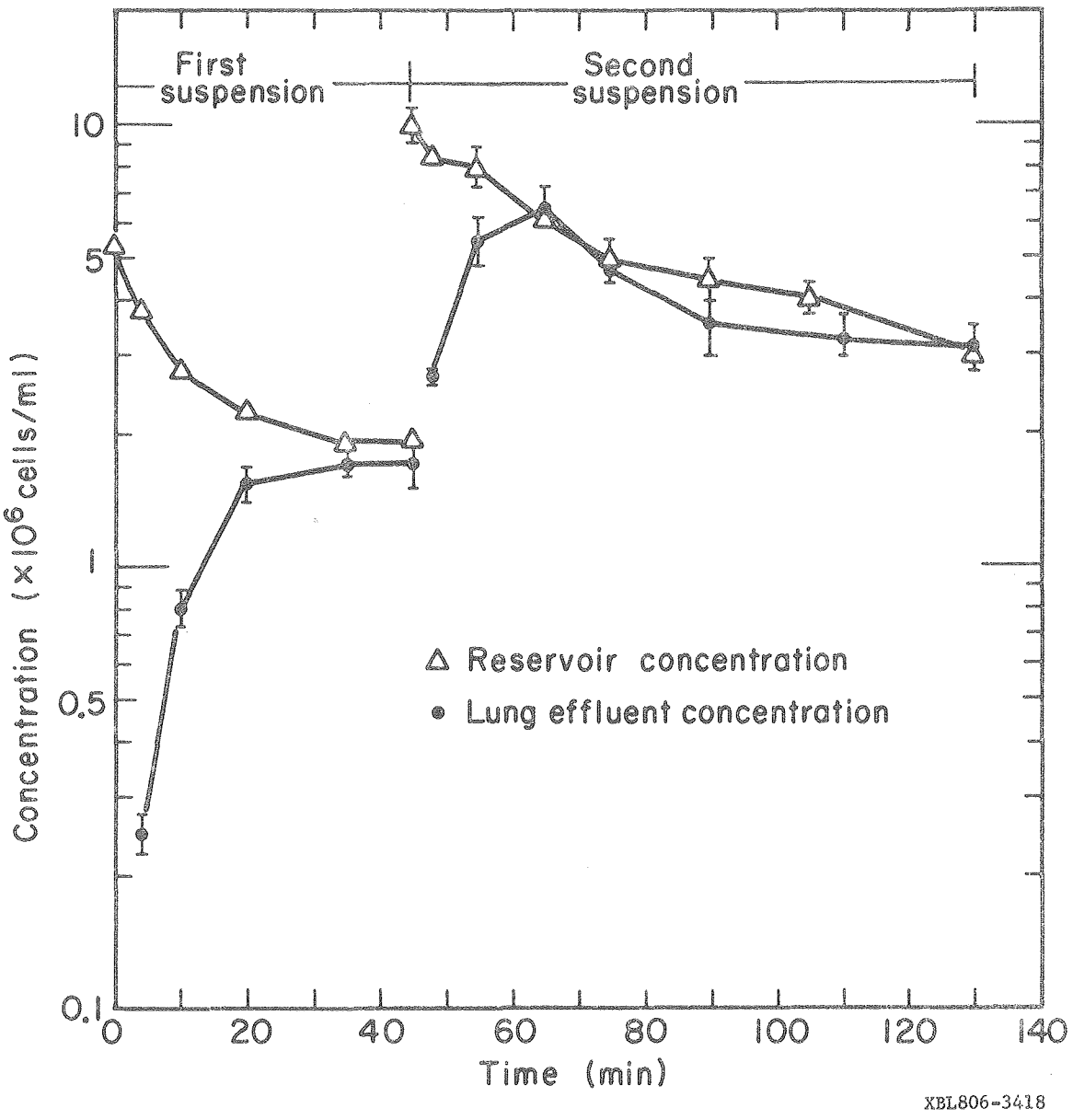
First suspension
Second suspension
10
5
1
0.5
0.1
Concentration (x10^6 cells/ml)
Δ Reservoir concentration
• Lung effluent concentration
0
20
40
60
80
100
120
140
Time (min)
XBL806-3418

Figure 11

Figure 12. Same as Figs. 9 and 11, but the second suspension has a lower concentration than the steady state concentration of the first suspension. Replacement of the circulating suspension with a new suspension with a concentration lower than the steady state concentration caused the lung to release some cells into circulation.

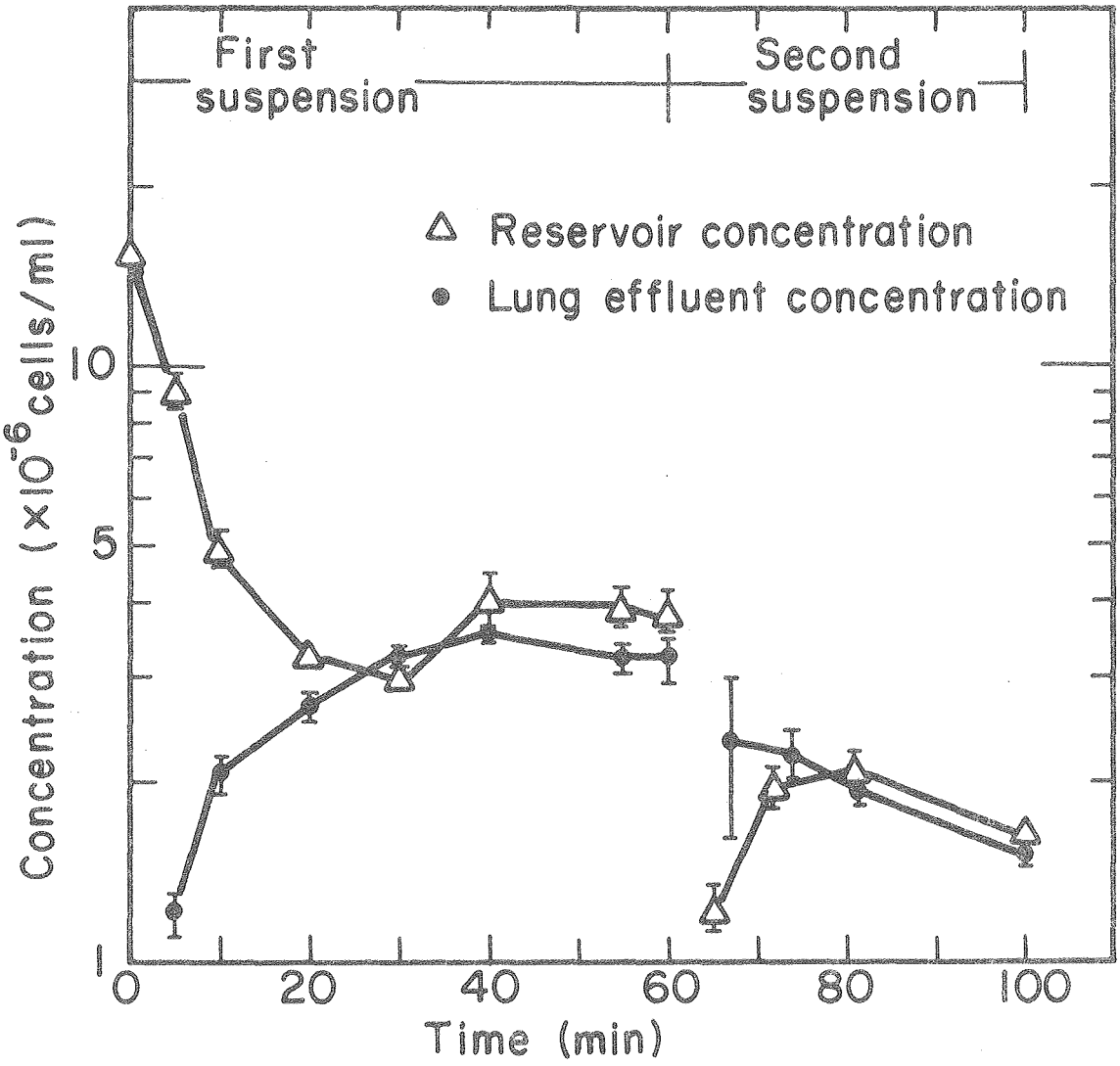

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Figure 12

Figure 13. Same data as in Fig. 12, but the Y axis in this figure is the ratio of the nucleated cell concentration in the samples from the reservoirs or lung effluent suspension to the initial concentration on a log scale. In the second suspension, the reservoir concentration increased by almost 220 percent, and the new plateau value was apprxoimately 140 percent of the starting concentration.

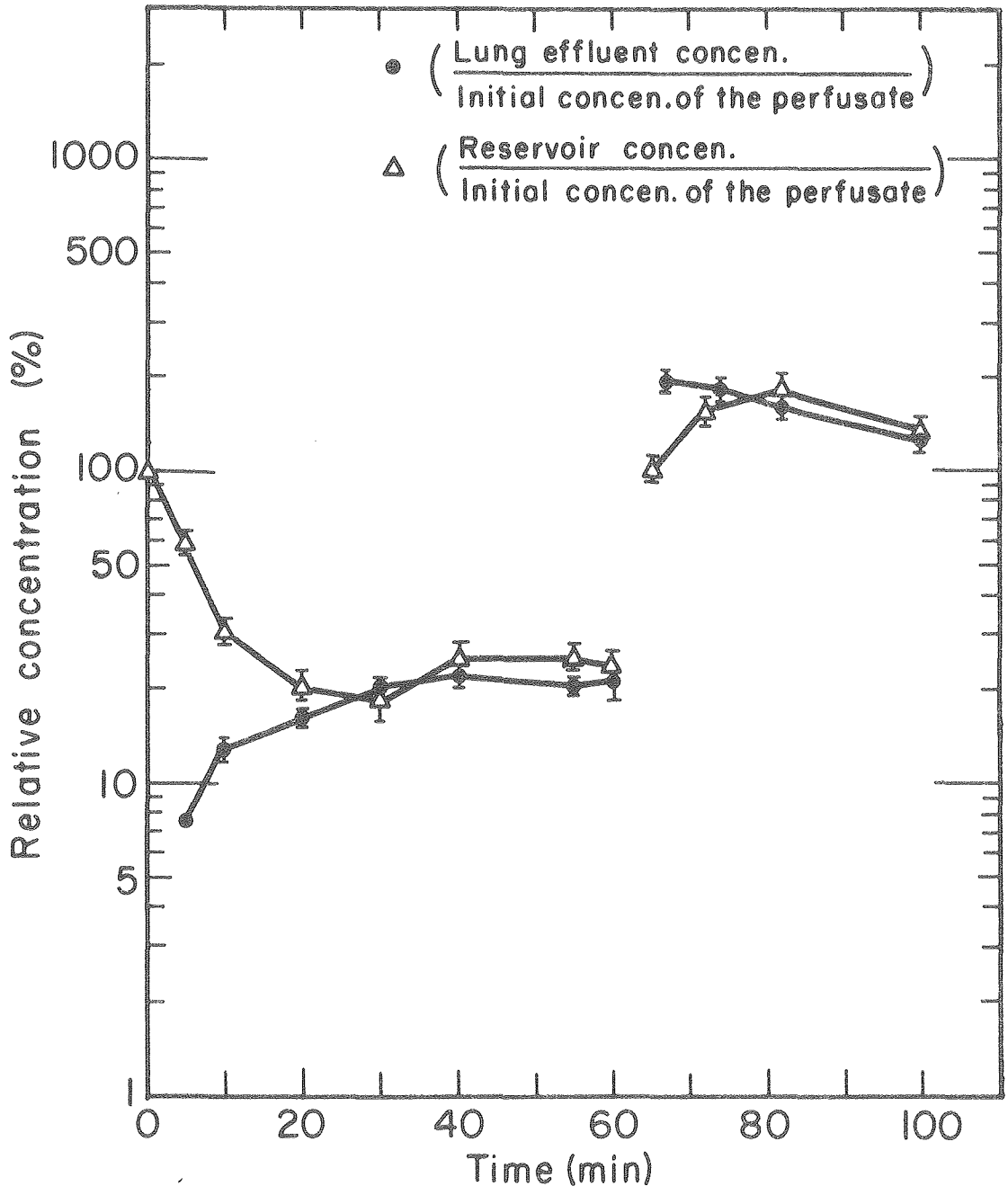

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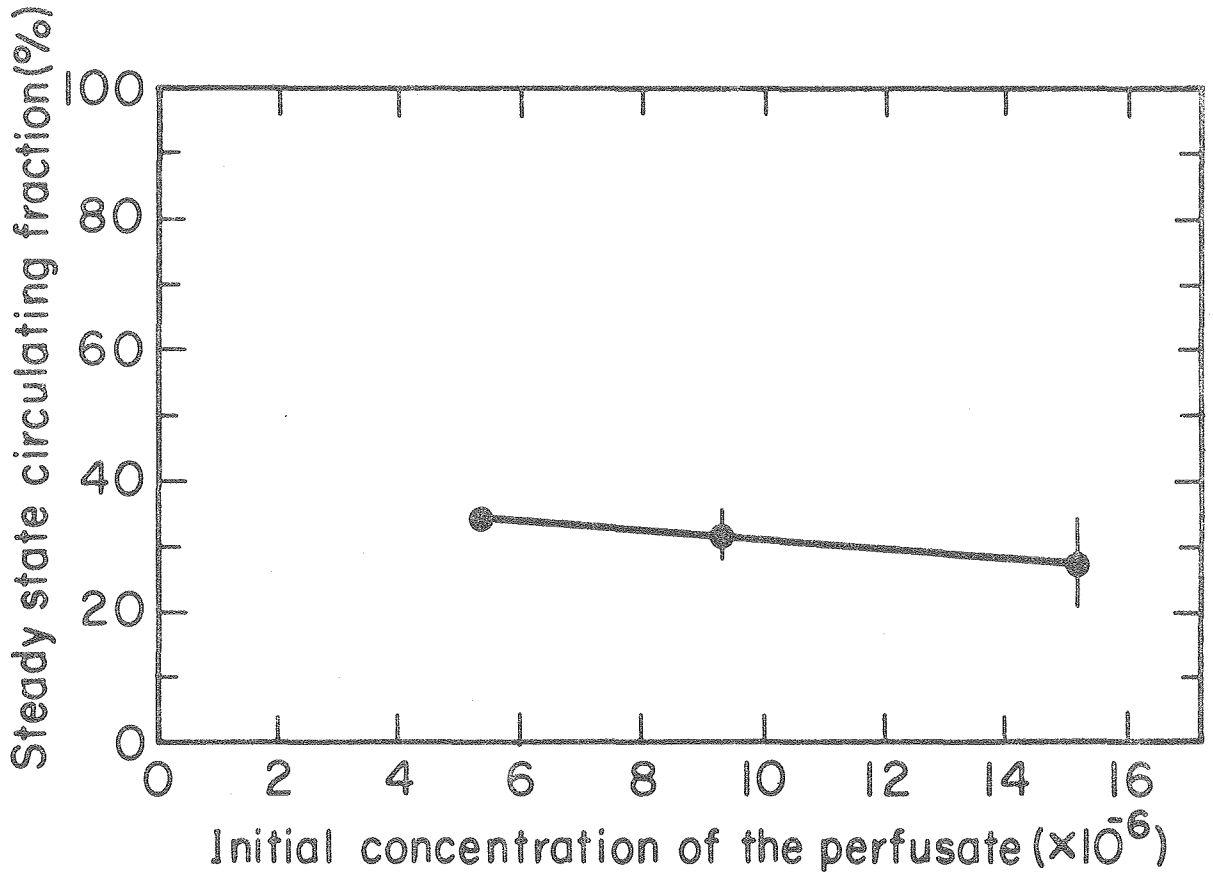
Figure 13

Figure 14. The effect of the initial cell concentration of the perfusate on the steady state concentration of circulating cells. Three bone marrow suspensions with different concentrations were perfused through isolated rat lungs in the continuous-circulation mode and their steady state concentration of circulating cells was determined. The Y axis in this graph is the ratio of the steady state circulating cell concentration to the corresponding initial concentration. The X axis is the initial concentration of each suspension. In the range of concentrations examined here, the fraction remaining in circulation at the steady state is always approximtely 30 percent of the initial concentration.



XBL806-3421

Figure 14

Figure 15. Sequestration of peritoneal neutrophils by isolated perfused rat lung. A suspension of peritoneal neutrophils was perfused through an isolated rat lung in the continuous-circulation mode and the nucleated cell concentration of the reservoir and the lung effluent suspension was determined. Also, a differential count was done on all samples to differentiate between neutrophils as one group and all mononuclear cells as another group. The Y axis is the ratio of the cell concentration in each sample to the initial concentration on a log scale. Neutrophils show the lowest plateau value, while the mononuclears have the highest. The plateau value of the mixed population (nucleated cells without differential count) lies between the two extreme values. Neutrophils as a homogeneous population of cells clearly follow the same pattern of sequestration observed for a heterogeneous population such as bone marrow.

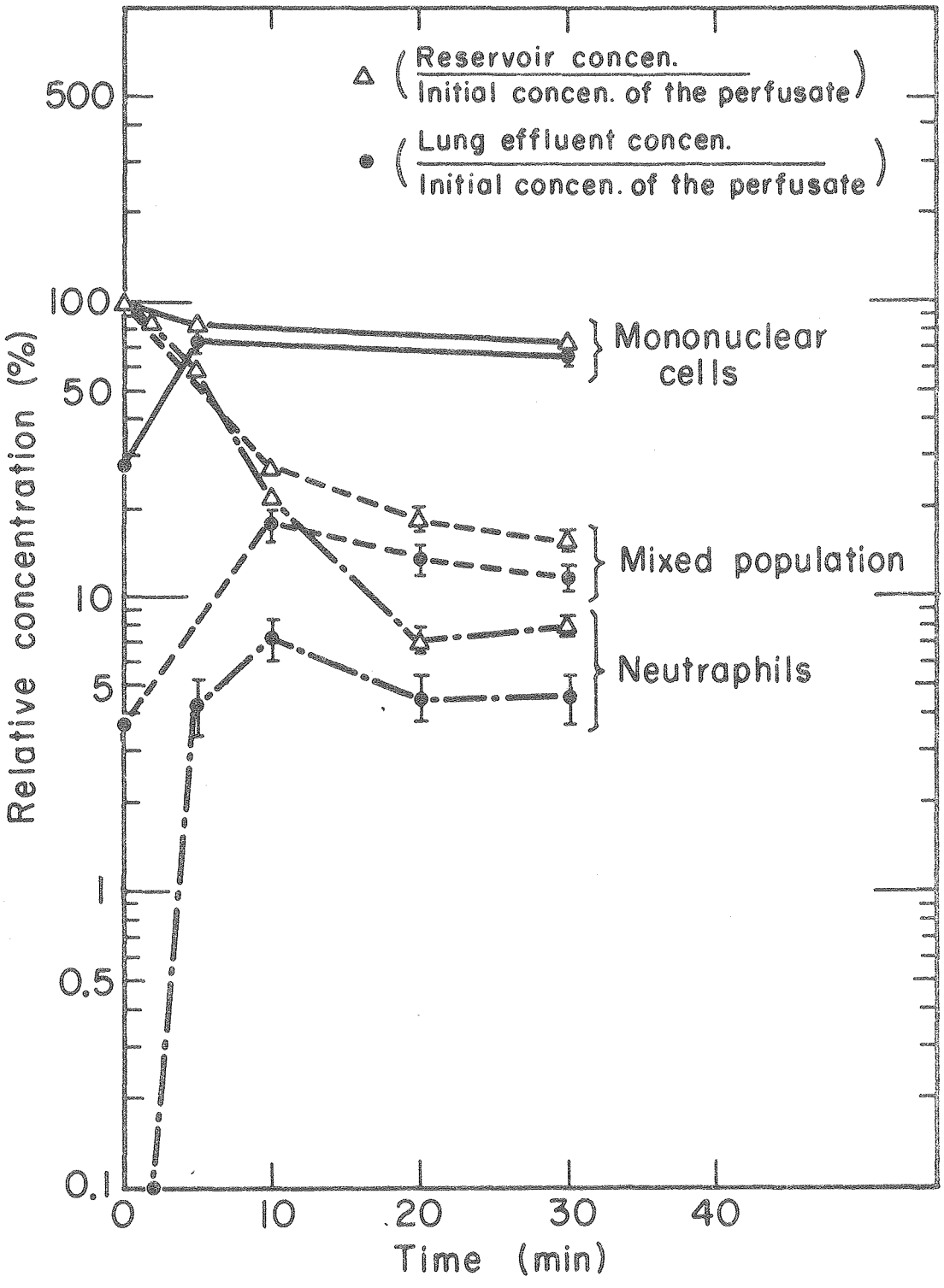
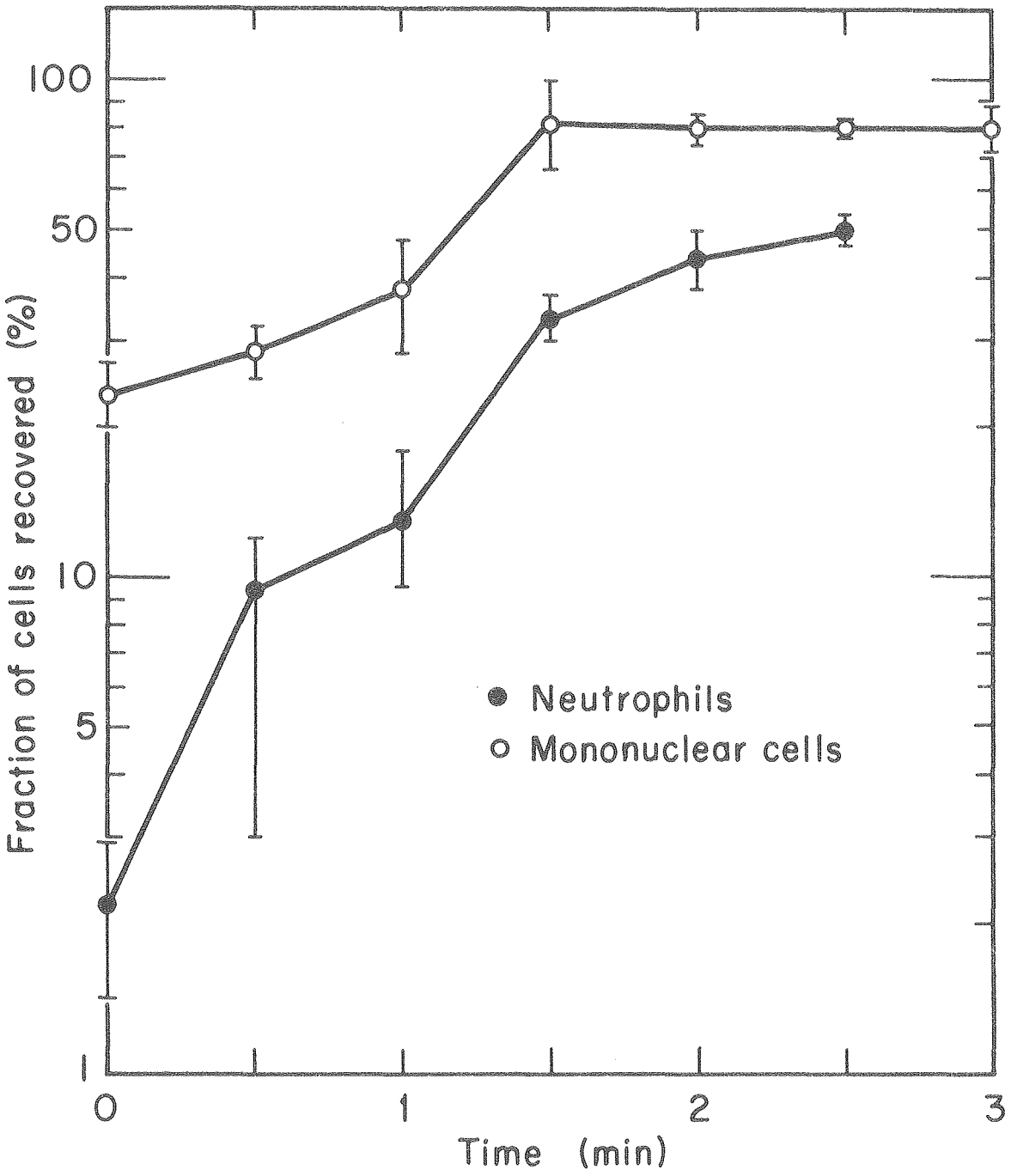

Δ (Reservoir concen. / Initial concen. of the perfusate)
● (Lung effluent concen. / Initial concen. of the perfusate)
Mononuclear cells
Mixed population
Neutraphils
Relative concentration (%)
500
100
50
10
5
1
0.5
0.1
0
10
20
30
40
Time (min)
XBL806-3422

Figure 15

Figure 16. Sequestration of individual blood leukocytes by isolated perfused rat lung in the single-pass mode. The data are essentially the same as was presented in Fig. 4. However, a differential count was done on each sample to differentiate neutrophils as one group and mononuclears as another group of cells. The Y axis is the ratio of leukocyte concentration recovered from the lung effluent to the perfused leukocyte concentration on a log scale. Neutrophils show a higher rate of sequestration than mononuclear cells.



XBL806-3423

Figure 16

B. Sites and Morphology of Sequestration

Sequestration of leukocytes by the isolated perfused rat lung is in many ways similar to the in-vivo phenomenon of leukocyte margination by the body's network of blood vessels. The results of kinetic and biochemical experiments reviewed in the previous section confirms the occurrance of sequestration phenomenon in the isolated perfused rat lung. However, they do not provide any information about the site and the morphology of this phenomenon. To address this aspect of the problem, I used scanning electron microscopy. Vascular perfusion fixation of the lung, followed by ethanol-cryofracturing, provides suitable specimens for examining the vascular network of the lung along with other morphological features. Figure 17 is an example of the well-preserved, fractured surface of a rat lung fixed by vascular perfusion and ethanol-cryofracturing, revealing a complex of alveoli and the capillaries surrounding them.

The original purpose of using scanning microscpy was to investigate and identify possible sites of leukocyte sequestration in the isolated perfused rat lung. However, before proceeding with such an investigation, one must be able to distinguish the venous side of the blood vessel network from the arterial side under the SEM. I took advantage of the results of experiments described in the Results section using latex particles (see Fig. 5) to establish surface criteria useful under SEM to differentiate veins from arteries in the rat lung. These studies revealed that latex particles, when perfused through the lung,

plug all of its capillaries and small arterioles. Thus, if the perfused lung is fixed by the tracheal infusion of a fixative and processed for the SEM, those vessels found to be clogged with or to contain particles should be on the arterial side of the network. If the characteristics of these vessels are then compared with those of the other vessels, one will be able to distinguish an arterial vessel from a venous one under SEM.

Figure 18 shows the fractured surface of a lung perfused with latex particles (see experiments for Fig. 5) and fixed at the end of perfusion by the tracheal infusion of the fixative. Several blood vessels of various diameters filled with particles are visible. Also visible are two larger arteries containing particles. Figure 19 is a higher magnification of one of the blood vessels in Fig. 18. Figure 20 shows another blood vessel of larger diameter also containing particles. In these figures, all of the blood vessels containing particles have a ridged endothelium. The nature of the ridges cannot be determined from these pictures, however, the ridges oriented in the direction of the blood flow in the vessel, i.e., they are elongated axially. The overall surface of the vessel is fairly smooth. On the other hand, in all other blood vessels which did not contain any latex particles, the supposed venous side of the network, the ridges were closer together and irregular. In additioon to the axially arranged ridges, circumferential ridges were also present. Figures 21 through 24 are typical examples of such blood vessels. Circumferential ridges were absent in blood vessels containing latex particles. It was

assumed, therefore, that fairly dispersed, axially elongated ridges and smooth luminal surfaces overall are characteristic of the arterial side of the rat lung vasculature, while vessels with compact and irregular axial ridges, as well as circumferential ridges are characteristic of the venous side of the network.

To identify the possible sites of the sequestration of leukocytes in the lung, a suspension of peritoneal neutrophils was perfused through a rat lung in the single-pass mode (see Method Section and control experiments for Table 1 in Results Section). At the end of perfusion, the lung was fixed by the tracheal infusion of fixative and processed for SEM. In all the samples examined, cells were invariably found in blood vessels which were assumed to belong to the venous side of the network. Figures 25 and 26 are examples of a typical blood vessel containing leukocytes.

Further examination reveals that those leukocytes that stick to the endothelium seem to put out various kinds of cellular projections. Figure 27 illustrates a group of leukocytes in a blood vessel. Three leukocytes have finger-like projections, those on one cell are short while projections on the other cells are much longer. The leukocyte in Fig. 28 has a leading lamella and a few short finger-like projections. Figure 29 is an illustration of a leukocyte with a leading lamella on one side and three or four retraction fibers on the opposite side of the cell. Figures 30 and 31 are higher magnifications of the leading lamella and the retraction fibers. This kind of morphoogy is characteristic of moving cells (101), which could mean that once the

leukocyte has stuck to the endothelium, it begins to move. Figure 32 is a fractured surface of a leukocyte stuck to the endothelium. At this level of resolution, it seems that the leukocyte has established close contact with the endothelium.

In summary, it seems that, similar to the in-vivo phenomenon of margination and attachment of leukocytes to blood vessels, the venous side of the vascular network of the isolated perfused rat lung is the main site of sequestration of perfused leukocytes. These leukocytes stick to endothelium and probably begin to migrate once they have attached to the vessel wall.

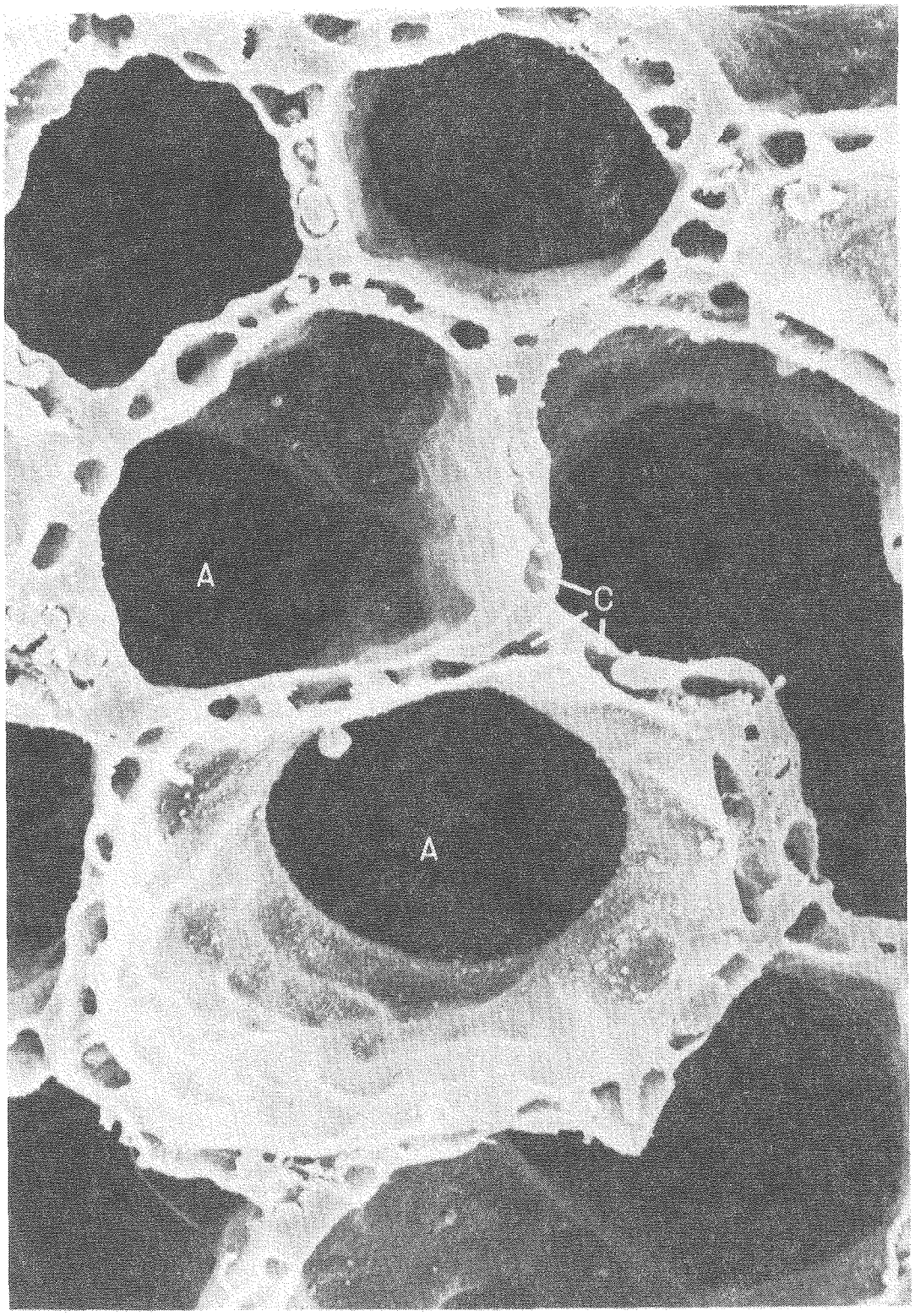


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Figure 17. Face of an ethanol-cryofractured rat lung. Several alveoli (A) and the capillaries (C) surrounding them are visible. Each capillary is shared by two alveoli. About 1600x.

Figure 18. A rat lung perfused through the vascular system with latex particles. In the center of the micrograph is a medium sized airway (AW). The surface of the secretary (Clara) cells can be seen bulging into the lumen of the airway. A number of blood vessels in which latex particles (P) can be observed are peripheral to the airway. About 350x.

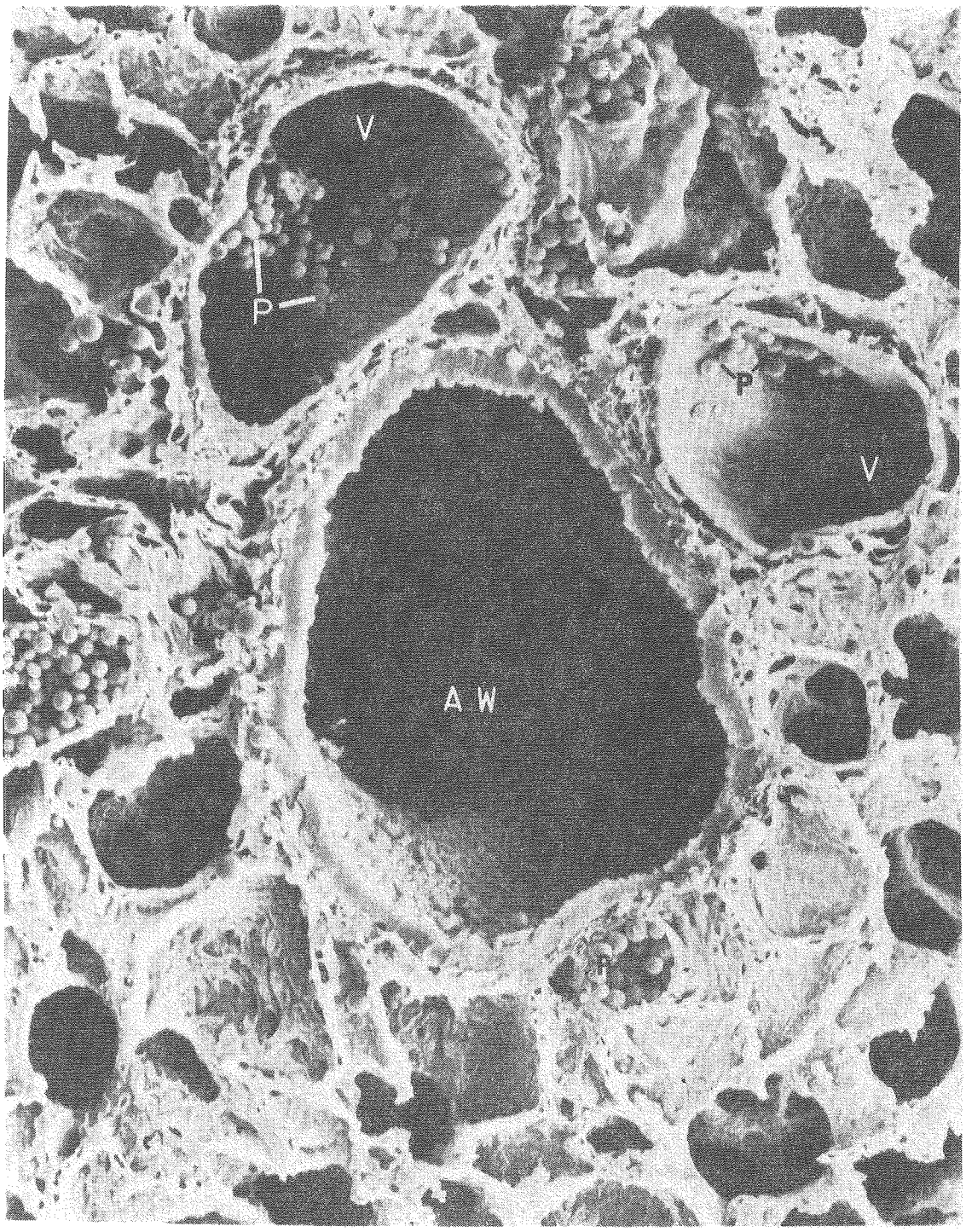

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Figure 18

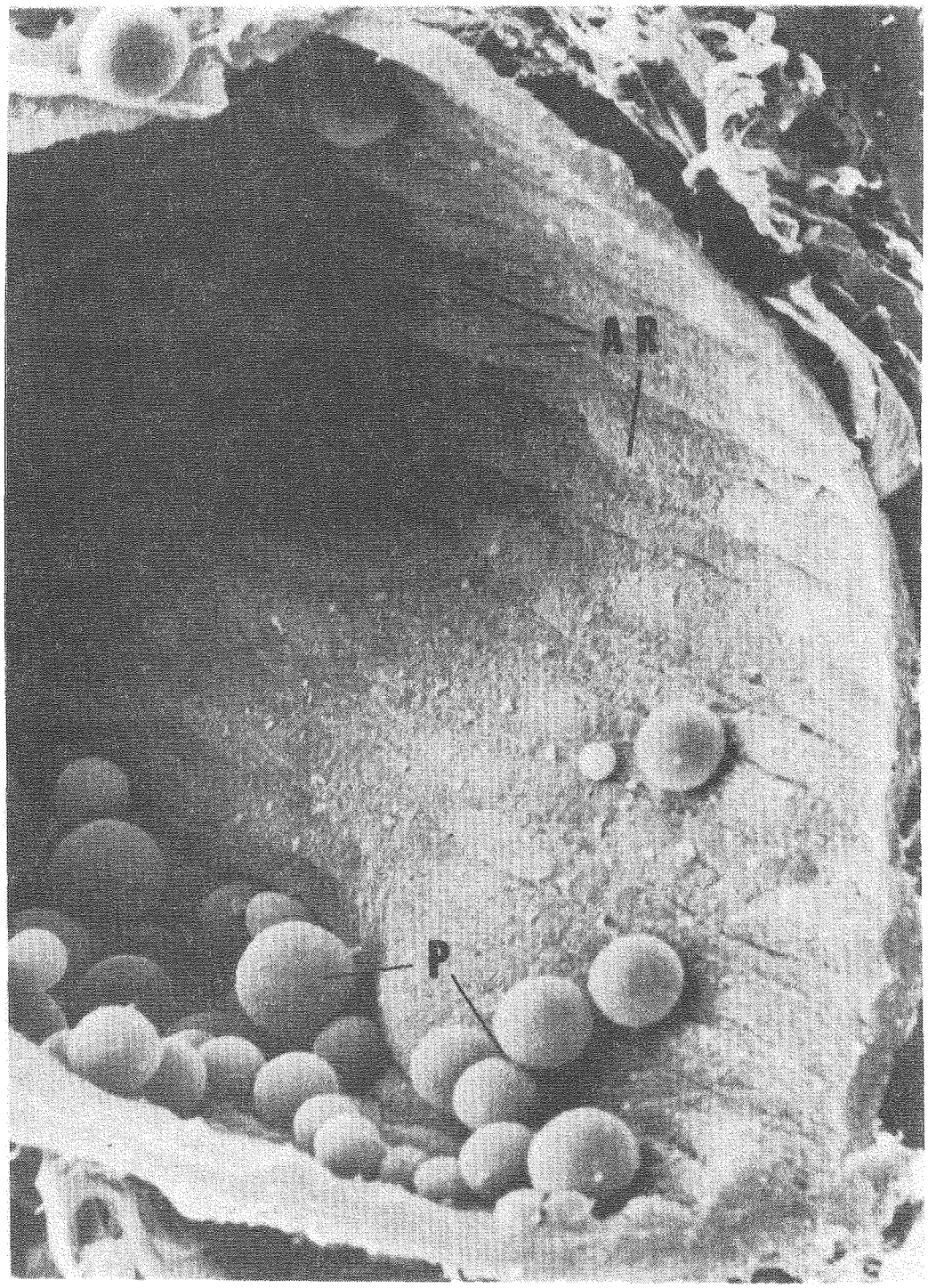

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Figure 19. Higher magnification of a blood vessel shown in Fig. 18 containing latex particles (P). Axial ridges (AR) are visible on the endothelial surface. About 1700x.

Figure 20. A large blood vessel with a smaller one branching from it (SV), both containing latex particles (P). Notice the regular axial ridges (AR) on the endothelium of the two vessels; the surface of the vessels is fairly smooth. In the upper right portion of the micrograph is a small vessel packed with latex particles. About 130x.

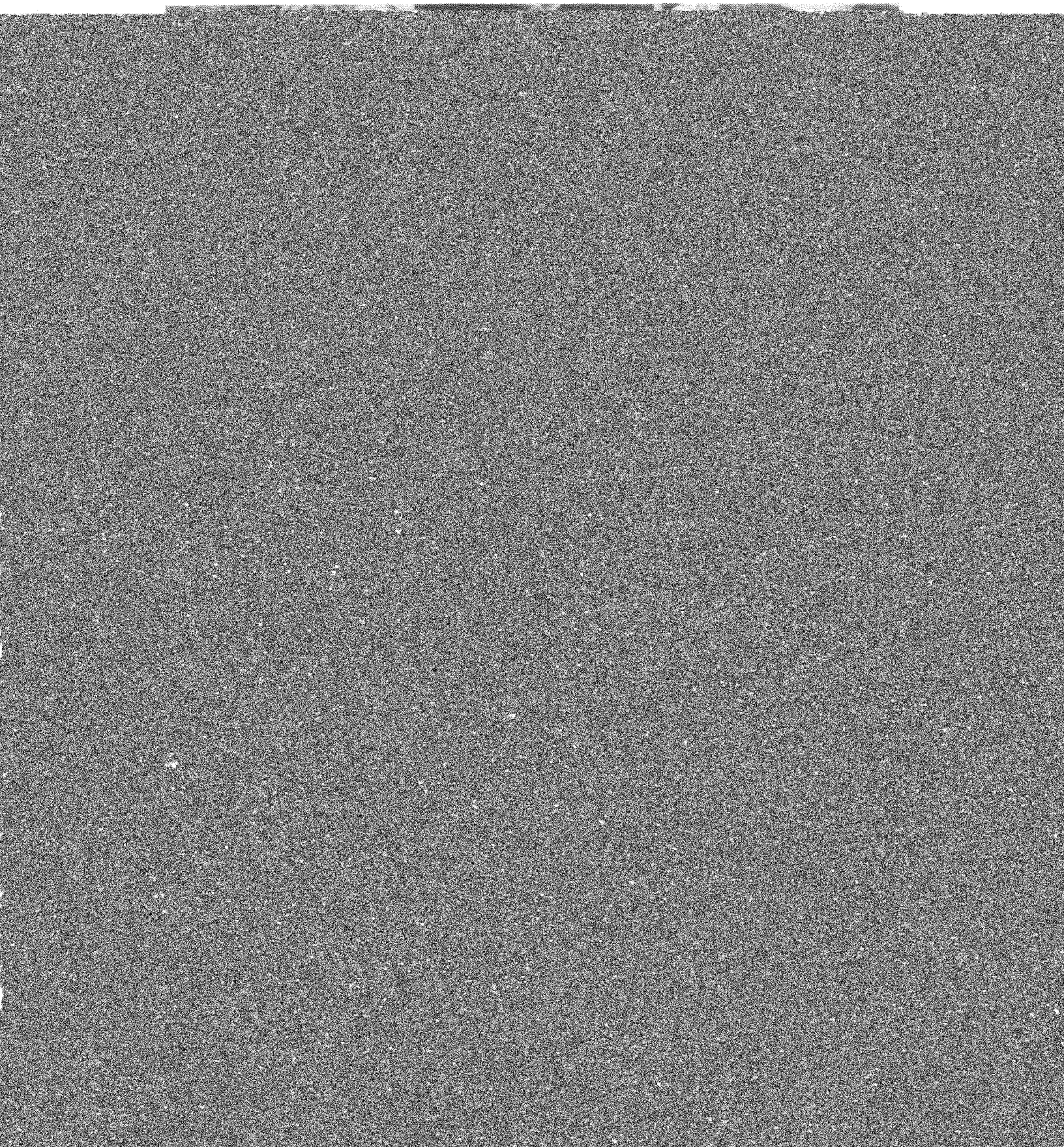

Figure 21. A portion of the same lung as in Figs. 18 and 20 showing a longitudinal fracture through a blood vessel (V) without any latex particles. Note the circumferenetial ridges (CR) and irregularities of the endothelium of this blood vessel. The overall surface of the vessel is rough. Numerous alveoli (A) surround the vessel and an airway (AW) is seen in the lower right portion of the micrograph. About 180x.

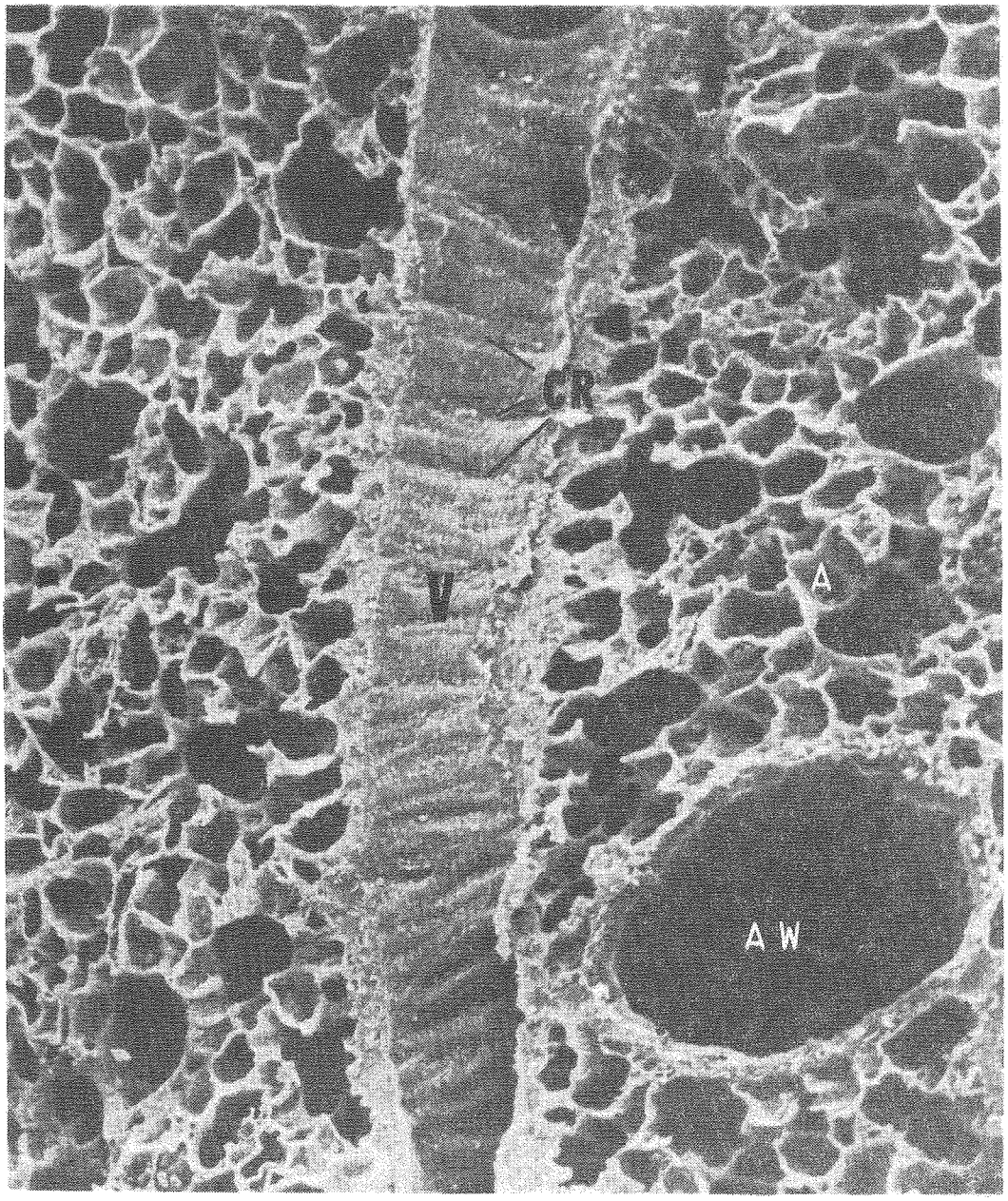

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Figure 21

Figure 22. Higher magnification of a portion of the vessel shown in Fig. 21. Notice the dense folding and bulging of the endothelium forming the axial ridges (AR). Circumferential ridges are also visible. About 1400x.

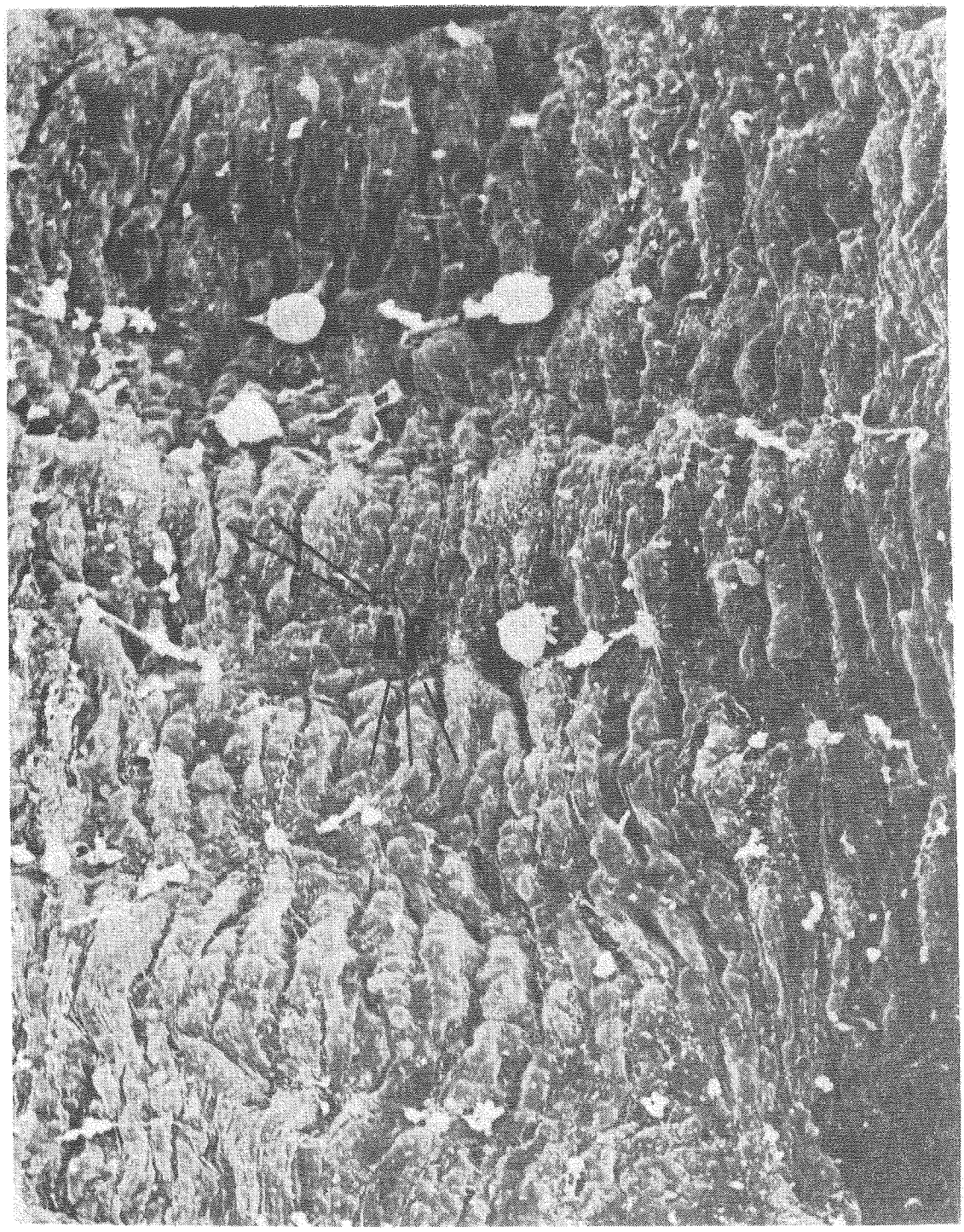

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Figure 22

Figure 23. Another part of the same lung showing the luminal surface of a different vessel without latex particles. Both axial (AR) and circumferential ridges (CR) are apparent. About 1300x.

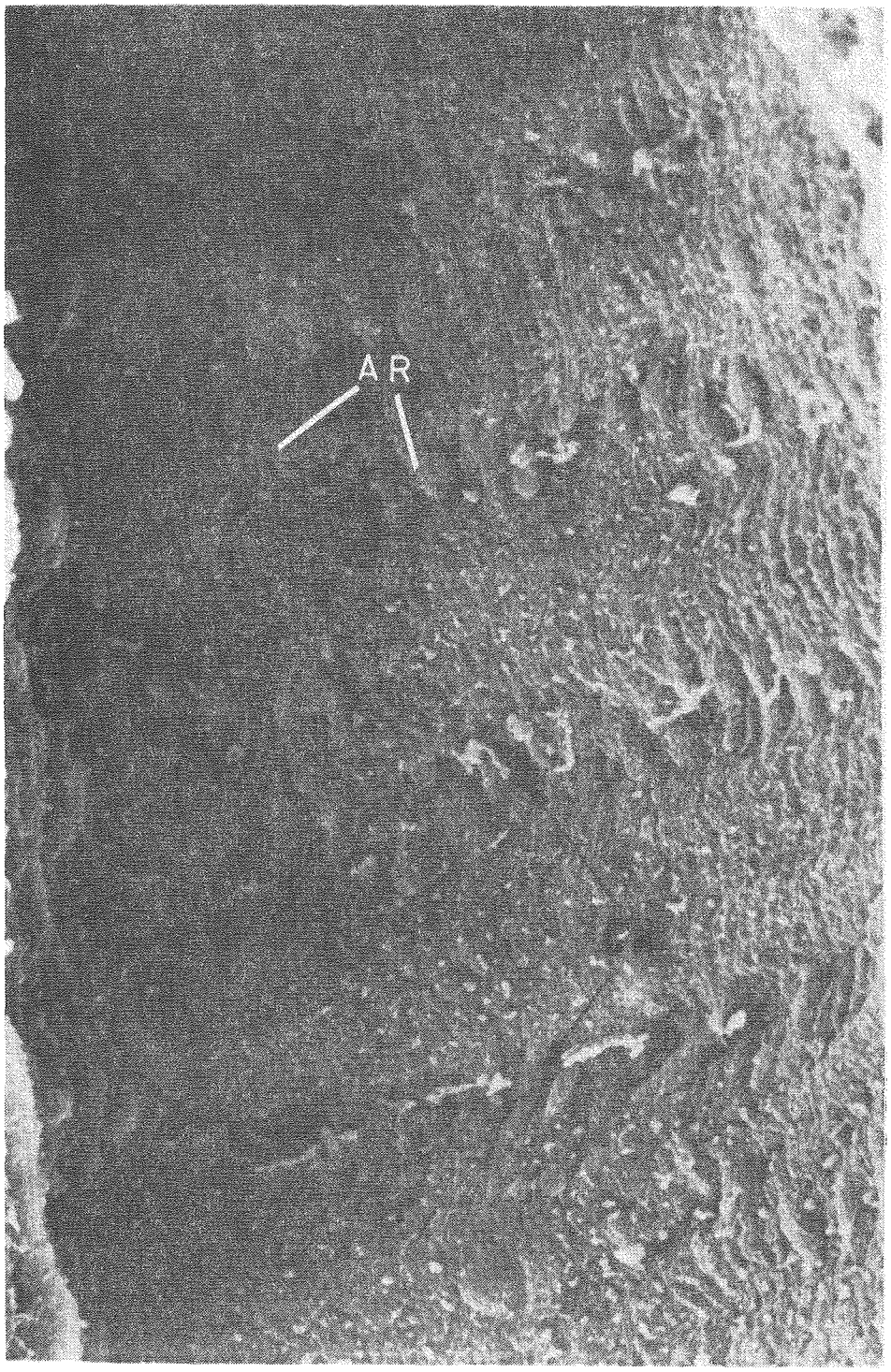

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Figure 23

Figure 24. High magnification of axial ridges (AR) of the endothelium of a blood vessel without latex particles from the same lung shown in Figs. 22 and 23. About 1300x.

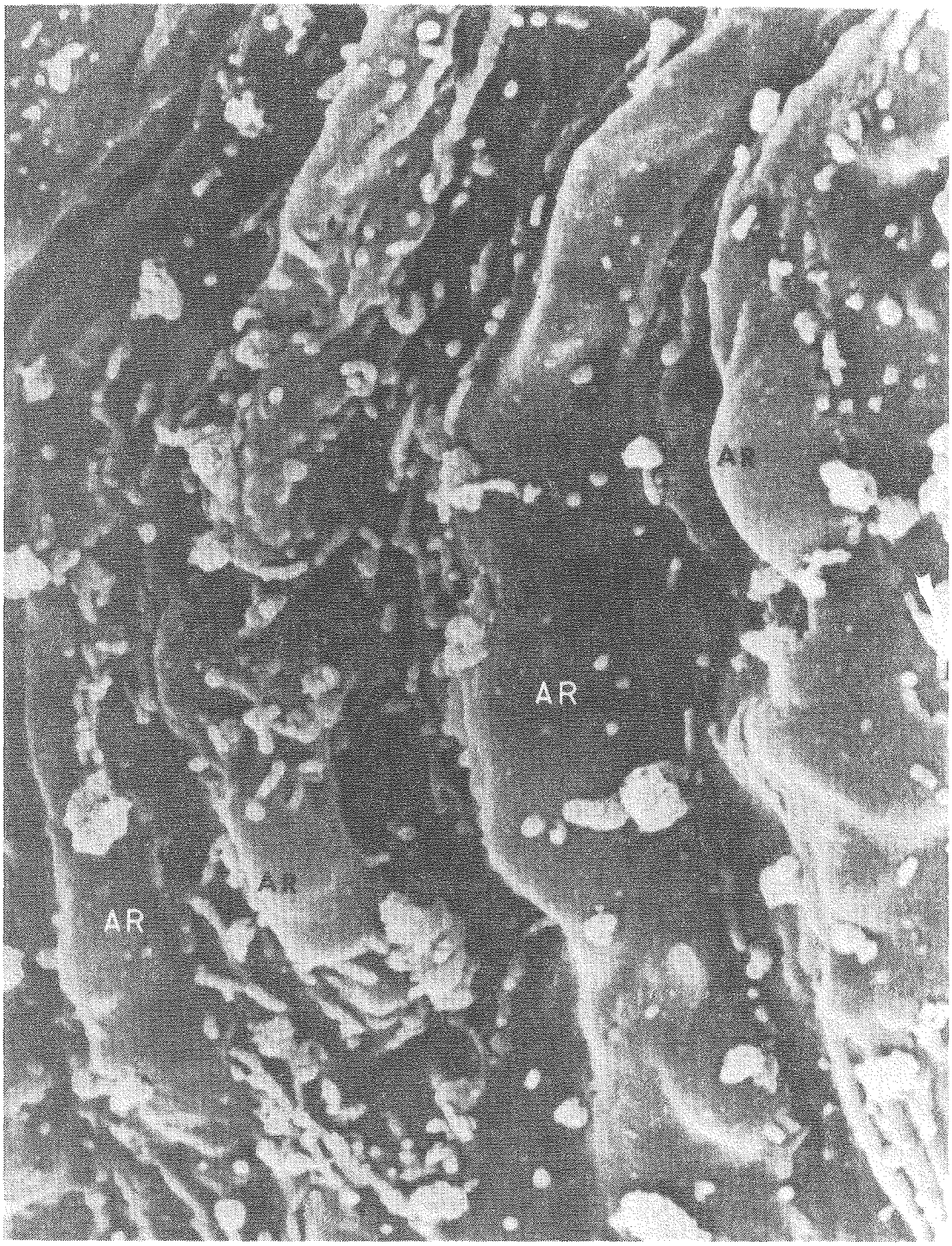

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Figure 24

Figure 25. Luminal surface of a large blood vessel with two smaller branches (SV). Leukocytes (L) and a few erythrocytes (E) are seen on the endothelial surface. This rat lung was perfused with a suspension of peritoneal neutrophils. Notice the generally rough surface and the axial and circumferential ridges in the endothelium of the vessel. About 520x.

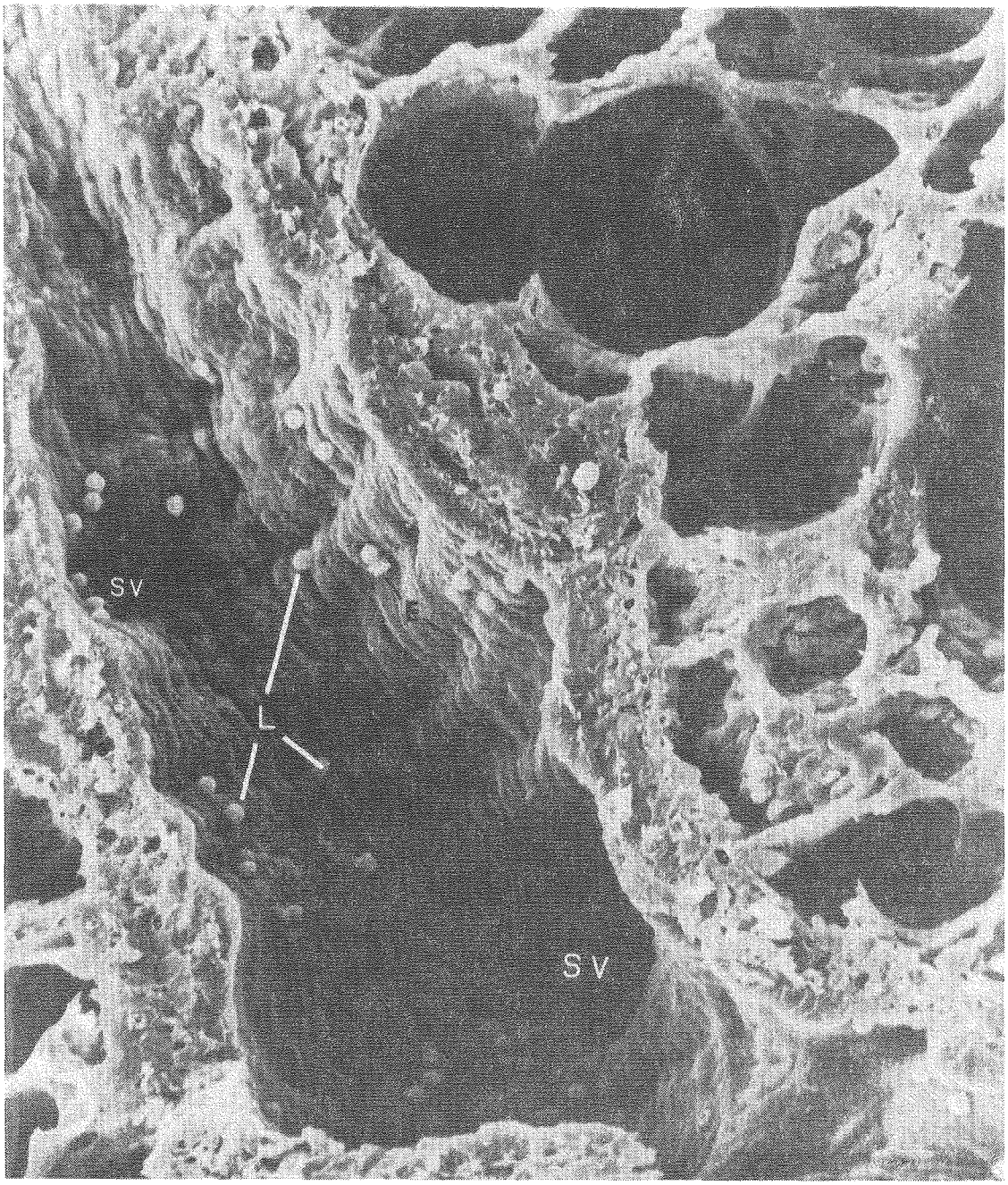

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Figure 25

Figure 26. Higher magnification of a portion of the same blood vessel shown in Fig. 25 which shows the ridges in more detail. In addition, numerous projections are visible on the surface of the leukocytes (L). About 1600x.

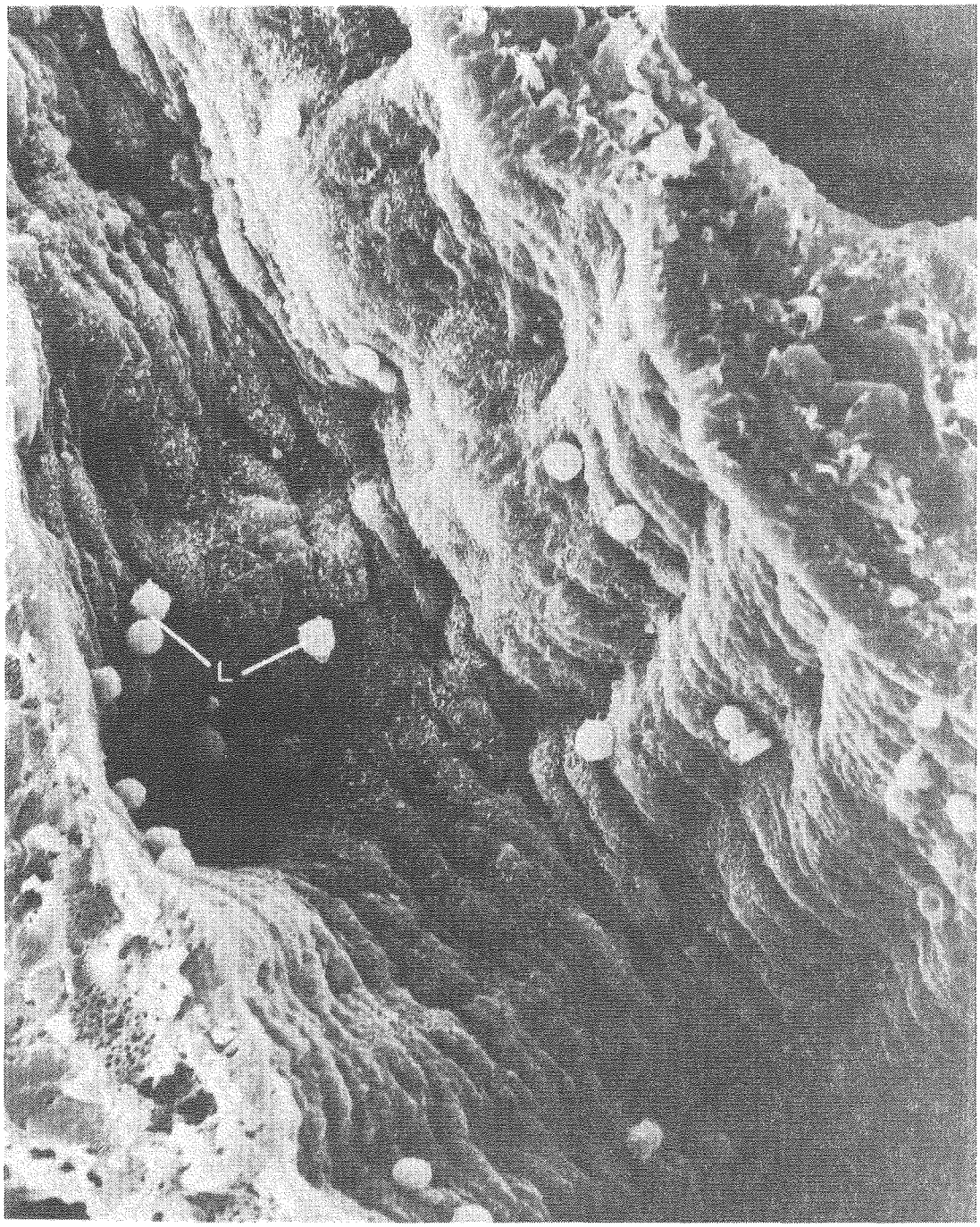

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Figure 26

Figure 27. A group of leukocytes in a blood vessel from a lung perfused with a suspension of peritoneal neutrophils. Finger-like projections (F) of varying length are present on most of the cells; some kind of interaction between the cells and the endothelial surface is suggested. About 10,000x.

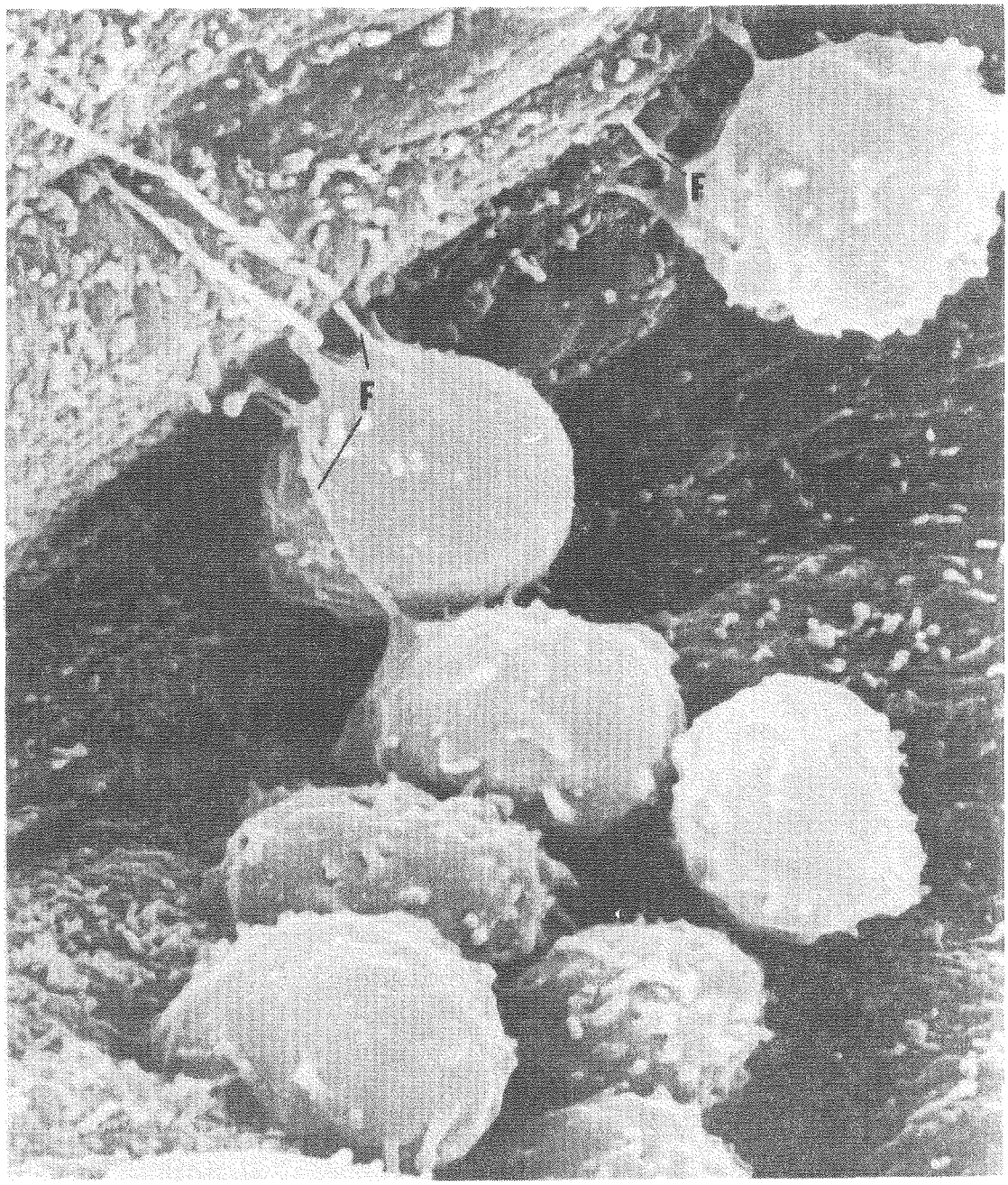

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Figure 27

Figure 28. Another leukocyte in a blood vessel from a lung perfused with a suspension of peritoneal neutrophils. Similarities in the morphology of this cell to cultured cells known to be in motion (101) suggests that this cell is moving across the endothelium (E_N) by extending the lamella (La) seen in the lower right portion of the micrograph; on the trailing edge of the cell, several retraction fibers (RF) are visible. About 10,000x.

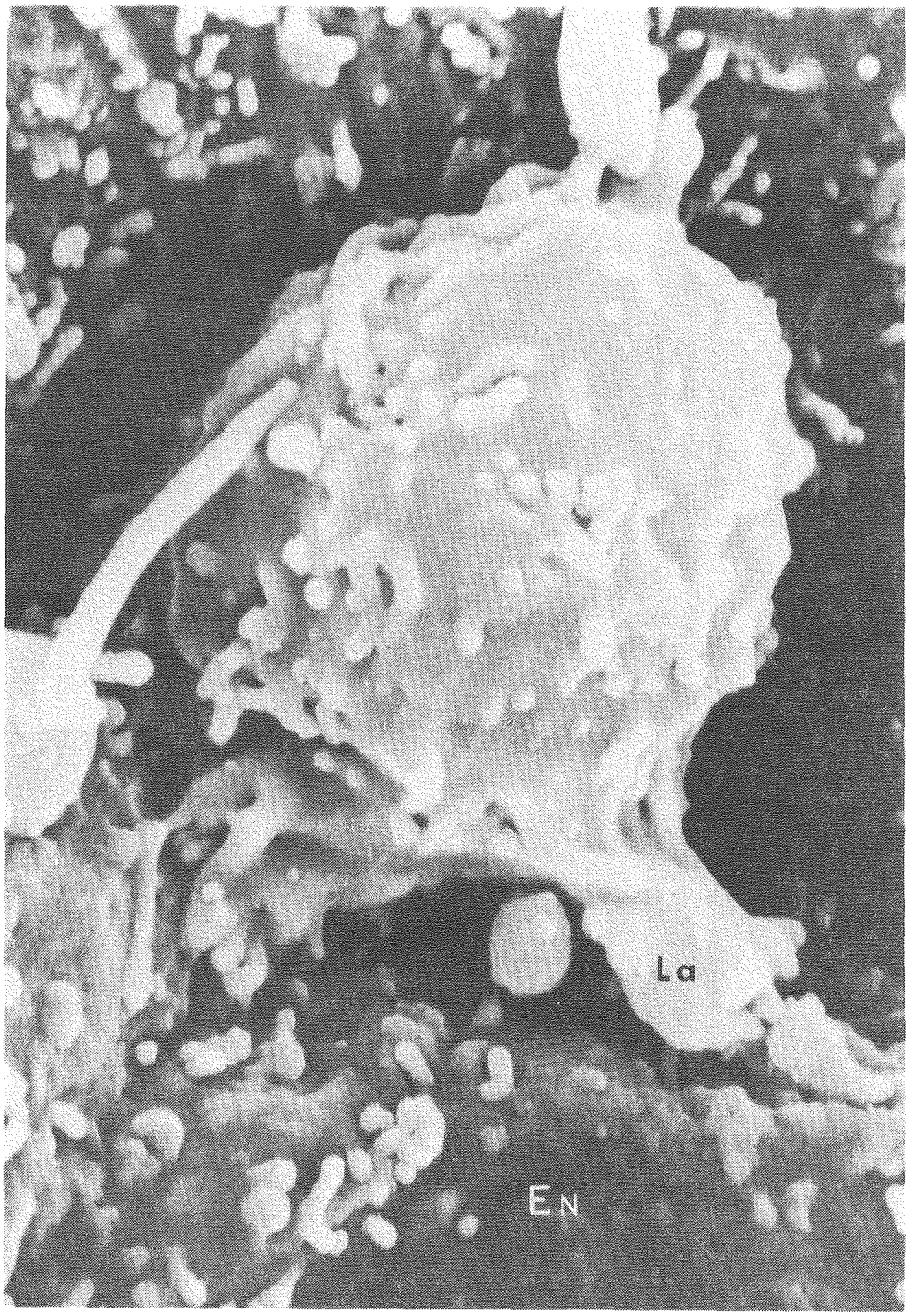

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Figure 28

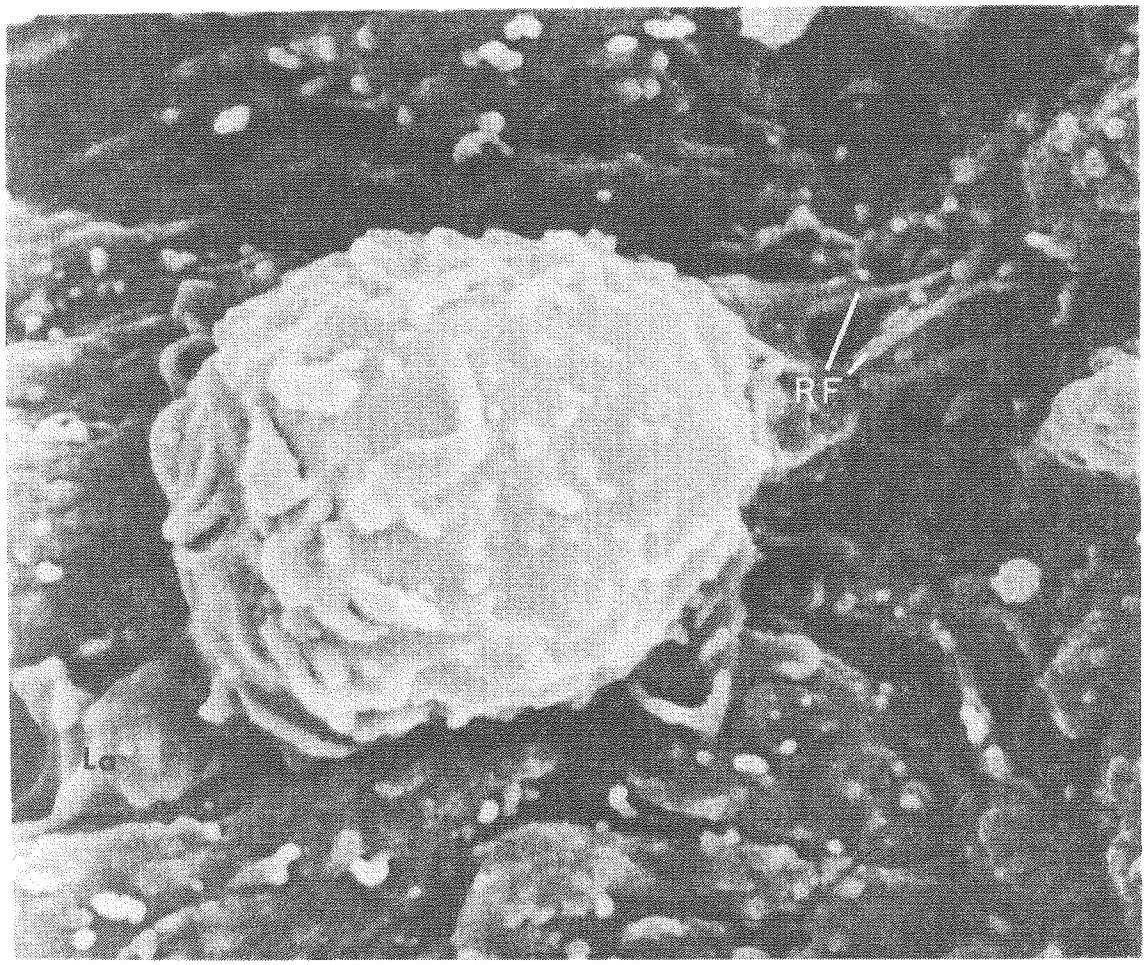

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Figure 29. A similar leukocyte from the same type of preparation. This cell is assumed to be moving toward the left by extension of the leading lamella (La) seen in the lower right portion of the micrograph. About 18,000x.

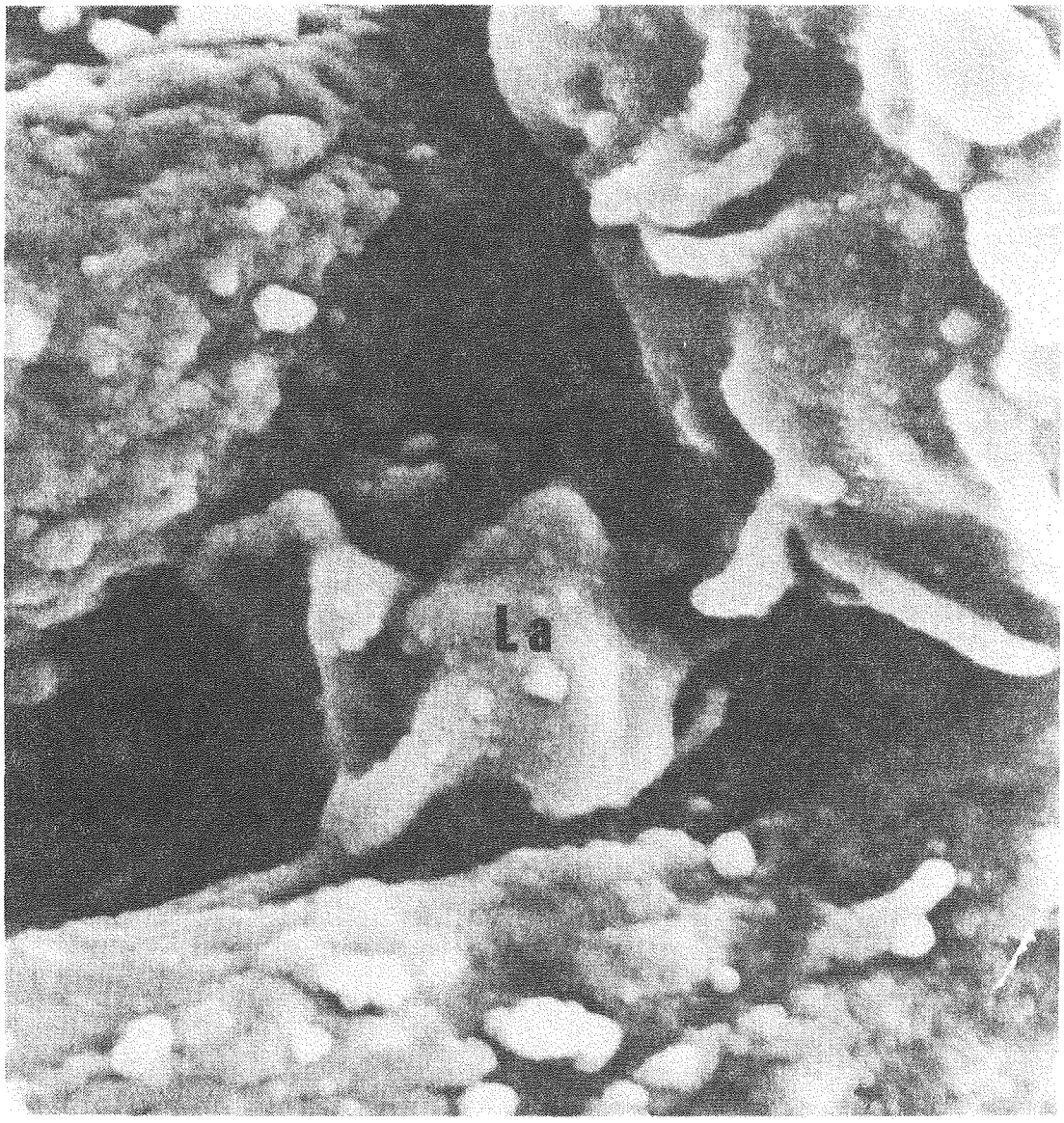

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Figure 30. Higher magnification of the leading lamella (La) of the same cell as in Fig. 29, showing the complex nature of this structure. About 30,000x.

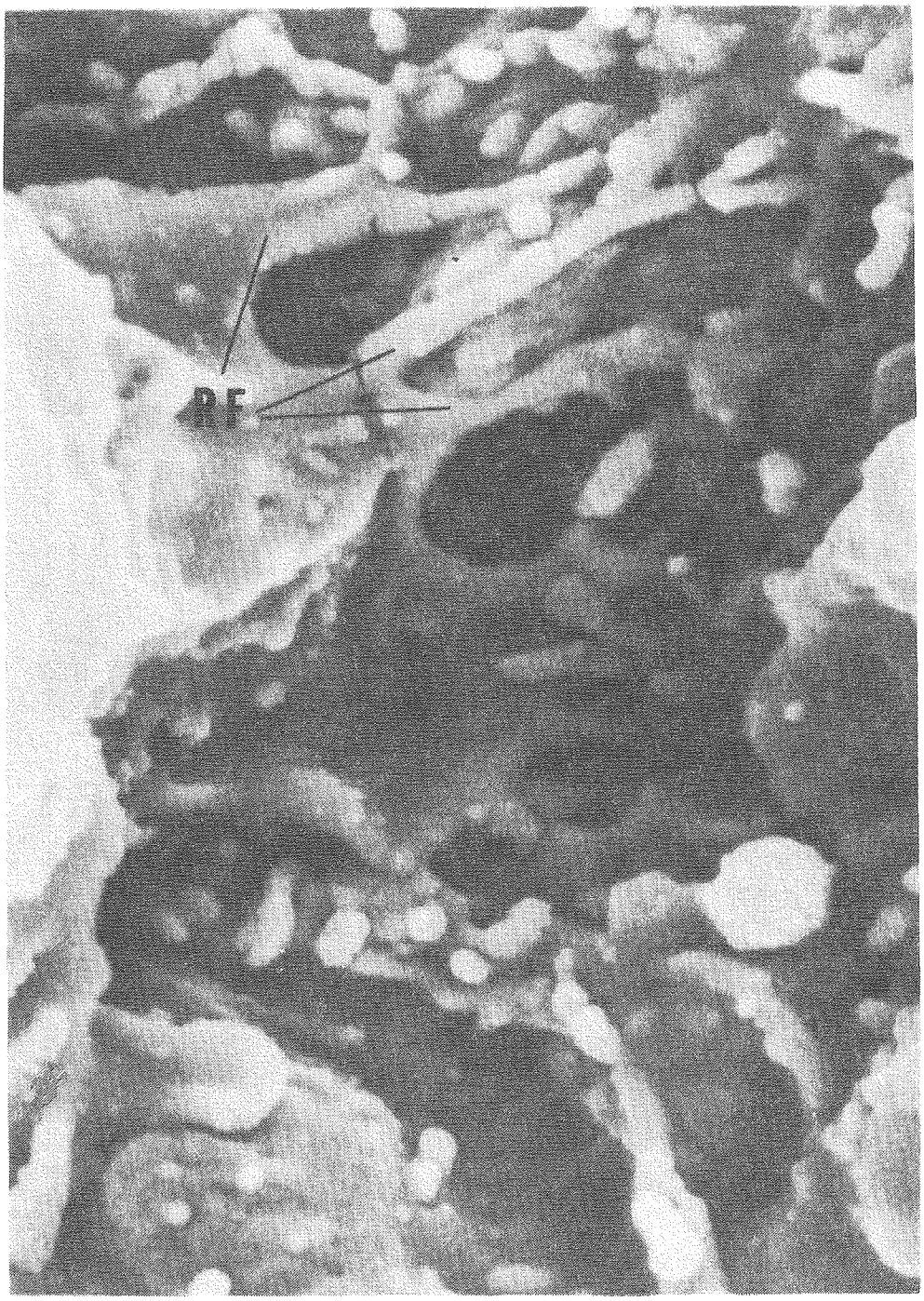

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Figure 31. Higher magnification of the retraction fibers (RF) of the same cell as in Fig. 29. About 34,000x.

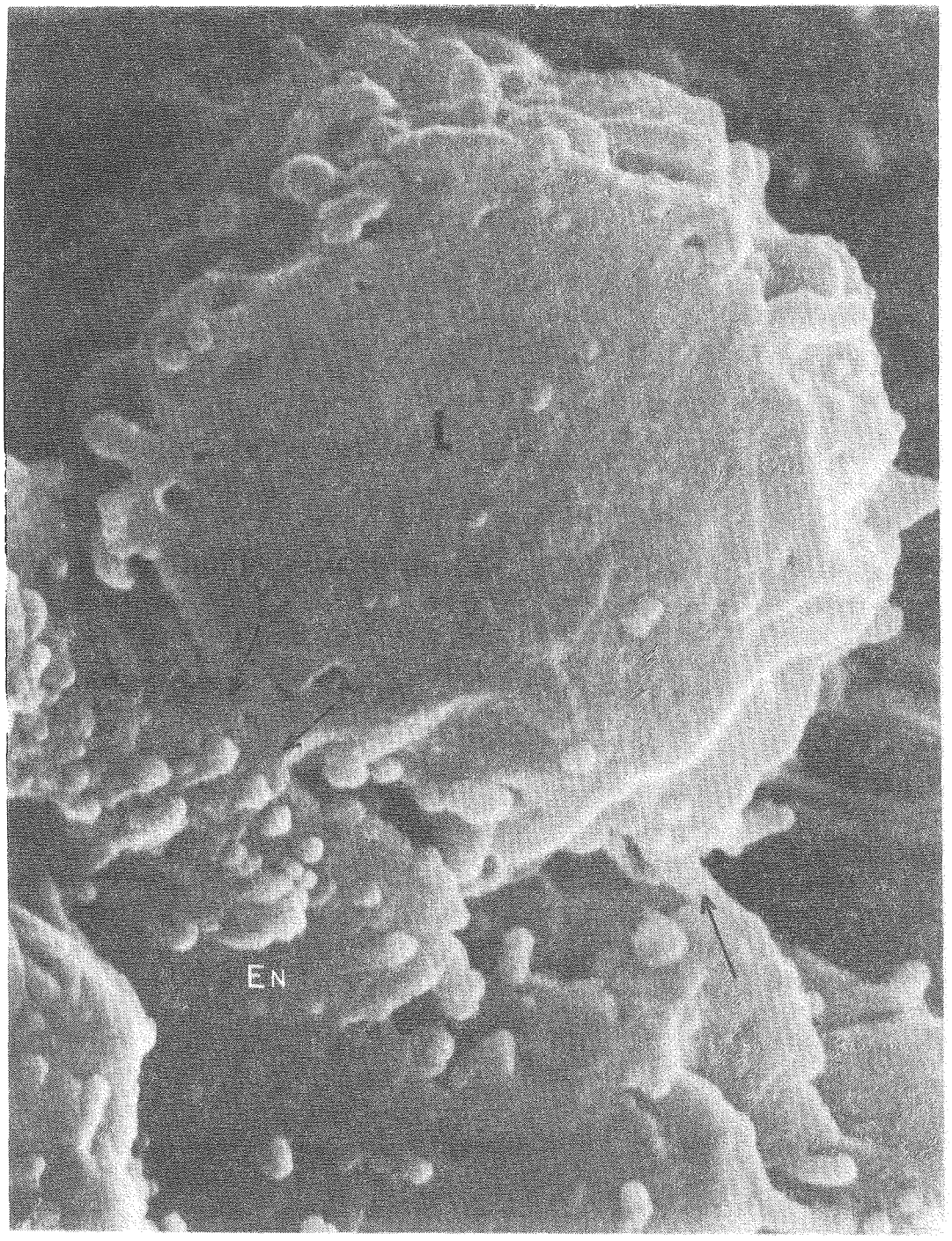

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Figure 32. Cross-fracture of a leukocyte (L) in a blood vessel. Points of close contact between the leukocyte and the endothelium are seen at the arrows. About 34,000x.

IV. DISCUSSION

A. Interpretation of Experimental Results

The in-vivo sequestration of leukocytes by the lung has been described by several investigators (52,59,60,84). The isolated perfused rat lung preparation offers the possibility of studying the sequestration of leukocytes by and the interaction of leukocytes with the vascular endothelium of the lung, within the lung, in the absence of other complicating factors present in the in-vivo studies.

Perfusing the lung for ten minutes with a cell free buffer before perfusion with the leukocyte suspension cleans the lung vascular bed of most of the blood and marginated cells in the lung and will allow the perfused leukocytes to be in direct interaction with a clean endothelium. This allows kinetic studies of the build up of the marginated pool of leukocytes in the lung.

The results of the experiments described clearly demonstrate that the isolated perfused lung sequesters the leukocytes traversing it. The change in the rate of sequestration (observed in Fig. 4), decreasing from an 80 percent level to a 10 percent level, indicates that the lung is acting as more than just a passive filter. If the lung were to act as a passive filter, removing cells at a constant rate, then one would have expected the number of cells successfully passing through the lung to be a constant fraction of cells entering it.

Of the three possible explanations for the variable rate of sequestration, that is, the opening of lateral shunts of larger diameter, the gradual death of the lung, and the physiological

saturation of the lung with leukocytes, the first two are ruled out by the results illustrated in Figs. 5 and 6.

The hypothesis of the opening of lateral shunts is based on the assumption that cells which enter the lung and are not able to pass through a capillary will gradually plug all the capillaries in the lung. The plugging of capillaries causes the opening of lateral shunts of larger diameter, through which cells can pass without retention. The result is a smaller rate of sequestration. This is a purely mechanical phenomenon, in which a particle, in this case a leukocyte, plugs a capillary and the closure of capillaries results in the opening of a lateral shunt. Such a mechanical event should not depend on the nature of the particle which plugs the capillary, i.e., the replacement of leukocytes with other particles of the same size should not change the outcome of the experiment. However, when the lung was perfused with latex particles of 7.6 ± 2.3 m in diameter, the particles were completely retained by the lung. Even though scanning microscopy of such lungs showed that particles had actually plugged all capillaries and small arterioles, no lateral shunt was ever opened in response to such a closure.

The presence and the functionality of arterio-venous shunts bypassing the pulmonary capillary network has been disputed before (83). My results, illustrated in Fig. 5, provide more evidence that no such shunts exist in the rat lung.

The functionality and useful life of an isolated perfused lung preparation has been assessed repeatedly by several investigators (98, 102-105), and the average reported useful life is between 1-1/2 to 2-1/2 hours. Although the duration of single-pass perfusion experiments is at most 30 minutes, well within the useful life of the preparation, the possibility that the decrease in sequestration is due to a loss in the sequestering ability of the lung should be considered.

Such a loss in sequestering ability may be due to the gradual death and malfunction of the isolated lung. This assumes that cell sequestration by the lung is an active process, and that early in the course of perfusion, when the lung is still healthy, it actively removes cells from circulation. But, as perfusion continues, the isolated lung loses its functional integrity and, failing to remove cells from circulation, will allow more cells to pass through it. The results of the double-perfusion experiment illustrated in Fig. 6 indicate that the decrease in sequestration observed in Fig. 4 is not due to the loss of sequestering ability of the lung. Similar results were reported by Ambrus and Ambrus (60). Their effort to exhaust the leukocyte filtering capacity of heart-lung preparation by introducing fresh blood very 30 minutes over a period of three hours was unsuccessful.

The decrease in sequestration with the continuation of whole blood perfusion, observed in the isolated lung in Fig. 4, is in contrast to the results of similar experiments with glass bead columns. Hopen (66) has reported that when whole blood was passed through a glass bead

column, the rate of leukocyte retention increased as more blood was passed through. This discrepancy is probably due to the fact that the lung vascular bed is a three dimensional structure and the endothelial cell surface encountered by leukocytes in the isolated perfused lung is totally different from the kind of structure and surface they meet in glass bead columns. This suggests that the results obtained with inert artificial surfaces like glass beads or nylon fibers should be evaluated more carefully.

With the exclusion of the first two alternatives discussed above, the physiological saturation of the lung with leukocytes remains as the most probable explanation. This assumes that as leukocytes pass over the endothelium of blood vessels, they attach to the luminal surface of the endothelium. As more and more cells enter the lung, the lung will gradually saturate with cells and consequently remove fewer and fewer cells from circulation. Attachment of leukocytes to endothelium can be either reversible or irreversible.

An irreversible attachment mechanism hypothesis assumes that there exists a large, but limited, number of receptors or receiving areas on the surface of the lung vascular endothelium. When the leukocytes pass over these areas, they gradually and irreversibly fill up all the vacancies and, therefore, the rate of cell removal decreases as the perfusion continues. On the other hand, a reversible attachment mechanism assumes that the cells attached to the endothelium can detach and return to circulation and the net sequestration rate is the sum of two different processes: the removal of cells from circulation

by the lung, and the detachment and release of sequestered cells from the lung into circulation. The decrease in sequestration is due to an increase in the rate of detachment and release of cells into circulation. Both mechanisms will result in a decreasing sequestration as more cells pass through the lung in the single-pass mode experiments. Therefore, the single-pass experiments were not capable of providing any information as to the reversibility of attachment. Depending on the reversibility, more explicit and yet different hypotheses can be formulated.

No evidence in the literature is available in support of the irreversible attachment of leukocytes to vascular endothelium under normal conditions. In contrast, several investigators, either by direct microscopic observation in live animals (49,50), or by kinetic studies (52) have provided evidence in support of reversible attachment of leukocytes to the endothelium under normal conditions. The results of my perfusion experiments in the continuous-circulation mode support the reversible attachment mechanism. These experiments are based on the idea that if a leukocyte is to attach irreversibly to the lung vascular endothelium when a suspension of leukocytes is recirculated through the lung, then the lung would eventually remove and deplete all the cells from circulation. That is, the circulating cell concentration would exponentially drop to zero.

The fact that the circulating cell concentration does not drop to zero in such experiments (see Fig. 7) does not exclusively support a reversible attachment mechanism. It could also mean an irreversible

removal of a specific group of cells from circulation, allowing the other cells to recirculate freely. This possibility was tested and ruled out by the results illustrated in Fig. 8. If the lung is exclusively filtering one cell type, when a suspension of leukocytes is recirculated long enough to reach the steady state, the recirculating cells remaining in the suspension should be the non-adherent cells, and if this suspension is recirculated through a second lung, no new filtration should occur by the second lung. This was not the case, as can be seen from Fig. 8. There was a net filtration and sequestration by the second lung. The fact that the second steady state established by the second lung has a different steady state concentration level than the first one suggests that different leukocytes are sequestered at different rates by the same lung.

The results illustrated in Fig. 7 are in agreement with the data published by Ambrus and Ambrus (59,60). They have observed that leukocytes from heparinized dog blood rapidly disappeared in their heart-lung preparation until a certain level was reached. This level was then maintained for the useful life of their preparation. However, my results and those published by Ambrus are in disagreement with the results obtained by MacGregor, et al (68) with nylon fiber columns. In a nylon fiber column, the fraction of cells retained by the column is dependent on the fiber weight; by choosing the right weight of nylon fiber, it is possible to remove all the leukocytes from the blood passing through the column.

Based on the results of my experiments, the following reversible attachment hypothesis can be formulated to explain the sequestration of leukocytes by the isolated perfused rat lung. As the leukocytes enter the lung, they reversibly attach to the endothelium of blood vessels. The rate of attachment is proportional to the incoming cell concentration, i.e., a constant, k_a, times the incoming cell concentration. Cells already attached can detach and return to circulation. The rate of detachment is proportional to the number of cells attached to the lung, i.e., a constant, k_b, times the number of cells already attached to the lung. The plateau region in Figs. 4 and 7 is the state at which the rate of detachment equals the rate of attachment, and therefore the net removal of cells from circulation approaches zero (not considering cell death and migration).

The reversibility of attachment is established by results illustrated in Figs. 4 and 8, as well as data published in the literature discussed earlier. The other two assumptions, that the rate of attachment is proportional to the incoming cell concentration and the rate of detachment is porportional to the number of cells attached to the lung were experimentally evaluated by studying the effect of incoming cell concentration on the sequestration and the steady state of the system.

The assumption that, at the plateau region, the rate of detachment is equal to the rate of attachment, which in turn is proportional to the incoming cell concentration, predicts that at the plateau region, replacement of the circulating suspension by a fresh suspension with a

concentration equal to the steady state concentration should not disturb the steady state. The results illustrated in Figs. 9 and 10 support this prediction. No detectable sequestration from the second suspension was observed when the circulating suspension was replaced by a fresh suspension of equal concentration.

The reversible-attachment hypothesis assumes that the attachment rate is proportional to the incoming cell concentration, i.e., changing the input cell concentration should disturb the steady state of the system. This predicts that an increase in the circulating cell concentration at the plateau should lead to an increase in the attachment rate and subsequently, to an increase in the detachment rate until a new steady state is again reached. This prediction was experimentally tested and the results illustrated in Fig. 11 confirm the prediction.

The assumption that the rate of detachment is proportional to the number of cells already attached to the lung could be tested by decreasing the circulating cell concentration at the steady state. In this case, the rate of attachment will decrease, however, since the number of cells in the lung has not changed, the rate of detachment will not change, and one would expect the lung to release some cells into circulation, raising the circulating cell concentration. This rise will result in an increase in the rate of attachment and a decrease in the rate of detachment. The overall result should be a new steady state with a new plateau level. This prediction was tested experimentally by replacing the circulating suspension with a fresh

suspension having a concentration equal to half that of the steady state concentration. As predicted by the hypothesis, the lung raised the circulating cell concentration by 50 percent by releasing cells into circulation (see Figs. 12 and 13).

Based on the reversible-attachment mechanism described above, it is possible to provide an explanation for the disappearance and distribution of infused DFP^{32}-labelled granulocytes in the body (52) and the inability to raise the leukocyte level in patients through transfusion of leukocyte suspensions (58,110).

Under normal conditions, the blood leukocytes are in a steady state, i.e., the same number of cells which marginate and attach to vessel walls are detached and released into circulation, so that the net removal of cells is equal to the number of cells which either permanently leave circulation and migrate out of the blood vessel network or die. The normal steady state concentration of leukocytes in the body varies in every individual according to the physiological condition of the body. When a suspension of leukocytes is infused, the steady state will be disturbed. This condition will be similar to the experimental conditions of Fig. 11. The infused cells will create a leukocyte suspension (blood in the body) with a higher concentration than the steady state concentration and, therefore, cells will be removed from circulation until a new steady state is reached.

The second steady state in Fig. 11 has a higher concentration than the first one. This is in agreement with clinical data. Perry (106) has reviewed the subject and has concluded that the efficiency of

infusion is at most 25 percent. My results indicate an increase of about 60 percent. This discrepency is understandable in light of the fact that the clinical data measure the distribution of these cells all over the body's blood vessels and the present system deals only with the isolated perfused lung. However, even a maximum yield of 25 percent suggests that, if leukocyte infusion is repeated several times, at intervals long enough for establishment of a new steady state, it should be possible to substantially raise the circulating leukocyte concentration of the body.

The differential affinity and stickiness of different blood cells towards the vascular endothelium is well documented. there is unaminous acceptance that neutrophils have the highest affinity for being marginated (49,52) and attached to the endothelium in-vivo (50), for sticking to artificial surfaces (63,68) or for being sequestered by the circulation of the various organs (59,61). The results of my experiments confirm these reports. When whole blood was passed through the isolated perfused lung in the single-pass mode, neutrophils was sequestered and retained more than were the mononuclear cells (Fig. 16). When a suspension of peritoneal neutrophils were recirculated through the isolated lung and brought into the steady state, both neutrophils and mononuclears followed the same pattern of interaction, however the neutrophils had a much lower steady state concentration than that of either the whole mixture or that of the mononuclear cells.

Data available in the literature indicate that the adhesion of granulocytes to glass beads (63) or to endothelial cells in tissue culture (77) is decreased in the absence of divalent cations. In agreement with the above data, neutrophil sequestration by the isolated perfused lung decreased by 92 percent in the absence of Ca^{++} and Mg^{++}. This suggests that the isolated perfused lung can be used as a test system to study the biochemical basis of leukocyte interaction with the vascular endothelium.

The reversible sequestration hypothesis described above, which is capable of explaining and predicting the behavior of leukocytes in interaction with blood vessel walls, can be formulated mathematically. By doing so, we can see how well the theoretical results, gleaned from solving the differential equations describing the system, agree with the experimental results described above, assuming that each leukocyte attaches to endothelium in a reversible manner. We can also ascertain whether or not the model is capable of making predictions which can be tested and confirmed experimentally. Finally, we can test the model's ability to provide a parameter by which individual blood cells could be characterized kinetically according to their affinity for blood vessel walls under a given condition.

B. Mathematical Model

The problem will be solved first for a simplified case in which a cell suspension is recirculated through a trap which can only remove cells from circulation. The results will then be extended to the more complex case of the isolated perfused lung in which the lung will be treated as a trap which is both a sink and a source of cells.

1. Simplifed Case

Consider a cell suspension with volume V and concentration C_o. This suspension is circulated through trap T, at flowrate F. The trap continuously removes a constant fraction, k, of cells which enter the trap (Fig. 33). We would like to find a function, C(t), to describe the cell concentration in the suspension at any time t, after the initiation of recirculation.

If $C_2(t)$ is the function describing the cell concentration in the suspension immediately after the trap, for instance at point X in the cell path, then the change in cell concentration of the reservoir will be given by:

$$\frac{dC(t)}{dt} = -\frac{F}{V} C(t) + \frac{F}{V} C_2(t) \qquad (1)$$

However, immediately before and after the trap we have:

$$C_2(t) = C(t) - kC(t) = (1 - k)C(t) \qquad (2)$$

By substituting equation (2) for $C_2(t)$ in equation (1), we have:

$$\frac{dC(t)}{dt} + \frac{F}{V} kC(t) = 0 \qquad (3)$$

Equation (3) has a general solution of the form:

$$C(t) = A \exp(-\alpha t) + B \text{ in which } \alpha = \frac{F}{V} k \qquad (4)$$

The boundary conditions of differential equation (3) are:

$$\text{at } t = 0,\ C(t) = C_0 \text{ and at } t = \infty,\ C(t) = 0 \tag{5}$$

$$C(0) = A + B = C_0 \tag{5a}$$

$$C(t=\infty) = B = 0 \tag{5b}$$

From equations (5a) and (5b) we have:

$$A = C_0 \tag{5c}$$

Therefore, by substituting A and B from equation (5) into equation (4), our desired function will be:

$$C(t) = C_0 \exp(-\frac{F}{V} kt) \tag{6}$$

2. Isolated Perfused Lung

The approach here is basically the same as described in the simplified mode. However, here the lung is considered as a bifunctional trap. It removes a constant fraction, k_a, of cells which enter it, and puts back into circulation a constant fraction ,k_b, of cells which have already been removed by it. In order to obtain a first approximate solution to the problem, two other phenomena, namely cell loss due to cell death and cell loss due to migration of cells through the endothelium are ignored in this treatment.

If $N_L(t)$ represents the total number of cells removed from the circulation by the lung at any time, t, after the initiation of circulation, we define the function $C_L(t)$ as:

$$C_L(t) = \frac{1}{V} N_L(t) \tag{7}$$

We also define the function X(t) as the change in cell concentration due to the lung presence, i.e., the change between the two points X and Y in the cells' pathway. Thus:

$$X(t) = k_a C(t) - k_b C_L(t) = \frac{dC_L(t)}{dt} \tag{8}$$

Also, if $C_2(t)$ represents the cell concentration at point X, between the two points X and Y we have:

$$X(t) = C(t) - C_2(t) \tag{9}$$

or

$$C_2(t) = C(t) - X(t) \tag{10}$$

From the law of conservation for the total number of cells we have:

$$C_o = C(t) + C_L(t) \tag{11}$$

From equation (11):

$$C_L(t) = C_o - C(t) \tag{12}$$

By substituting equation (12) for $C_2(t)$ in equation (8), we get:

$$X(t) = (K_a + k_b)C(t) - k_b C_o \tag{13}$$

By substituting equation (13) for $X(t)$ in equation (10), we get:

$$C_2(t) = (1 - k_a - k_b)C(t) + k_b C_o \tag{14}$$

Substituting equation (14) for $C_2(t)$ in equation (1) gives us:

$$\frac{dC(t)}{dt} + \frac{F}{V}(k_a + k_b)C(t) - \frac{F}{V} k_b C_o = 0 \tag{15}$$

The general solution of the above differential equation is:

$$C(t) = A \exp(-\tau t) + B \tag{16}$$

After substituting equation (16) for $C(t)$ in equation (15), we have:

$$A[\frac{F}{V}(k_a + k_b) - \tau] \exp(-\tau t) + \frac{F}{V}[B(k_a + k_b) - k_b C_o] = 0 \tag{17}$$

Equation (17) should hold for all values of t. At $t = \infty$, we have:

$$\frac{F}{V}\left[B(k_a + k_b) - k_b C_o\right] = 0 \qquad (18a)$$

therefore

$$B = \frac{k_b C_o}{k_a + k_b} \qquad (18b)$$

By substituting equation (18b) for B in equation (17), we get:

$$A\left[\frac{F}{V}(k_a + k_b) - \tau\right] \exp(-t) = 0 \qquad (19)$$

Equation (19) should also hold for all values of t. Therefore:

$$\frac{F}{V}(k_a + k_b) - \tau = 0 \qquad (20a)$$

$$\tau = \frac{F}{V}(k_a + k_b) \qquad (20b)$$

The initial condition for differential equation (15) is:

at $t = 0$, $C(t) = C_o$. Therefore:

$$C(0) = A + B = C_o \qquad (21a)$$

$$A + B = C_o \qquad (21b)$$

When we substitute equation (18) for B in equation (21b), we get:

$$A = \frac{k_a C_o}{k_a + k_b} \tag{22}$$

therefore, the final form of the function C(t) is:

$$C(t) = \frac{C_o}{k_a + k_b} [k_a \exp (- \frac{F}{V} (k_a + k_b)t) + k_b] \tag{23}$$

Substituting equation (23) for C(t) in equation (14), we get:

$$C_2(t) = \frac{C_o}{k_a + k_b} [k_a(1 - k_a - k_b) \exp (- \frac{F}{V} (k_a + k_b)t) + k_b] \tag{24}$$

We will define functions $P_1(t)$ and $P_2(t)$ as follows:

$$P_1(t) = \frac{C(t)}{C_o} 100 = \frac{100}{k_a + k_b} [k_a \exp(-\tau t) + k_b] \tag{25}$$

$$P_2(t) = \frac{C_2(t)}{C_o} 100 = \frac{100}{k_a + k_b} [k_a(1 - k_a - k_b) \exp(-\tau t) + k_b] \tag{26}$$

Functions $P_1(t)$ and $P_2(t)$ represent the ratio of cell concentration in the reservoir and the lung effluent suspension to the initial concentration of the perfusate, respectively, at any time, t, after initiation of perfusion.

If $k_b = 0$, then equation (23) will reduce to:

$$C(t) = C_o \exp (- \frac{F}{V} k_a t) \tag{27}$$

which is the same as equation (6) obtained for the simplified model.

According to equations (25) and (26), for all possible values of k_b in which $k_b > 0$, we should have:

$$P_1(t=\infty) = P_2(t=\infty) = \frac{100k_b}{k_a + k_b} > 0 \qquad (28)$$

Equation (28) shows that for all possible values of k_b in which $k_b > 0$, a state of equilibrium is reached between circulating cells and the lung at which no more cells are removed from circulation by the lung, i.e., there will be a time after which the number of cells removed from circulation by the lung will equal the number of cells put back into circulation by the lung. The exact location of this point of equilibrium depends on the ratio of k_a/k_b. This also provides a way to experimentally evaluate k_a/k_b. Figures 34 and 35 show graphs of $P_1(t)$ and $P_2(t)$ as a function of time for selected values of k_a and k_b.

Dresch (107) and other investigators (52,61) have reported that in human beings, the blood granulocytes are composed of two pools, a circulating pool and a marginated pool which is temporarily out of circulation through attachment to vessel walls. These pools are in rapid equilibrium and, under normal conditions, are of about the same size.

The present model is able to provide an explanation to the above phenomenon. By assuming $k_a = k_b$, the model predicts that at equilibrium $C(t) = 1/2C_o$, i.e., the number of circulating cells

should be half of the total number of cells and equal to the total number of cells removed from circulation.

The model predicts that for a given value of k_a and k_b, the actual value of equilibrium concentration, $C(t = \infty)$, is dependent upon the initial concentration, C_o. This can be seen from equation (23). However, this equilibrium value is always a constant fraction of the initial concentration, as shown by equation (28). In other words, the model predicts that, on a ratio basis, the equilibrium value should be independent of the initial concentration and only a function of k_a and k_b (or k_a/k_b). Values of k_a and k_b depend on cell type, lung condition, cell condition and/or the chemical composition of the perfusion medium.

3. Calculation of k_a and k_b from the Experimental Results

a) Bone Marrow Suspension

As indicated in the Material and Method section, two different parameters were measured in the recirculation experients. These were the reservoir cell concentration for which the samples were taken from the suspension reservoir at various times and the lung effluent cell concentration for which samples were collected from suspension drops dripping from the lung.

In order to calculate k_a and k_b, the method of least-square fit was used to fit a function of the form $C(t) = A \exp(-\theta t) + B$ as predicted by the model, to the experimental data. Since A, B, and θ are all unknowns, the approach taken was to assign an arbitrary value to θ, and then calculate A and B for the value of θ. Then, a new

value for θ was calculated from A aand B and the whole sequence was repeated again until the best values for A, B and θ were obtained. The values for k_a and k_b were then calculated from A and B. The mathematical details are discussed in the following section (108).

1) Mathematical Treatment

Let f be a continuous function which in the interval (a,b) is to be approximated by a linear combination:

$$\rho(x) = c_o\rho_o + c_1\rho_1(x) + \ldots\ldots + c_n\rho_n(x) \tag{29}$$

We should determine the coefficients $c_o, c_1, \ldots c_n$ such that a weighted Euclidean norm of the error function $\rho(x) - f$ is minimized. That is, such that

$$\int_a^b \left| \rho(x) - f(x) \right|^2 w(x)dx \quad \text{(continuous case)} \tag{30a}$$

$$\sum_{i=o}^{m} \rho(x) - f(x_i)\ ^2 w_i \quad \text{(discrete case)} \tag{30b}$$

is minimized ($w(x)$ or w_i are weight factors).

When $\rho_o, \rho_1, \ldots \rho_n$ are linearly independent, the least-squares approximation problem will have a unique solution;

$$\rho^*(x) = c_o^*\rho_o + c_1^*\rho_1(x) + \ldots\ldots + c_n^*\rho n(x) \tag{31}$$

where the coefficients c_j^* satisfy the so-called normal equation (35). The solution is also characterized by the orthogonality property that $\rho^*(x) - f$ is orthogonal to all $\rho_j(x)$, $(j = 0,1,....n)$.

The orthogonality condition is:

$$(\sum_{j=1}^{n} c_j^* \rho_j - f(x),\ \rho_k) = 0,\ (k = 0,1,...,n) \tag{32}$$

where (f,g) stands for the inner product or scalar product of two real-valued continuous functions and is identified by the relation:

$$(f,g) = \begin{cases} \int_a^b f(x)g(x)w(x)dx & \text{(continuous case)} \\ \sum f(x_i)g(x_i)w_i & \text{(discrete case)} \end{cases} \tag{33}$$

In the discrete case, if all weights $w_i = 1$, then (f,g) is the same as the vector inner product of vectors f and g. It is easy to show that, in general, if f, g, ρ are functions and c_1 and c_2 real numbers, we have:

$$(c_1 f + c_2 g,\ \rho) = c_1(f,\rho) + c_2(g,f) \tag{34}$$

Therefore, the orthogonality condition given by equation (32) will have the form:

$$\sum_{j=i}^{n} (\rho_j,\rho_k)c_j^* = (f(x),\rho_k),\ (k = 0,\ 1,...,n) \tag{35}$$

The above compact system of linear equations (called normal equations) is equivalent to the system of equations

$$(\rho_o,\rho_1)c_o^* + (\rho_o,\rho_1)c_1^* + \ldots + (\rho_o,\rho_n)c_n^* = (\rho_o, f) \qquad (36a)$$

$$(\rho_1,\rho_o)c_o^* + (\rho_1,\rho_1)c_1^* + \ldots + (\rho_1,\rho_n)c_n^* = (\rho_1, f) \qquad (36b)$$

$$\ldots$$

$$(\rho_n,\rho_o)c_o^* + (\rho_1,\rho_n)c_1^* + \ldots + (\rho_n,\rho_n)c_n^* = (\rho_n, f) \qquad (36n)$$

2) Application to the Experimental Data

In this case of our experiment, we have the following measured data (all weights are equal to one):

x	0	6	10	20	30	40	Reservoir cells
f(x)	100	65	50	41	38	31	

x	3	6	10	20	30	40	Lung effluent suspension
f(x)	18	24.8	35.4	32	32	29	

We would like to fit a function of the form $\rho(x) = A \exp(-\alpha x) + B$ to the above data. Using the notation of section 1, $\rho(x)$ can be written as:

$$\rho(x) = c_0\rho_0 + c_1\rho_1(x) \tag{37}$$

in which

$$c_0 = B \text{ and } c_1 = A \tag{38a}$$

$$\rho_0 = 1 \text{ and } \rho_1(x) = \exp(-\alpha x) \tag{38b}$$

System of equations (36) will then become:

$$(\rho_0,\rho_0)c_0 + (\rho_1,\rho_0)c_1 = (f,\rho_0) \tag{39a}$$

$$(\rho_0,\rho_1)c_0 + (\rho_1,\rho_1)c_1 = (f,\rho_1) \tag{39b}$$

Since f is a discrete function, we will have:

$$nc_0 + c_1 \sum \exp(-\alpha x_i) = \sum f(x_i) \tag{40a}$$

$$c_0 \sum \exp(-\alpha x_i) + c_1 \sum \exp(-2\alpha x_i) = \sum f(x_i) \exp(-\alpha x_i) \tag{40b}$$

Using matirx notation, equation (40b) can be written as:

$$E \cdot C = F \tag{41}$$

in which

$$E = \begin{pmatrix} n & \Sigma\exp(-\alpha x_i) \\ \Sigma\exp(-\alpha x_i) & \Sigma\exp(-2\alpha x_i) \end{pmatrix}$$

$$C = \begin{pmatrix} c_0 \\ c_1 \end{pmatrix} \quad \text{and} \quad F = \begin{pmatrix} \Sigma f(x_i) \\ \Sigma f(x_i)\ \exp(-\alpha x_i) \end{pmatrix}$$

Using matrix algebra, equation (41) can be solved for c by multiplying both sides of the equation with, E, the inverse matrix of E. That is:

$$\overline{E} \cdot E \cdot C = \overline{E} \cdot F \tag{42}$$

but, $\overline{E} \cdot E = 1$. Therefore:

$$C = \overline{E} \cdot F \tag{43}$$

A program was developed to calculate c_0 and c_1 using matrix algebra. As mentioned earlier, since both c_0 and c_1 are themselves functions of α, and α is also unknown, an arbitrary value had to be assigned to α to initiate the calculation. The program used calculated values of c_0 and c_1 to recalculate α and then the whole

sequence was repeated again until the best value of α was found. The goodness of fit was evaluated by using R^2, the coefficient of determination defined as:

$$R^2 = \frac{[\Sigma f(x_i)\rho_1(x_i) - (\Sigma f(x_i)\Sigma\rho_1(x_i))/\Sigma\rho_o]^2}{[\Sigma\rho_1{}^2(x_i) - (\Sigma\rho_1(x_i))^2/(\Sigma\rho_o)][\Sigma f^2(x_i) - (\Sigma f(x_i))^2/\Sigma\rho_o]} \quad (44)$$

The values of R^2 are between 0 and 1 and indicate how closely the equation fit the experimental data; the closer R^2 is to 1, the better the fit. Values that give the best fit to both sets of experimental data were:

$$\alpha = 0.17614$$

$$A = 63.5$$

$$B = 37$$

From equation (25) we have:

$$\alpha = (k_a + k_b)\frac{F}{V} = 0.17614 \quad (45a)$$

$$A = 100k_a/(k_a + k_b) = 63.5 \quad (45b)$$

$$B = 100k_b/(k_a + k_b) = 37 \quad (45c)$$

Solving equation (45) for k_a and k_b with experimental condtion $F = 9$ ml/min and $V = 70$ ml, we get:

$$k_a = 0.87 \tag{46a}$$

$$k_b = 0.50 \tag{46b}$$

Therefore, the equations describing the experimental system will have the forms:

$$P_1(t) = 63.5 \exp(-0.176t) + 37 \tag{47}$$

$$P_2(t) = -23.5 \exp(-0.176t) + 37 \tag{48}$$

In Fig. 36, $P_1(t)$ and $P_2(t)$ are plotted as a function of time. Also shown are experimental data for both reservoir and lung effluent suspension cells.

b) Other Cell Populations

The above procedure was applied to results illustrated in Fig. 15 to calculate k_a and k_b for neutrophils, mononuclear cells, and the mixed population. Due to the larger number of cells present in the reservoir samples as compared to the lung effluent suspension samples, cell counts in the reservoir have much better statistics than the lung effluent does. Thus, only the data from the reservoir group counts are used for the final calculation of k_a and k_b. Equations 25 and

26 were fitted to data given below and k_a and k_b were calculated from best-fit coefficient.

	x	0	2	5	10	20	30	k_a/k_b
neutrophils	$f_1(x)$	100	86.4	58	22.7	6.9	8	53
mononuclear cells	$f_1(x)$	100	---	87	---	---	71	0.42
moxed population	$f_1(x)$	100	---	---	29	18	16	5.6

4. Coefficient of Stickiness

The ratio of k_a/k_b can be used as a convenient measure of stickiness and retention of leukocytes. Let's define $k_s = k_a/k_b$ as the coefficient of stickiness. The k_s values for different cell types are compared below.

Cell Population	k_s
mononuclear cells from the suspension of peritoneal neutrophils	0.29
bone marrow suspension	1.74
mixed population of peritoneal neutrophil suspension	5.6
neutrophils	53

The value of k_s for each cell population depends on the composition of the population and the media of interaction. The higher the k_s value, the stickier the cell population. Neutrophils have the highest k_s value compared to the mixed or the mononuclear cell populations. The mixed population from the peritoneal cavity has a higher k_s value than the bone marrow population because the former contains about 94 percent neutrophils while the latter usually contains about 65 percent mature neutrophils.

5. Cell Loss Factor

Granulocytes continuously migrate through the endothelium into the interstitial space (109). Cells leave circulation randomly, as indicated by the disappearance curves of infused labelled leukocytes in-vivo (107). The magnitude and rate of cell loss demonstrated by these curves includes cell loss due to cell senescence. The random loss of cells from circulation can be represented by an exponential function. In my previous treatment of the model, the cell loss factor was ignored so that a first approximate solution of the problem could be obtained. Here, the loss factor will be included and the half-life of circulation in the isolated perfused lung will be calculated from this loss factor.

If the cells leave circulation at a rate of k_L cell/min, we can define the functions $F_1(t)$ and $F_2(t)$ as

$$F_1(t) = P_1(t)\exp(-k_3 t) \tag{50}$$

$$F_2(t) = P_2(t)\exp(-k_3 t) \tag{51}$$

$F_1(t)$ and $F_2(t)$ represent the ratio of leukocyte concentration in the reservoir and the lung effluent, respectively, to the initial concentration of the perfusate. Substituting for $P_1(t)$ and $P_2(t)$ from equatons (48) and (49) we obtain:

$$F_1(t) = 63.5\ \exp[(k_L - 0.176)t] + 37\ \exp(-k_L t) \tag{52}$$

$$F_2(t) = -23.5\ \exp[(k_L - 0.176)t] + 37\ \exp(-k_L t) \tag{53}$$

If equations (52) and (53) are fitted to the data illustrated in Fig. 4, the value of k_L that gives the best fit (judged by eye) is:

$$k_L = 0.0045 \tag{54}$$

Substituting this value of k_L into equations (52) and (53), we have:

$$F_1(t) = 63.5\ \exp(-0.1805t) + 37\ \exp(-0.0045t) \tag{55}$$

$$F_2(t) = -23.5\ \exp(-0.1805t) + 37\ \exp(-0.0045t) \tag{56}$$

A graph of $F_1(t)$ and $F_2(t)$ plotted as a function of time is illustrated in Fig. 37.

The half-life of the circulation of leukocytes in the isolated perfused rat lung is approximately 2-1/2 hours, which is of the same order of magnitude as the in-vivo value of 5-6.7 hours (107,52). The difference is most probably due to the greater rate of cell death in the perfusion system than in the body.

6. Experimental Evaluation of the Model

The reversible-attachment model described above provides predictions which can be tested experimentally. The model predicts that for all values of k_b for which $k_b > 0$, given enough time, the system in the continuous-circulation mode will reach a steady state at which the net cell removal from circulation by the lung will approach zero (disregarding the cell loss factor). Such a steady state was indeed demonstrated in all cell perfusion experiments in the continuous-circulation mode. The steady state concentration of circulating cells will be given by:

$$C_s = C_2(t = \infty) = C_1(t = \infty) = C_o \frac{k_b}{k_a + k_b} \tag{57}$$

This equation provides a simple, although less accurate, way to calculate the coefficient of stickiness, k_s, from experimental results. By determining the steady state concentration we will have:

$$k_s = \frac{k_a}{k_b} = \frac{C_o - C_s}{C_s} \tag{58}$$

The model also predicts that, at the steady state, the ratio of circulating cell concentration over the initial perfusate concentration is a constant number, independent of the initial perfusate concentration. This can be seen from equation (57) and was also demonstrated in equation (28). If P_s is the ratio of the circulating cell concentration at the steady state to the initial concentration of the perfusate, then we have:

$$P_s = 100x \frac{C_s}{C_o} = \frac{100\ k_b}{k_a + k_b} \tag{59}$$

This prediction was tested experimentally by perfusing the lung, in the continuous-circulation mode, with three leukocyte suspensions with different initial concentrations. The result of this experiment is illustrated in Fig. 14. For the range of concentrations tested, 5.3×10^6 to 15×10^6 cells/ml, the ratio of the concentration of circulating cells at the steady state to the initial perfusate concentration was fairly constant, 31.2 ± 3.9 in agreement with the prediction made by the model. If the coefficient of stickiness of the bone marrow suspension is calculated from this number using equation (59), we have:

$$k_s = 2.25 \pm 0.57 \tag{60}$$

which is in agreement with the value of 1.74 calculated by curve fitting.

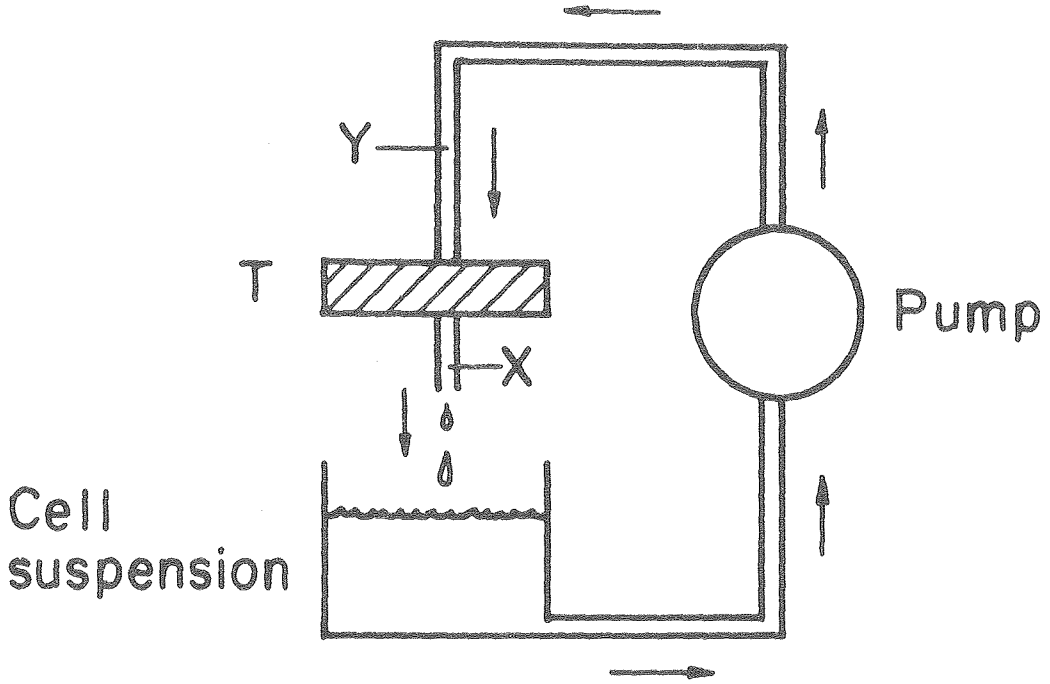

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Figure 33. Schematic representation of the simplified case. T = cell trap.

Figure 34. Graphic representation of the ratio of reservoir cell concentration (———) and the lung effluent cells (– – –) to the initial concentration of the perfusate as a function of time for arbitrary values of k_a and k_b. In this graph, k_a is kept constant at .5, and three values of k_b, 0, 0.5, and 1 are plotted.

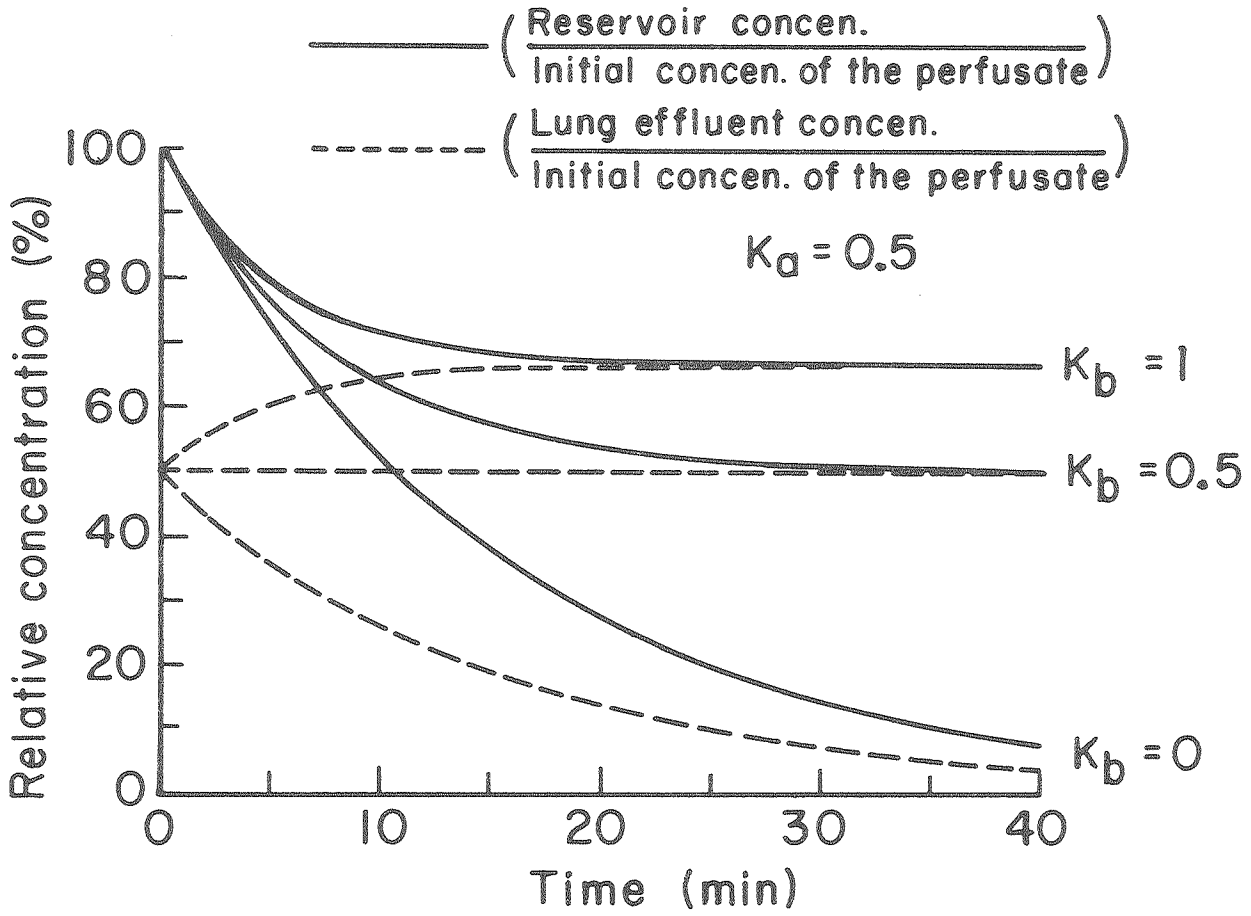

Figure 34

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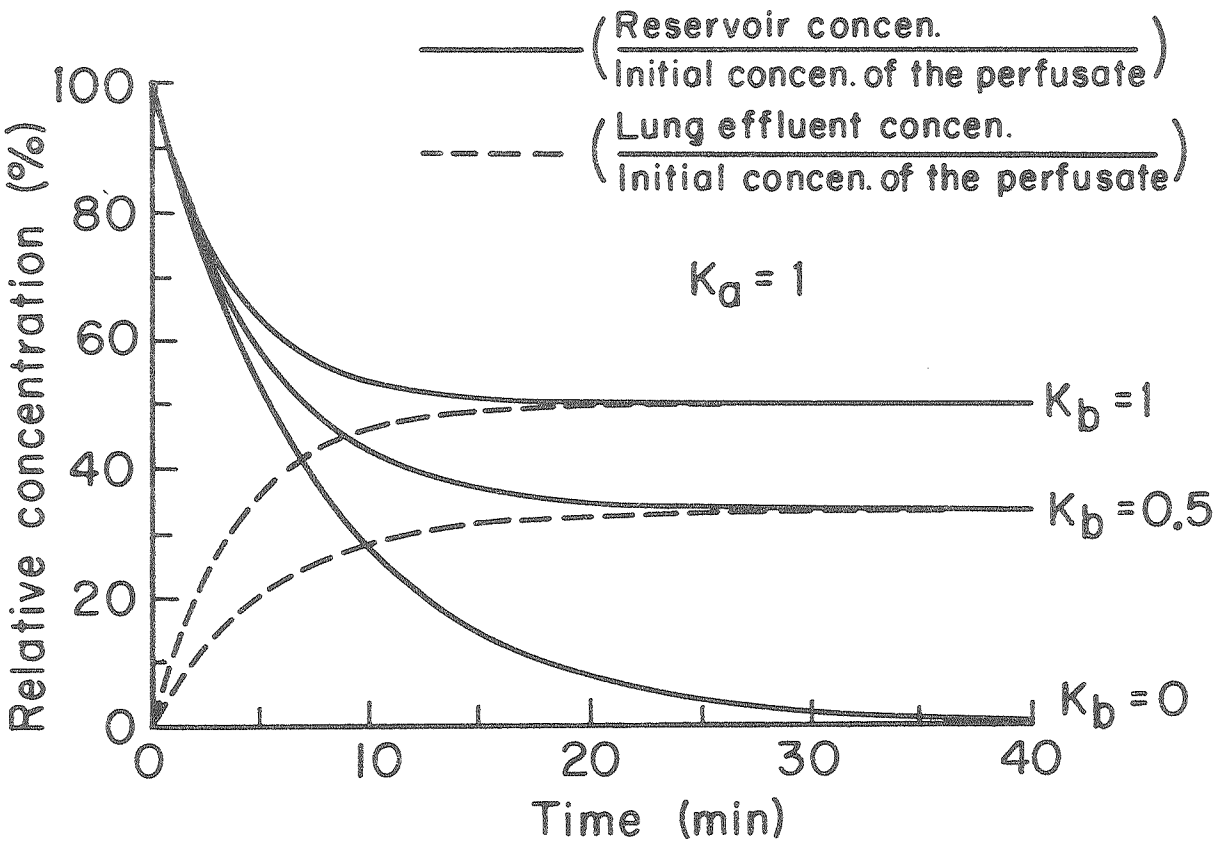

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Figure 35. Same as Fig. 34 except for k_a value. In this graph, k_a is kept constant and equal to 1.

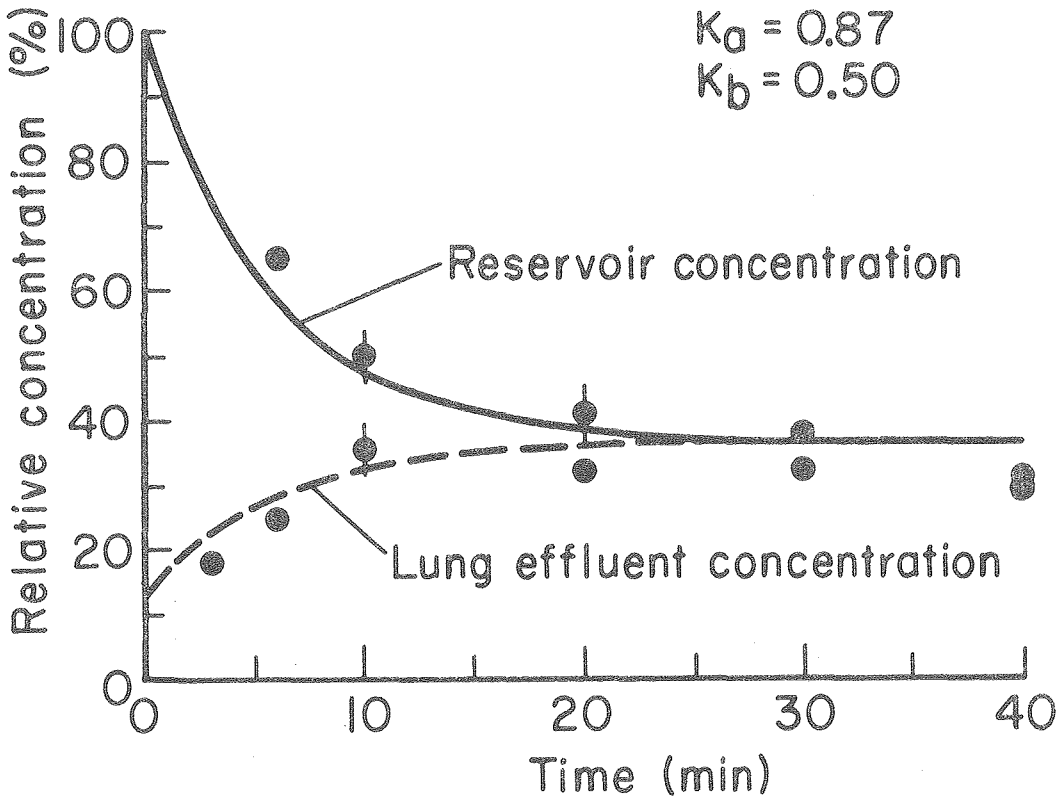

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Figure 36. The best-fit of the model prediction for the ratio of the reservoir cell concentration (——) and the lung effluent cell concentration (----) to the initial concentration of the perfusate is plotted as a function of time in the presence of experimental data.

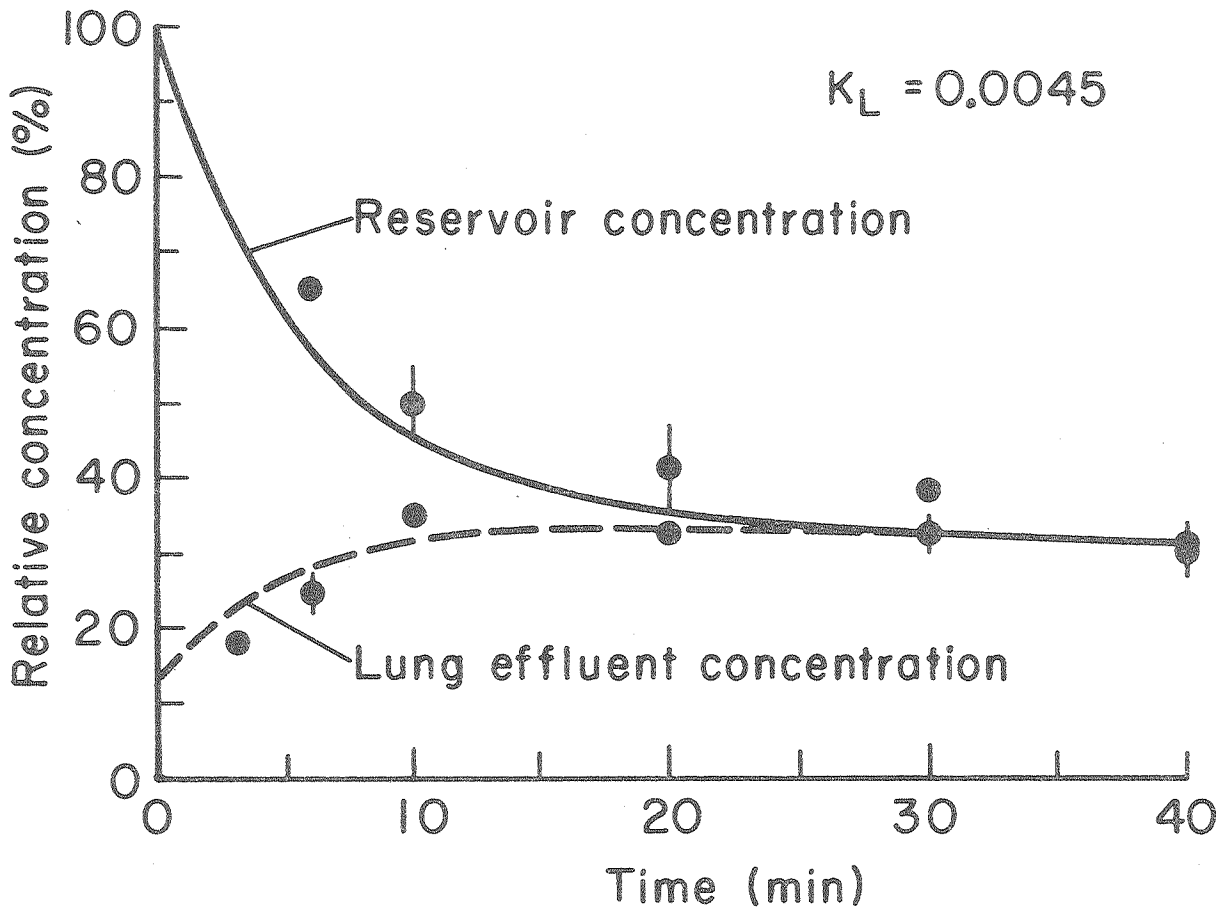

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Figure 37. The model prediction corrected for loss factor for the same data as in Fig. 36.

V. CONCLUDING REMARKS

The phenomenon of leukocyte margination and attachment to blood vessel walls, although known for a long time, is still a challenging and poorly understood area of investigation. In this report, the problem of the kinetics of leukocyte sequestration by the lung and their attachment to blood vessel walls was approached using an isolated perfused rat lung preparation.

In this paper, I have attempted to first, establish the occurance of leukocyte sequestration by the isolated perfused lung, and then, examine the various possible mechanisms responsible for sequestration. By excluding alternative hypotheses, the reversible-attachment mechanism is shown to be a possible underlying mechanism for cell sequestration. The rate of attachment of cells to endothelium is proportional to their input concentration and the rate of detachment is proportional to the number of cells attached to the lung.

The site and morphology of sequestration is examined by scanning electron microscope, which shows the post-capillary venules and veins to be the site of attachment of leukocytes to vessel walls. These morphological studies suggest that once the cells attach to the endothelium of a vein or a post-capillary venule, they extend finger-like projections and establish close contact with the endothelium. Some cells possibly move along the endothelium.

The reversible-attachment interpretation of the data is consistent with previously reported observations concerning the normal distribution of leukocytes in the body, the distribution of infused, labelled

leukocytes in the body and the failure to raise the leukocyte concentration in pateints with deficient leukocyte systems through transfusion of leukocyte suspensions.

These data suggest that the major factor determining whether or not leukocytes traversing the lung vascular network are going to be sequestered is the leukocyte concentration in the suspension relative to the number of cells already attached to the walls of the network. Under normal conditions, circulating leukocytes are in equilibrium with the attached cells. Any variations in the concentration of circulating cells, such as the infusion of labelled cells or of normal cells for therapy or leukapheresis, or in the number of attached cells, as in the case of inflammation which results in accelerated cell migration across the endothelium, will disturb the equilibrium and cause cells to shift from one pool to another in order to reestablish equilibrium.

A mathematical model based on the reversible-attachment mechanism is developed to explain the experimental results and predict the behavior of the system under various conditions. The model provides a parameter, coefficient of stickiness, k_s, by which the individual blood cells can be characterized according to their affinity for sticking to vascular walls. The higher the value of k_s, the stickier the cell. Neutrophils have the highest k_s and the mononuclear cells (mostly leukocytes) have the lowest. The k_s value of mixed populations of neutrophils and mononuclear cells falls somewhere in between, according to the proportion of these cells in the population. The

model envisions the sequestration of leukocytes by the lung as an active process. Divalent cations are required, to some extent, for the process, i.e., their absence does not totally abolish sequestration.

Much more work needs to be done to understand the vital process of margination and attachment of leukocytes to blood vessel walls. The biochemical factors and cellular components mediating attachment of leukocytes to vascular endothelium need to be identified. Although some of these factors can be studied in-vitro, using tissue culture of endothelial cells, their net contribution should eventually be evaluated under a condition in which cells flow over the endothelial surface of a blood vessel, preferably in an organ such as the isolated lung.

The k_s value of individual blood cells can be calculated by infusing labelled cells and then carefully monitoring their disappearance from the blood every five minutes so that a detailed disappearance curve of the first hour of post-infusion kinetics can be obtained. The model developed here can then be applied to calculate k_s from such a curve.

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