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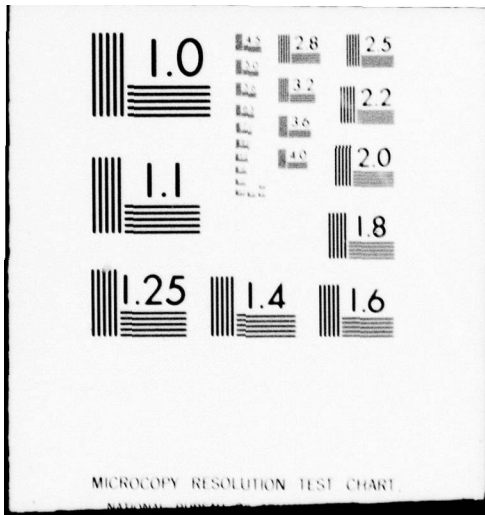
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Versus
Saline vs Colloid.

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A. Studies Completed This Year

1. Introduction

INTRODUCTION

The thrust of our research has been to explore clinical surgical problems that would have great relevance to military medicine. For example, we have just completed a series of studies involving hemorrhagic shock in the baboon followed by clinical studies in man. These studies indicated that Ringer's solution is equally effective as albumin solution for initial resuscitation from hemorrhage, yet albumin costs approximately 100 times as much as Ringer's solution. The decision to use Ringer's rather than albumin would have great impact on military medicine.

In our current project, we are developing new ways to examine what happens to lungs in septic shock. We believe septic shock remains an unsolved clinical problem. Our long range goal is to examine changes in interstitial albumin and sodium plus changes in alveolar surfactant during septic shock. We also hope to compare the pulmonary effects of Ringer's lactate versus albumin infusion in septic shock in baboons and then in man.

A. Studies Completed This Year

2. Changes in Lung Ultrastructure Following Heterologous and Homologous Serum Albumin Infusion in the Treatment of Hemorrhagic Shock.

CHANGES IN LUNG ULTRASTRUCTURE FOLLOWING
HETEROLOGOUS AND HOMOLOGOUS SERUM ALBUMIN INFUSION
IN THE TREATMENT OF HEMORRHAGIC SHOCK

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We have previously shown by electron microscopic studies that interstitial pulmonary edema can be observed in the lungs of baboons infused with human serum albumin in the treatment of hemorrhagic shock.⁷ Similar treatment with Ringer's lactate solution produces no such changes in this animal preparation. The development of interstitial pulmonary edema following human serum albumin administration in the baboon has several possible explanations. One is that the infused albumin accumulates in the lung interstitium. Another is that the changes seen in the lung ultrastructure are related to the infusion of a heterologous protein solution.

The object of this study was to compare the ultrastructural pulmonary effects of homologous and heterologous serum albumin infusion in the treatment of hemorrhagic shock in baboons.

Methods and Materials

Thirteen baboons were the test animals. Two were controls, two were sacrificed after hemorrhage, three were sacrificed after each of the following therapies - Ringer's lactate infusion, human serum albumin infusion, and baboon serum albumin infusion.

Each animal was tranquilized while still in his cage with phencyclidine hydrochloride (0.8 mg/kg). Under local anesthesia, a siliconized plastic catheter was placed in one femoral artery for blood withdrawal and arterial pressure measurements. Another catheter was inserted into the inferior vena cava via the femoral vein for infusion therapy and venous pressure measurements. An endotracheal tube was inserted and the animal was then loosely restrained in the prone position.

Hemorrhage: The arterial pressure was reduced to 60 mm Hg for one hour and then to 40 mm Hg for a second hour by femoral artery bleeding into a plastic bag containing 67.5 cc of citrate-phosphate-dextrose.

Resuscitation: Each animal was resuscitated over a four-hour period. A particular test fluid was given in volumes sufficient to restore and maintain arterial pressure and venous pressure to prehemorrhage levels.

Electron Microscope: At the end of each study, the pulmonary artery was catheterized via a median sternotomy under sodium pentobarbital anesthesia. The lungs were fixed by in vivo dual perfusion via a pulmonary artery and bronchus by a technique previously described.⁵ The perfusate was potassium pyroantimonate. This solution forms an electron-opaque complex with sodium, thus providing a method for identifying the distribution of pulmonary interstitial sodium. This is an established technique which has been utilized in prior studies.³

Fixed tissues were dehydrated in acetone and embedded in Araldite or Epon-812. Ultra-thin sections were mounted on a copper grid, stained with uranyl acetate, and viewed under an RCA EMU 312-4 microscope. Each specimen was read and evaluated by one of us (TKD) without knowledge of the treatment regimen.

Purification of Baboon Serum Albumin: The procedure, a modification of the Cohn fractionation technique² was adapted to permit the purification of 400 ml of citrated plasma at a time. Purity of the preparation was checked by electrophoresis, which showed a single uniform peak (Figure 1).

Results

A. Clinical Observations

All 13 animals survived shock and resuscitation. Table 1 shows the volumes of fluid infused in the groups. Animals in the Ringer's lactate group received a significantly larger volume of fluid than those that received albumin ($p < 0.05$).

B. Electron Microscopic Findings

Normal Lung: In the normal baboon lung (Figure 2a), the thin portion of the alveolar capillary membrane is composed of an epithelial and endothelial layer with an intervening narrow interstitial zone. In the thicker portion of the alveolar capillary membrane (Figure 2b), the interstitial zone becomes more complex. This zone contains collagen fibers which are of special interest, since the earliest electron microscopic findings of interstitial edema is dispersion of the individual fibers. In the interstitium, occasional sodium precipitates can be seen in relation to individual collagen fibers, indicating an inherent affinity between collagen and sodium.

Effects of Hemorrhagic Shock: Striking alterations in lung tissue were seen following hemorrhagic shock, the most profound being in the interstitium (Figure 3a). Here, large accumulations of edema fluid were noted. The interstitial edema fluid stained intensely, indicating the presence of sodium (Figure 3b). In addition, the concentration of sodium in and around the dispersed collagen fibers was remarkably increased.

C. Effects of Resuscitation:

Ringer's Lactate: The lung tissue of animals resuscitated with Ringer's lactate was practically indistinguishable from that of normal lungs (Figure 4a). The evidence for interstitial edema was minimal. There were few empty spaces, the smudge areas were rare, and the collagen fibers were tightly packed as in the normal state. Sodium precipitates (Figure 4b) were occasionally seen in relationship to the collagen fibers.

Human Serum Albumin: In the lungs from animals resuscitated with human serum albumin (Figure 5a), the tissues appeared similar to those seen in the untreated animals subjected to hemorrhage. Interstitial edema persisted, as manifested by empty spaces in the interstitium, smudging, and collagen fiber dispersion. In addition, abnormal concentrations of interstitial sodium (Figure 5b) could be seen especially in proximity to collagen fibers.

Baboon Serum Albumin: We could find no difference in the electron microscopic appearance of lungs from animals treated with baboon serum albumin and those treated with human serum albumin. Evidence of interstitial

edema was present in all sections studied (Figure 6a). There were multiple empty spaces observed in the interstitium. Smudging was frequently observed. There was gross dispersion of interstitial collagen fibers. Increased concentrations of sodium (Figure 6b) were noted in the edema fluid, especially in proximity to collagen fibers.

Discussion

In this study, baboons resuscitated from hemorrhagic shock with either homologous or heterologous serum albumin developed ultrastructural evidence of pulmonary edema. Animals resuscitated with Ringer's lactate solution showed no major changes in lung ultrastructure despite the significantly larger infusion volumes required by this group. These results suggest that the infused albumin accumulated in the baboon lung interstitium and produced interstitial edema.

These results are difficult to reconcile with the traditional Starling hypothesis.⁸ The hypothesis states that the movement of water across a semipermeable membrane is dependent upon the algebraic sum of the hydrostatic and colloid osmotic pressures on both sides of the membrane. If this hypothesis were correct for the lung, the infusion of albumin during hemorrhagic shock would maintain plasma colloid osmotic pressure and therefore inhibit the movement of plasma water into the lung interstitium. On the other hand, the infusion of Ringer's lactate will lower plasma oncotic pressure and therefore favor the filtration of plasma water into the lung interstitium.

The best explanation for the discrepancy between our results and the predicted results based on the Starling hypothesis involves the permeability of the pulmonary capillary membrane to albumin. The Starling hypothesis assumes that the pulmonary capillary membrane is impermeable to albumin. It is now clear that this is incorrect. In fact, large amounts of albumin pass through the pulmonary capillary membrane and are cleared from the lung presumably through the lymphatic system. The evidence for this point of view is as follows:

In studies in sheep¹ and baboons¹⁰, lung lymph was collected and analyzed for albumin levels. If the pulmonary capillary membranes were impermeable to albumin, lung lymph albumin levels should be low compared to plasma. In both sheep and baboons, the lung lymph albumin levels were approximately 80% of the plasma value, indicating a high degree of permeability for albumin.

The effects of elevated pulmonary microvascular pressure on pulmonary lung albumin in sheep was reported by Brigham.¹ When lung vascular pressures were increased, lung lymph flow increased, and lung lymph albumin levels fell. The fall in lung interstitial albumin levels offset the effect of increased intravascular pressure. Thus, the high level of basal pulmonary interstitial albumin represents a safety factor against increased intravascular pressure.

The effect of reduced plasma colloid osmotic pressure on pulmonary lymphatic kinetics was also studied. Lung lymph was collected from baboons subjected to profound plasmaphoresis with Ringer's lactate.¹⁰ Lung lymph albumin levels declined in a similar fashion to plasma levels. The lowest

levels were approximately 1 gram % for both fluids. In addition, lung lymph flow increased fourfold. Despite a 15% gain in body weight during the plasmaphoresis, there was no evidence of pulmonary edema. There were no changes from control levels in blood gases, intrapulmonary shunt, dynamic compliance, or lung water content. The results of this study illustrate again the safety factor provided by high levels of pulmonary interstitial albumin in the normal state. A fall in plasma colloid osmotic pressure is offset by a corresponding decline in pulmonary interstitial colloid osmotic pressure.

The effect of lowered plasma colloid osmotic pressure on lung function in man has been reported.⁹ A group of 43 patients undergoing major abdominal aortic surgery was resuscitated with either Ringer's lactate or 5% albumin solution. The relationship between changes in the gradient between colloid osmotic pressure (COP) and pulmonary capillary wedge pressure (PCWP) versus intrapulmonary shunt was studied. The base line gradient (COP-PCWP) was nine torr and the intrapulmonary shunt was 15%. No relationship could be found after resuscitation between changes in gradient and changes in intrapulmonary shunt. In several patients resuscitated with Ringer's lactate, the gradient became negative as the colloid osmotic pressure declined and PCWP remained constant. Despite marked weight gain and peripheral edema in these patients, no evidence of pulmonary edema was noted. Presumably, increased lymphatic clearance of pulmonary interstitial albumin protected the lungs from the effects of reduced plasma oncotic pressure.

In another clinical study, Lowe et al., studied 137 patients undergoing laparotomy for trauma who were resuscitated with either Ringer's lactate or 5% albumin solution. Since red cell losses were replaced with washed red cells, the patients in the Ringer's lactate group were resuscitated without any albumin. No differences were noted between the two treatment groups in death rates or in the incidence of respiratory failure.

Changes in lung capillary permeability to albumin have also been studied. Lung lymph was collected from sheep treated with either histamine or pseudomonas bacteria.¹ The lung lymph flow increased in a fashion similar to the results of the increased vascular pressure studies. However, the concentration of albumin in the lung lymph remained unchanged, in contrast to the results of the increased intravascular pressure studied. These results suggest that the safety factor of declining pulmonary interstitial colloid osmotic pressure is lost when the pulmonary capillary permeability to albumin is increased.

Increased capillary permeability was demonstrated clinically by Robin et al.⁶ They studied alveolar fluid contents in patients suffering from pulmonary edema and reported the levels of albumin and fibrinogen in the alveolar fluid were in equilibrium with plasma levels. High molecular weight Dextran (5000,000 M.W.), when injected intravenously, appeared in the alveolar fluid within minutes. The authors termed this "capillary leak syndrome".

In summary, we found that homologous serum albumin, when infused into the hemorrhaged baboon, produced ultrastructural evidence of interstitial edema. The best explanation for these findings is that the infused albumin accumulates in the lung interstitium.

Summary

The object of this study was to compare the ultrastructure pulmonary effects of the infusion of homologous and heterologous serum albumin solution in the treatment of hemorrhagic shock in baboons.

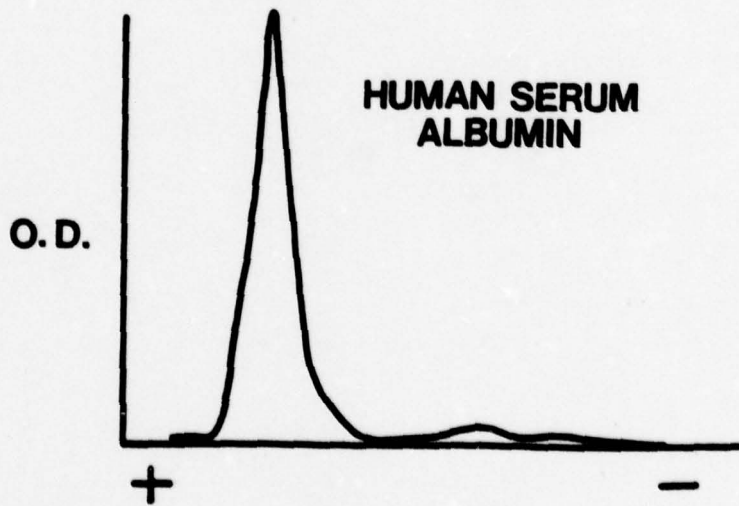
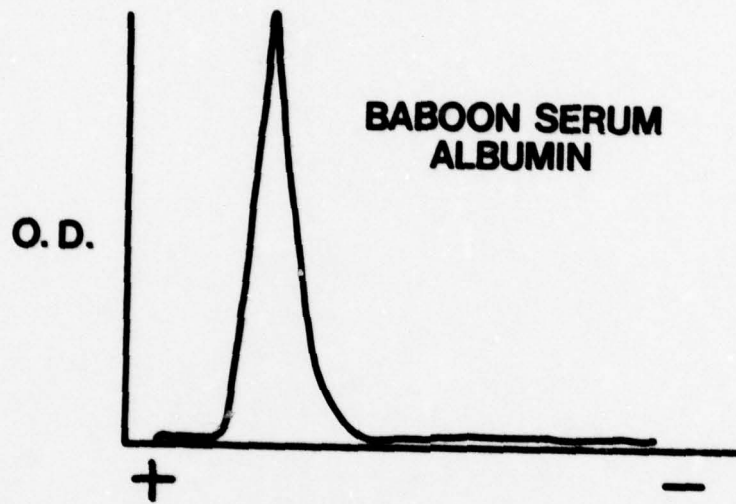
Adult baboons subjected to hemorrhagic shock were resuscitated with either baboon serum albumin, human serum albumin, or Ringer's lactate solution. The lungs were fixed in vivo with potassium pyroantimony, a solution which produces electron dense interstitial precipitation of sodium.

The lungs from animals resuscitated with baboon serum albumin showed evidence of interstitial edema, including dispersion of collagen fibers, interstitial smudging and increased interstitial sodium concentrations. Similar changes were soon following human serum albumin infusions. Lung tissue from animals treated with Ringer's lactate solution showed minimal changes from normal.

These results suggest that interstitial pulmonary edema develops after either homologous or heterologous serum albumin infusion in the treatment of hemorrhagic shock in baboons.

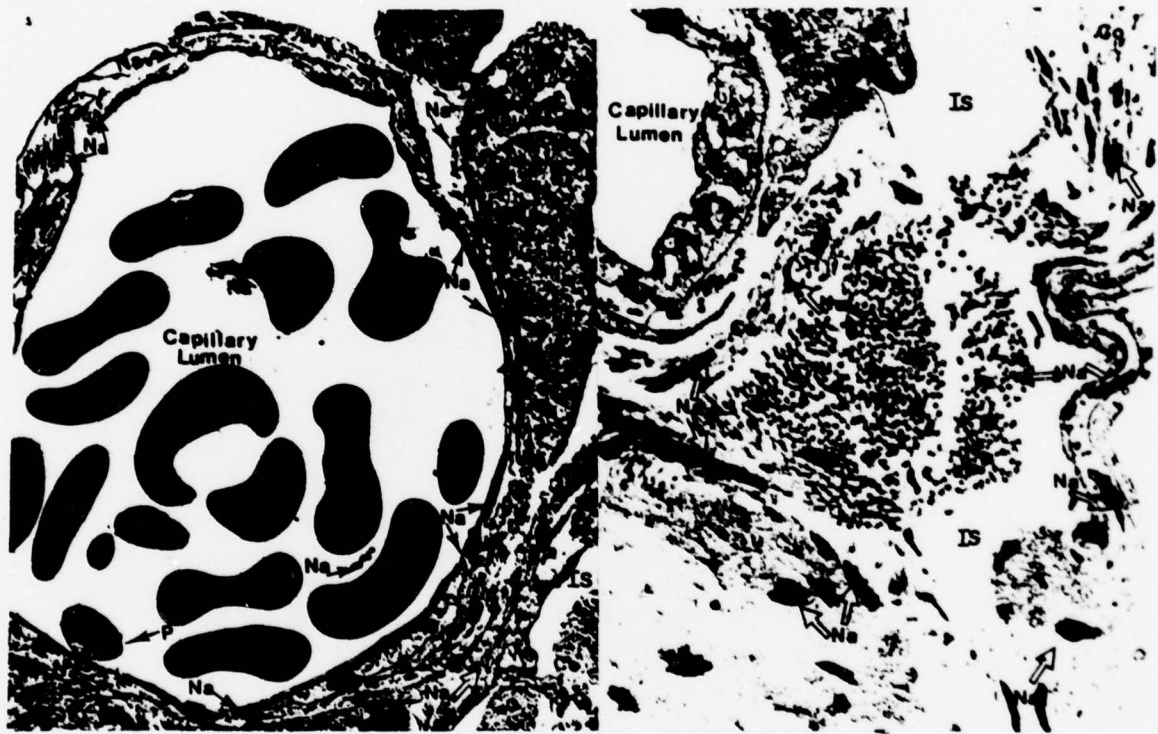
FIGURE 1:

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"Changes in Lung Ultrastructure..."



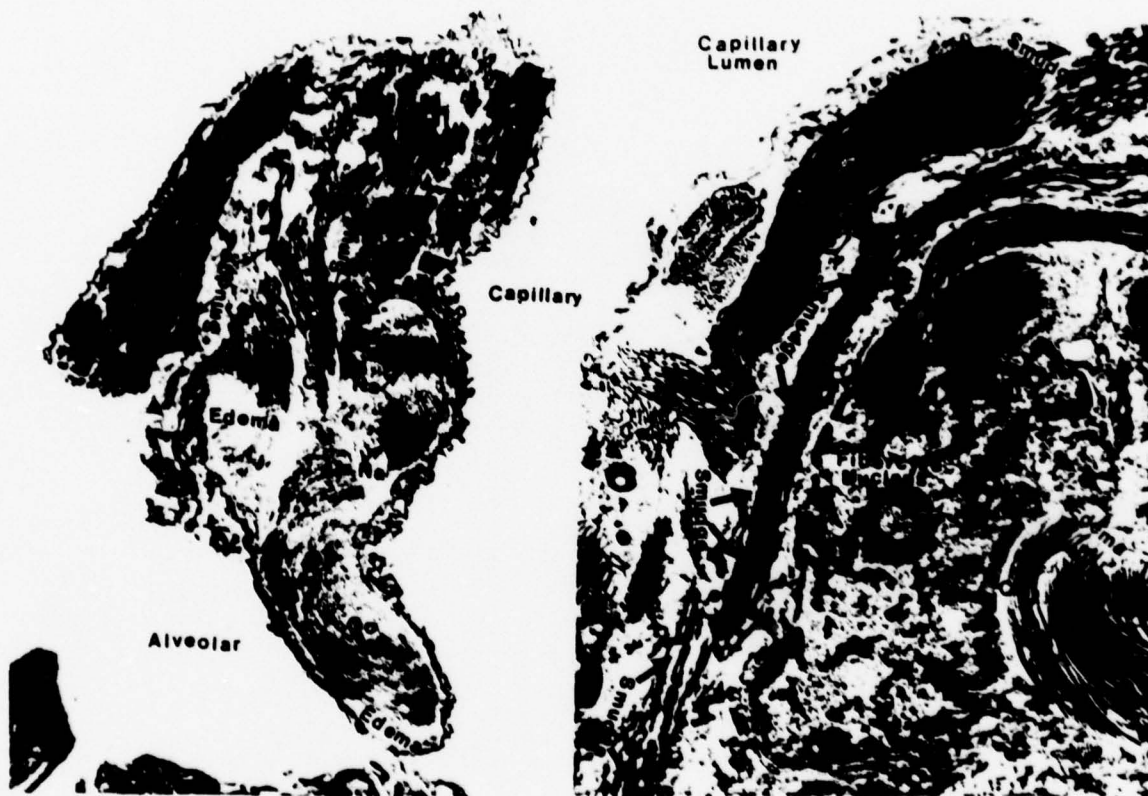
FIGURES 2a and 2b:

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"Changes in Lung Ultrastructure..."



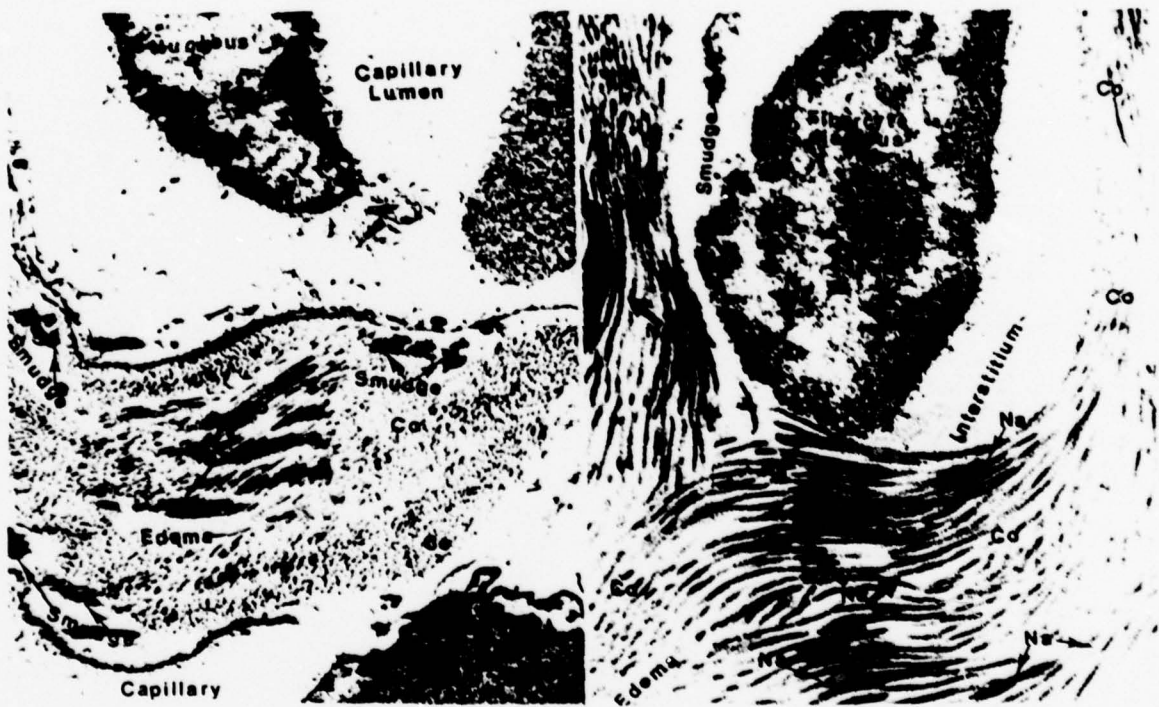
FIGURES 3a and 3b:

MOSS ET AL.
"Changes in Lung Ultrastructure..."



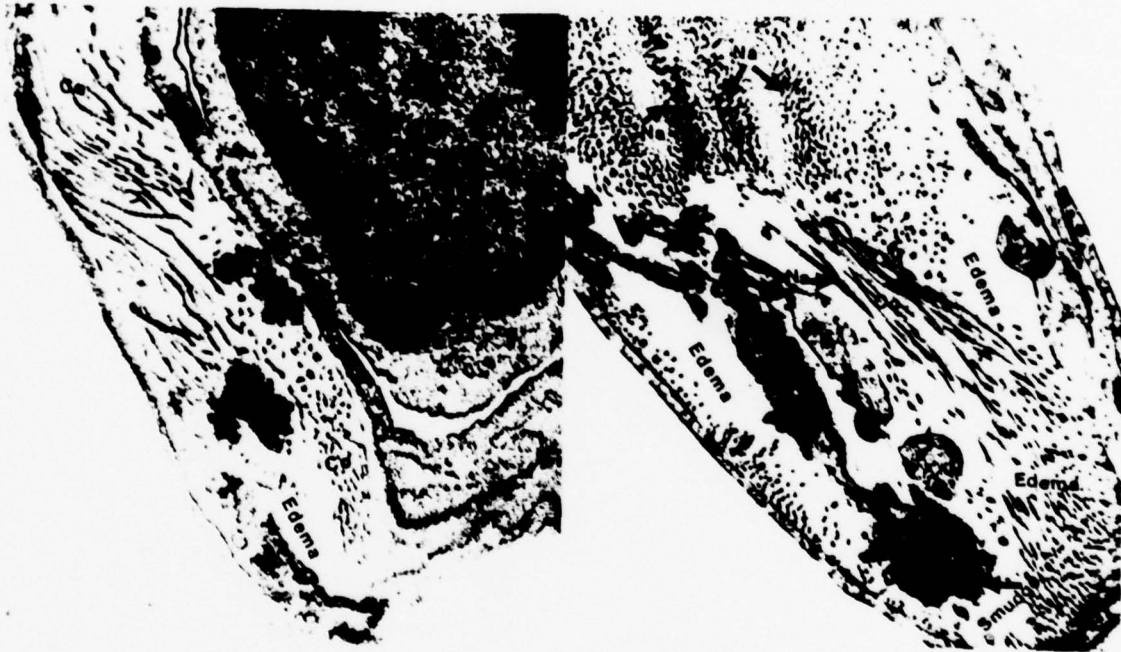
FIGURES 4a and 4b:

MOSS ET AL.
"Changes in Lung Ultrastructure..."



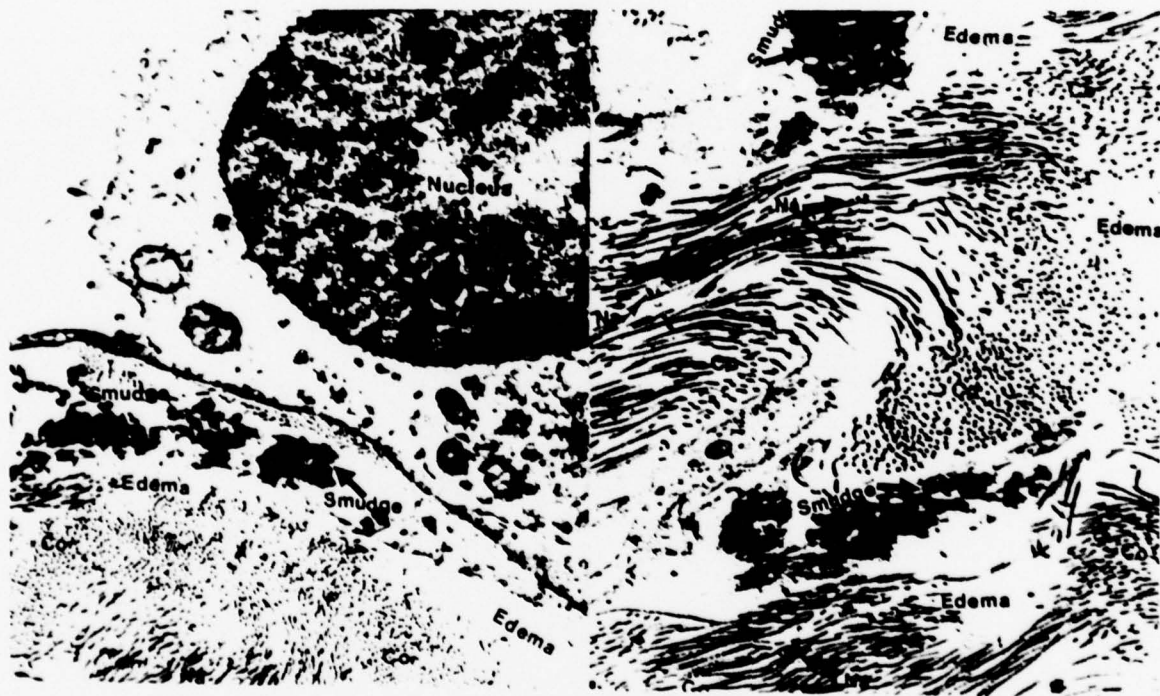
FIGURES 5a and 5b:

MOSS ET AL.
"Changes in Lung Ultrastructure..."



FIGURES 6a and 6b:

MOSS ET AL.
"Changes in Lung Ultrastructure..."



A. Studies Completed This Year

3. Alterations in Pulmonary Surfactant Following Sympathectomy.

Surfactant can be defined as the surface-active material which forms the alveolar layer. The dynamic properties of surfactant which contribute to the normal mechanics of the lung are known as the surfactant system.¹ Without surfactant, the alveolus would behave like an ordinary bubble and would collapse with the high surface tensions generated by the contracting lung.

Today it is well accepted that an impairment of the surfactant system is involved in several types of pulmonary pathology; for example, the infant respiratory distress syndrome (hyaline membrane disease)² and the adult respiratory distress syndrome³, associated with cardiopulmonary bypass, lung transplantation, trauma, pancreatitis, or long-term mechanical ventilation.

Of these, only the infant respiratory distress syndrome has been clearly connected with a surfactant deficiency. In the others, a disturbance in the surfactant system of the lung is recognized, but the exact mechanism by which this disturbance is mediated still remains unknown. Therefore, a systematic investigation of the morphologic, physiologic, and biochemical properties, along with the controlling factors in surfactant function, is in order.

The study of surfactant began in 1929 when von Neergaard¹ recognized the need for a surfact-active substance in the alveolus. He compared fluid-filled lungs with air-filled lungs and found that they contracted differently. This difference he attributed to what he believed were surface forces in the air-filled lung. A surface force is created when two substances of different densities, such as air and alveolus, come into contact with each other. However, von Neergaard was unable to make exact measurements of these surface forces or describe in detail the surface-active substance which he thought was present in the lung.

Little experimentation on the surfactant system was done for the next 25 years, until Pattle⁴ in 1955 described a surface-active material in the lung. He washed animal lungs with saline and found that he obtained a foam which possessed unique characteristics. The bubbles of the foam were highly resistant to antifoaming agents and were of a constant size. When the bubbles were placed in air-free water, they disappeared, thereby demonstrating their permeability to air, but they also left behind in the water a transparent film or "ghost" which Pattle interpreted as being the surface-active material which had been lining the bubble of pulmonary foam.

Following the work of Pattle, Clements⁵ in 1957 made the next significant contribution in the field of surfactant research. He was actually able to measure the surface tension of the lung tissue. Clements demonstrated that the surface-active material found in the lung was capable of reducing the surface tension of the air-lung interface when the alveolus was compressed or contracted.

The research of von Neergaard, Pattle and Clements established the basic principles of surfactant research. They found that there was a powerful surface-active material in the lining of the alveolus which was capable of influencing the surface tension in the alveolus. By altering the surface tension, the surfactant system was able to affect the mechanical properties of the lung.

Having established these principles, research was then directed to the morphology and biochemistry of the surfactant system. Using the electron microscope, lung tissue was studied extensively. At the level of the alveolus two types of epithelial cells were identified. The type I pneumocyte (Type A) is a small cell (0.7 microns in diameter) located directly on the alveolus (Figure 1). The function of this cell remains unknown. The type II (type B)

or granular II pneumocyte, is larger (7-14 microns) and its cytoplasm is filled with large cytosomes or lamellated bodies.⁶ Studies now indicate that this granular II pneumocyte may be an integral part of the surfactant system. Examination of various mammalian fetuses (for example, the rabbit fetus) shows that there is simultaneous development of both the alveolar lining layer and surface activity in the lung several days after the appearance of lamellated bodies within the granular II pneumocyte.⁷ Research in ontogeny also indicates that a surface active pulmonary lining is present in those animals with granular pneumocytes.¹

In addition to defining cellular structure in the lung, the electron microscope was used to demonstrate an alveolar acellular lining layer. This layer is located between the alveolar epithelial cells and the air in the alveolus. The lining layer was described by Gil and Weibel^{8,9} and it stains with osmium tetrozide, appearing as a dark alveolar lining under the electron microscope. Bolande and Klaus¹⁰ were able to demonstrate a similar alveolar lining with their fluorescent studies on the light microscopic level.

Extensive biochemical analyses of the alveolar lining layer demonstrated that the major surface active material was phospholipid, dipalmitoyl lecithin.¹ Buckingham and co-workers,¹¹ using various radioactive isotopes, then began to label fatty acids and determined the synthesis of phospholipid in the lung was relatively rapid. After synthesis in the lung, the phospholipid could then be traced to a location in the alveolar lining layer. It was found that radio-actively labeled precursor of lecithin appeared first within the granular II pneumocyte and then in the alveolar lining layer.

Although thorough morphologic, physiologic, and biochemical experiments have been performed, the control mechanism of the surfactant system has not

yet been discovered. There has been much speculation during the past ten years as to a neural or a neurohumoral control of surfactant production. This work has mainly been directed at the granular II pneumocyte since radioisotope studies indicate this cell as the probable source of pulmonary phospholipid. However, no nerve endings have yet been described in direct contact with these cells. There has been some research directed to the theory that the granular pneumocyte resembles a secretory cell and may be under the control of the vagus nerve.¹² However, the experiments on the vagus have been inconclusive and the effect of the parasympathetic nerves on the granular II pneumocyte and the surfactant system remains uncertain. In contrast, few studies have been done on the relationship between the sympathetic nervous system and surfactant. Some investigations have attempted to link the sympathetic system to pulmonary insufficiency in conditions such as head trauma.^{13, 14} Wyszogrodski et al.¹⁵ suggested the use of catecholamines in the treatment of respiratory insufficiency in premature rabbits. However, none of this research has defined the exact role of the sympathetic system in surfactant production.

The purpose of this study was to determine the relationship between the nervous system and both the granular II pneumocyte and the phospholipid lining of the alveolus. In order to treat the clinical conditions resulting from surfactant dysfunction, it is first necessary to understand what controls the surfactant system of the lung.

MATERIALS AND METHODS

Fifty neonatal Sprague-Dawley rats of either sex were divided equally into two groups. Beginning within six hours after birth, the experimental

group received intraperitoneal injections daily for eight days of 50 g/gm of body weight of 6-hydroxydopamine, an agent that selectively destroys adrenergic nerve endings and produces chemical sympathectomy.¹⁶ The control intervals by cervical dislocation between the ages of three and eight weeks. Lung tissue was obtained immediately for light and electron microscopy. The tissue for electron microscopy was fixed in 4% glutaraldehyde, stained with osmium tetroxide, and processed.

Additional lung tissue was obtained for phospholipid staining by the method of Weller, Bayiss and Abdulla¹⁷, developed in 1965. This technique utilized ammoniacal silver nitrate and hydroxamic acid to localize phospholipids as a final reaction product of dark silver granules.

The glutaraldehyde-fixed tissue was cut between 30-5- microns and treated in Gallya's ammoniacal silver nitrate solution for 24 hours. The sections were then stained in an alcoholic phosphotungstic acid solution and embedded in Araldite. Thin sections were then cut and mounted for the electron microscope. Tissue for light microscopy was routinely fixed in neutral formaldehyde, processed and cut in 7-micron sections. Both hematoxylin-eosin and periodic acid-Schiff stains were used.

Lung tissue was also analyzed for total lipids, phospholipid content and fatty acids. Lipids were extracted by Folch's method (2:1 Chloroform-methanol). Water-soluble nonlipid contents were removed and the amount of lipid was determined gravimetrically. Phospholipid assay was done by determining the amount of phosphorus in a specific volume (Bartlett's method). Fatty acids were determined by gas chromatography.

In evaluating the data from the experiment concerning the lamellated bodies of the sympathectomized animals, a scoring system was developed.

The scores ranged from one to five based on the content and organization of the lamellated bodies. Seven animals were selected from the control group and five from the sympathectomized group. Fifty cells with 400 lamellated bodies were scored from each group. The p value was 0.005.

RESULTS

Control Group:

The low power views from the control lungs showed well-preserved alveoli, normal capillaries containing red blood cells, and intact pulmonary interstitium. The epithelial cells occupied their usual position on the alveolar lumen and their membranes were smooth. The granular II pneumocyte had its characteristic oval eccentric nucleus and its granular cytoplasm, with well-developed endoplasmic reticulum. The most prominent cytoplasmic organelle was the lamellated body which contained concentric layers of densely osmiophilic material. Also, numerous mitochondria and multivesicular bodies could be seen. The multivesicular bodies were small clusters of tubules which were distributed throughout the cytoplasm.

Structures in the cytoplasm of the granular II pneumocytes resembling transition forms between the numerous multivesicular bodies and the lamellated bodies were encountered. The lamellated bodies were located in various positions from the perinuclear area to the periphery of the cytoplasm. Some lamellated bodies were found to be covered with only a thin rim of cytoplasm. Others appeared to be extruded into the alveolar space.

The lamellated bodies were also found free in the alveolus directly adjacent to the granular II pneumocytes. Occasionally a lattice arrangement was noted in the alveoli. This ordered structure is known as tubular myelin

figure and occurs in normal animals.⁹

Control Group Phospholipid Stain. An electron micrograph from a control animal demonstrated the normal distribution of phospholipid.¹⁸ With this staining technique the phospholipid occurred as small dark granules. Within the lamellated bodies, the phospholipid was distributed as fine granules around the periphery of the body. Lining the alveolus, the phospholipid seemed to occur as a rather continuous line of small granules.

Control Group Biochemical Analysis. The phospholipid phosphorus per gram of total lipid in the control lungs showed a mean of 656 micromoles with SD 62.

Sympathectomized Group:

Peripheral and hilar axonal degeneration validated the technique of sympathectomy in the 6-hydroxydopamine treated lungs. The alveoli were well preserved, but contained bizarre, elongated tubular myelin forms the capillaries were intact. However, many of the granular pneumocytes showed extensive cytoplasmic degradation with a dilation of the endoplasmic reticula. The most prominent structural alteration following sympathectomy occurred in the lamellated bodies. In a high power view of a granular pneumocyte the lamellated bodies demonstrated a thinning of the lamellae, a loss of the concentric pattern within the body, and a decrease in the osmiophilic content. Some of the lamellated bodies appeared almost empty.

In an attempt to quantitate the differences observed in the lamellated bodies, the following scoring system was developed. A lamellated body with a score of five had thick concentric rings of dense osmiophilic material. A score of four represented a thinning of the lamellae. A score of three meant a loss of the concentric pattern and with a score of two, only peripheral

coarse osmiophilia remained. A score of one was a lamellated body which was almost empty, with only a thin rim of osmiophilia.

Using this scoring system, the following data was accumulated (Table). The mean score from the control animals was 4.43 and the mean in the sympathectomized group was 3.77 was a p value of < 0.005 . These figures indicate that there was a significant difference in the lamellated bodies of the granular II pneumocyte following sympathectomy.

Sympathectomized Group Phospholipid Stain. After sympathectomy, three major patterns of phospholipid distribution were noted. In one group, the lamellated bodies of the granular II pneumocyte contained large clumps of phospholipid with some of the reaction product located in the center of the body, with a loss of even distribution of dark granules along the alveolus. In the second group the lamellated bodies within the granular pneumocytes were empty, and there was absence of the dark phospholipid granules lining the alveolus. In the third category, the phospholipid lining was clumped and fragmented in appearance.

Sympathectomized Group Lipid Biochemical Analysis. The phospholipid phosphorus per gram of total lipid in the sympathectomized lungs showed a mean of 892 micromoles with SD 31 ($P \leq 0.001$). Analysis of fatty acids demonstrated that unsaturated fatty acids were unaffected by sympathectomy.

DISCUSSION

The control animal lungs in this study were found to have the typical morphology of the rat lung. The only variations concerned the lamellated bodies of the granular II pneumocyte. The lamellated bodies seemed to originate from the multivesicular bodies and numerous transition forms were

seen in the control animals. Once completely formed, the lamellated bodies appeared to migrate to the periphery of the cell. From there, they were extruded into the alveolus. In addition to lamellated bodies, other structures such as the lattice tubular myelin figures were found in the alveoli and could represent another form of the lamellated phospholipid material in the alveolus. From these observations, it can be postulated that the lamellated body arises from the multivesicular body, migrates through the granular pneumocyte and is extruded into the alveolus. Once in the alveolus, the phospholipid of the lamellated body becomes applied to the alveolus as surfactant---the alveolar lining layer.

The hydroxylamine staining technique showed the control lungs to contain a fine, even distribution of phospholipid both in the lamellated bodies and along the surface of the alveolus.

Following sympathectomy there were no transitional forms between the multivesicular and lamellated bodies. Only an occasional lamellated body was found to be extruded into the alveolus and there were no structured tubular myelin figures. Instead, there were bizarre, elongated tubular myelin forms in the alveoli. There were also changes within the lamellated bodies consisting of a decrease in both the osmiophilic content and organization of the bodies.

Using the scoring system previously described, it was possible to quantitate these changes and establish that there is a significant difference in the lamellated bodies after sympathectomy. In addition, there was a change of the phospholipid pattern within the lamellated bodies and fragmentation of the alveolar lining layer.

From these observations, it can be seen that sympathectomy in the rat produces changes in the granular II pneumocyte and the surfactant layer of the lung. The mechanism for these changes is still undetermined but there are several unexplored possibilities. There may be direct sympathetic innervation of the granular pneumocyte but as yet there have been no nerve endings described in contact with this particular cell. There could also be an indirect effect on either the pulmonary vasculature or bronchi. Since both these structures have sympathetic innervation, it might be possible to influence granular pneumocyte function through its blood supply or ventilation. The third mechanism is that of a sympathetically innervated neurosecretory cell on which the granular II pneumocyte is hormonally dependent. A type of neurosecretory cell has already been described in the lungs of several mammals, including man.^{19, 20} These cells are known as neuroepithelial bodies and their function in the lung has not yet been determined.

The most likely of these three mechanisms is that of a neurosecretory type since the change noted in the granular pneumocyte is a selective one. The sympathectomy affects the pattern of phospholipid distribution in the lamellated bodies and along the alveolus. This suggests a disturbance in fatty acid metabolism and transport within the granular pneumocyte. Such a defect in the surfactant system would be difficult to create by simple denervation. These observations imply that normal surfactant production may be dependent on a neural or neurohumoral mechanism.

This study has both research and clinical implications. If it is possible to produce an alteration in the surfactant system by performing a sympathectomy it may be practical to use a denervated lung as a model

to study surfactant production. In many clinical syndromes such as the infant and adult respiratory distress syndromes, a defect in the surfactant system has been hypothesized. However, until the mechanism of surfactant synthesis and maintenance is understood, it will continue to be impossible to treat these forms of respiratory insufficiency. By defining exactly where the defect occurs in a deranged surfactant system such as the one created in this study, it may be possible to correct this defect by administering a sympathetic agent such as a biogenic amine.

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TABLE --- LAMELLATED BODY DATA

	No. of Animals	No. of Cells	No. of Lamellated Bodies	Mean Score \pm S.E.
Control	7	50	400	4.43 \pm 0.03
6-HODA	5	50	400	3.77 \pm 0.16

P < 0.005

B. On-Going Studies

1. Introduction

INTRODUCTION

Controversy still exists over the proper choice of fluids for resuscitation of patients in shock following trauma or sepsis, with particular regard to the incidence of posttraumatic pulmonary insufficiency. Previous work from our laboratory has demonstrated distinct morphologic changes in the pulmonary ultrastructure following hemorrhagic shock.^{1,2} We have compared the changes following resuscitation with crystalloid and colloid, and our interest has continued to focus on the differences between the two solutions.

Staub has made significant contributions to the understanding of the fluid and protein composition of lung fluids.³ We can now classify the two major types of pulmonary edema as that due to increased driving pressure (high pressure edema), and that due to altered integrity of the capillary membrane (permeability edema).⁴ An important development has been the recognition that in the normal state, the microvasculature of the lung is not impermeable to proteins. This observation has led to a number of physiologic studies that have examined the effects of hemorrhagic and septic shock on pulmonary capillary membrane permeability.^{5,6,7} Although not all in agreement, the general impression has been that protein permeability is increased following sepsis, but not hemorrhage.

Morphologic studies have failed to satisfactorily demonstrate the transfer of plasma proteins across the capillary membrane.^{8,9,10,11} Tracers such as ferritin, catalase, hemoglobin, and horseradish peroxidase have been used, and although the endothelial cell has been permeable to many of these macromolecules, it has been shown that some of the substances can alter membrane permeability by themselves.^{8,11,12}

Bignon has recently demonstrated autologous albumin in the alveolar capillary membrane and the alveolar lining material using the principles of immunoelectron microscopy.^{13,14} The mode of transcapillary transport, however, has still not been conclusively demonstrated.

We have become interested in the techniques of fluorescent microscopy and autoradiography as methods to study the mechanism of protein transport.^{15,16} An Evans blue-albumin solution is able to identify the general area of leakage from the microvascular bed, but does not allow any quantitative interpretations. We are also investigating the properties of fluorescein labeled dextrans (FITC-Dextrans) as tracers in morphologic studies of vascular permeability,¹⁷ and plan to use these as markers for use with the electron microscope. Autoradiography with ¹²⁵I-albumin permits examination of the pinocytotic vesicles and intercellular junctions, and should lead to a better understanding of the transvascular escape of protein. After identifying the normal mechanism, application of the techniques in the hemorrhagic and septic models should demonstrate any altered permeability and help clarify the proper methods of resuscitation in the clinical situation.

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B. On-Going Studies

2. Radioautography of ¹²⁵-Tagged Albumin in the Lung.

RADIOAUTOGRAPHY

OBJECT: To define the pulmonary vascular and extravascular distribution of albumin in the control state and following shock.

METHODS

In order to inject an appropriate amount of isotope for quantifiable radioautography in a small enough fluid volume, a specially prepared, high specific activity 0.25 m Ci/mg 125 -labeled albumin (New England Nuclear NEX 076, I-125-BSA bovine serum albumin), with an albumin concentration of 20 mg/ml was used. This is five times the usual specific activity.

A. Fixation

Three anesthetized female mice weighing approximately 25 g. were injected with 0.15 - 0.25 ml of the tracer solution through the tail vein. The tracer solution contained less than 2% free iodide when tested by trichloroacetic acid precipitation of the protein.

The lungs of three mice were fixed at thirty, sixty, and ninety minutes after the tracer injection. A few minutes before fixation, each mouse was anesthetized with pentobarbital (100 mg/kg) intraperitoneally. The chest was quickly opened, and a 4% glutaraldehyde solution in a cacodylate buffer was instilled into the pleural cavities until a minimal fixation was obtained. The lungs were then excised, and samples taken from various areas. The specimens were sliced into blocks about 1mm^3 , and immersed in the glutaraldehyde at 4° C. for four hours. Postfixation in 2% osmium tetroxide was then performed for two hours, followed by dehydration in graded acetone solutions and propylene oxide. The blocks were embedded overnight in Araldite.

B. Sectioning

The tissue blocks were cut into 1 μ m thick sections using glass knives in a microtome (LKB-Huxley or a Sorvall JB4). The sections were stained with toluidine blue, and the appropriate blocks selected for further examination. Ultrathin sections were cut, and after being mounted on copper grids, were dip-coated with a nuclear emulsion (Ilford K2) with a 1:1 emulsion water ratio. After appropriate exposure (21-25 days), the emulsion was developed and the sections examined under an RCA EMU 312-4 microscope.

The following figures are the results of these preliminary experiments, and represent a validation of this technique as performed in our laboratory:

Figure 1: Cluster of ¹²⁵I-labeled material in the alveolar lumen.

Figure 2: The capillary lumen shows decreased quantities of material. Most of the tagged material is in the interstitium. We have no lymphatic lumen visualized in this micrograph.

Figure 3: Shows the tagged material in a Type II granular pneumocyte.

C. Shock

The next phase of the project is designed to further characterize the time course of the passage of the albumin from the vascular to the extravascular space, and to delineate the mode of transport. Different time intervals following injection, and various methods of fixation will be used. When this has been completed, the model will be used to study the effects of both hemorrhagic and septic shock on the handling of albumin by the lung.

I. Hemorrhage

Hemorrhagic shock will be induced by judicious bleeding from a tail vein. The level of shock will be monitored by a small pneumatic cuff on the tail connected to an electromagnetic flowmeter, a technique shown to be as accurate in the mouse as intraarterial monitoring.

II. Sepsis

The septic model involves ligation and puncture of the cecum, with subsequent peritonitis. Further experience will help determine the proper timing of the fixation process after the septic event.

LEGENDS

- FIG. 1 A tracing of the densitometric scan of a gel electrophoresis strip spotted with baboon serum albumin (upper panel) and human serum albumin (lower panel).
- FIG. 2a Low power view of normal baboon lung showing localization of sodium pyroantimony. The capillary contains a number of red blood cells and a portion of a platelet (P). The interstitium shows regular banding of collagen (CO) with discrete crystalline localization of sodium pyroantimony (NA). A portion of a granular pneumocyte with dense lamillate bodies within the cytoplasm can be seen in the upper right of the micrograph. x 3,200.
- FIG. 2b High power view of normal lung. The precise localization of the sodiumpyroantimony (NA) in and around the collagen (CO) fibers within the interstitium (IS) can be seen. Note the distribution in two planes: in transverse section sodium can be found along the outer rims of the individual collagen fibers; in longitudinal sections the sodium pyroantimony deposits have aggregated in geometric precision along the silhouette of the fibers. x 29,500
- FIG. 3a Lower power view of a baboon lung in hemorrhagic shock, showing distribution of sodium pyroantimony. Note the characteristic interstitial edema with smudging of sodium pyroantimony along the collagen fibers (CO). It appears that collagen (CO) has a spongelike property for the sodium-rich edema fluid. x 12,000

- FIG. 3b. Higher power view of a similar field as in Fig. 3a. The smudging and disruption of the interstitial space are magnified. x 20,000
- FIG. 4a. Baboon lung resuscitated with Ringer's lactate. The interstitial space is relatively normal. Collagen fibers do not appear disrupted. Small areas of edema-rich smudges are still seen. x 20,000
- FIG. 4b. Another area from the same animal (Fig. 4a) showing the collagen. Note the distribution of sodium appears similar to that in the control (Figs. 2a and 2b). Although minor amounts of edema and smudge still persist, the general appearance of the pulmonary interstitium is similar to the control baboon lungs. x 27,000
- FIG. 5a. Baboon lung after resuscitation with human serum albumin. Note the marked interstitial edema, dispersal of collagen fibers, (CO) and patchy areas of smudging of sodium pyroantimony. x 28,500
- FIG. 5b. Another area of the pulmonary interstitium from the same lung as in Fig. 5a. The pattern of architectural disintegration of the interstitium is similar to Fig. 3b. The distribution of sodium-rich edema smudges are more spectacular. Furthermore, disorganization of collagen periodicity is apparent x 28,500.

FIG. 6a. Lung tissue after resuscitation with baboon albumin. The interstitial space shows edema, disintegration of periodicity of collagen, and edema-rich smudges. A granular pneumocyte can be seen on the upper right of the micrograph. x 19,500

FIG. 6b. A higher view of the interstitium from the same baboon (Fig. 6a). The interstitial edema, collagen disruption, and nongeometric localization of sodium can be easily appreciated. x 28,500

TABLE 1. Infusion Volumes (cc/kg)

<u>Group</u>	<u>Mean</u>	<u>S.D.</u>	<u>N</u>
Ringer lactate	148	17	3
Human serum albumin	63	11	3
Baboon Serum albumin	65	14	3

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FIGURE 1:

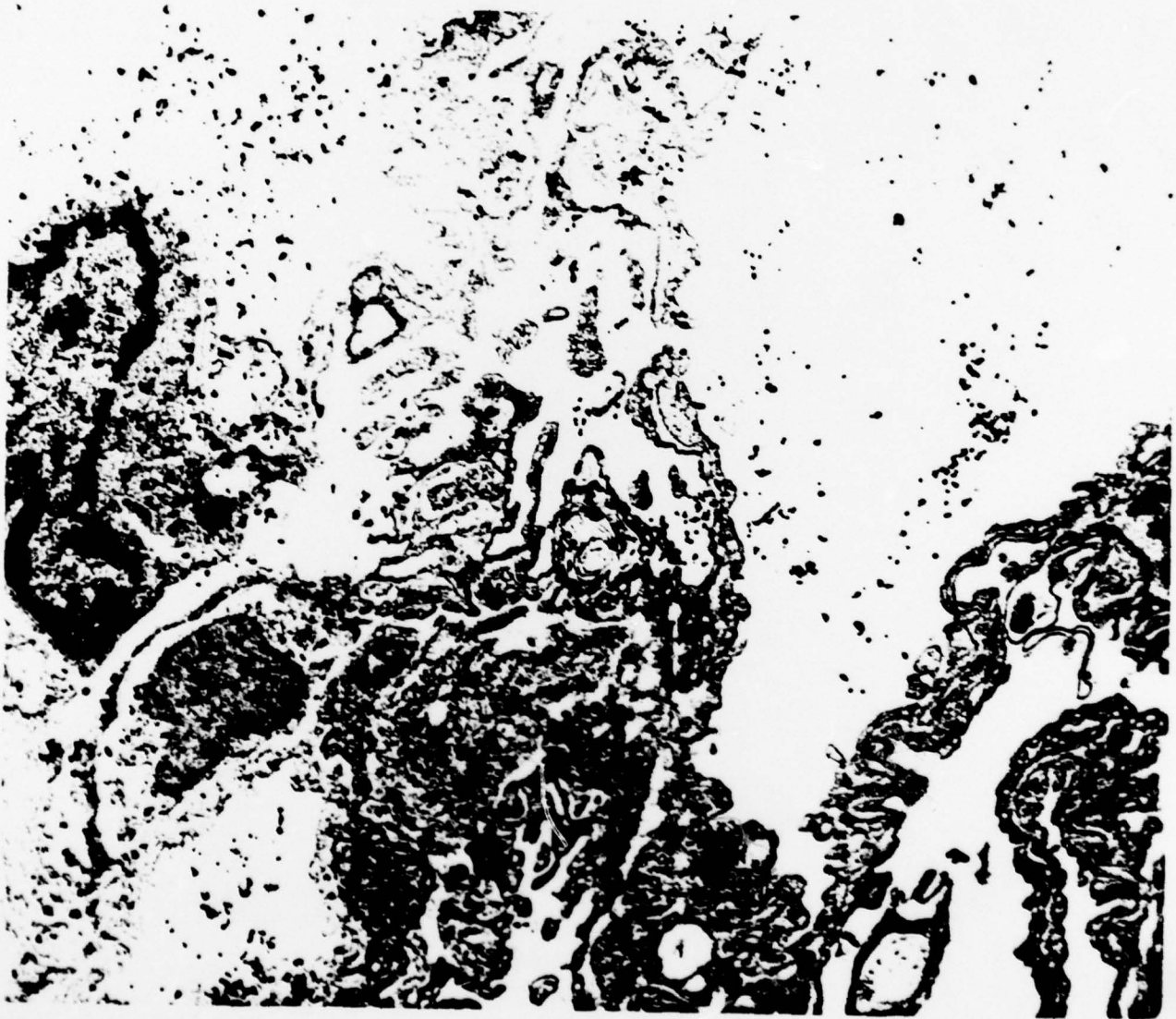
MOSS ET AL.
"Radioautography of $^{125}\text{-I}$ Albumin..."

FIGURE 2:



FIGURE 3:



B. On-Going Studies

3. Fluorescent Micrography in the Lung.

OBJECTIVE: To obtain more direct information regarding the permeability of the lung microvasculature to plasma proteins, using fluorescent labeled tracer substances.

METHODS

A. Evans Blue Albumin:

I. Fixation

0.6g of Evans blue (J.T. Baker Co.) and 4g of bovine serum albumin (B.S.A.) (Sigma) are dissolved in 100 ml 0.9% Na Cl. 0.1 ml of the labeled albumin is injected into the tail vein of unanesthetized mice (body weight 20-25g, Charles River, SPF). At selected intervals between ten minutes and five hours after injection, each animal is anesthetized with pentobarbital (100 mg/kg) intraperitoneally. A tracheostomy is performed, the chest is opened, and the lungs are inflated to a constant transpulmonary pressure of 10 cm H₂O. The inflated lungs are frozen in the open thorax using liquid propane cooled to -180° C. in liquid nitrogen. The excised lungs are freeze-dried at -35° C. for 24 hours, and suitable tissue blocks are vacuum infiltrated and embedded in paraffin.

II. Sectioning and Microscopy

Sections of 10 μ m thickness are cut on a sliding microtome, and after removal of the paraffin by heat, are mounted in nonfluorescing oil. The sections will be examined using a fluorescence microscope with a mercury lamp, using dark field illumination, a BG 12 excitation filter, and a barrier filter with cut-off at 530 mn.

Preliminary evaluation will establish suitable criteria for recording the time course and distribution of the fluorescence. Sections will then be presented in random, unidentified fashion to two different investigators,

and the appearance and distribution of the fluorescence will be graded on a 0 - 3+ scale. The results will help determine the areas of the lung architecture to be examined more carefully using techniques suitable for electron microscopy.

III. Shock

When the technique has been satisfactorily performed in the control animals, the model will be used in both hemorrhagic and septic shock.

A. Hemorrhage

Hemorrhagic shock will be induced by judicious bleeding from a tail vein. The level of shock will be monitored by a small pneumatic cuff on the tail connected to an electromagnetic flowmeter, a technique shown to be as accurate in the mouse as intraarterial monitoring.

B. Sepsis

The septic model involves ligation and puncture of the cecum, with subsequent peritonitis. Further experience will help determine the proper timing of the fixation process after the septic event.

B. Fluorescein Labeled Dextrans

I. Technique

Fluorescein isothiocyanate labeled dextrans (FITC-Dextran) have previously been used for direct observations of vascular permeability in living animals, and there is recent evidence to suggest their value as electron microscopical tracers. The advantage of these molecules as tracers is their wide range of molecular sizes. Efforts are currently underway to obtain these labeled compounds for use as tracers in the baboon.

II. Shock

Following the initial experience with two control animals in the normal state, the influence of both hemorrhagic and septic shock will be examined.

A. Hemorrhage

Hemorrhagic shock will be induced in four baboons by a standard model of bleeding from the femoral artery into a sterile plastic bag to lower the mean arterial pressure to 60 mm Hg for one hour, and to 40 mm Hg for the second hour. The animals will then be sacrificed and the lungs examined under the electron microscope after standard tissue processing.

B. Sepsis

Sepsis will be induced in four baboons by infusion of live *E. Coli* organisms, Dunwald strain B15, (obtained from Dr. L.B. Hinshaw, V.A. Hospital, Oklahoma City). Lyophilized samples are reconstituted, cultured, and recovered for injection. A standard suspension is obtained using optical density read at 550 nm, and subsequent colony counts determine the bacterial concentration. After early dissatisfaction with bolus injection,

efforts are now being focused on a four hour infusion of the bacterial suspension. Hemodynamic and respiratory parameters are being monitored in an attempt to establish a dose-response curve and thus a reproducible model. Sepsis will then be induced for the four hours, followed by sacrifice of the animals and examination of the lungs under the electron microscope.

C. Budget

MICHAEL REESE HOSPITAL AND MEDICAL CENTER

Treatment of Hemorrhagic Shock
Saline vs. ColloidPrincipal Investigator - Gerald S. Moss, M.D.
Contract No. N00014-75-C-1086
Task No. NR-105-539Budget: 12/1/78 - 11/30/79

1. Salaries

a. Gerald S. Moss, M.D. (Principal Investigator, 20% time effort)	\$ -0-
b. Biochemist (100% time and effort)	\$ 10,260.00
c. Fringe Benefits (FICA 6.13%)	\$ 629.00
d. Indirect Cost (75% of salary and wages)	\$ 7,695.00
	<u>\$ 18,584.00</u>

2. Supplies and Reagents

a. Electronmicroscopy Stains	\$ 800.00
b. Photographic Paper and Emulsions	\$ 285.00
c. I.V. Fluids and Tubing	\$ 350.00
d. 125 I-Albumin @ \$250.00 for 5mCi x 9 doses	\$ 2,250.00
	<u>\$ 3,685.00</u>

3. Experimental Animals

a. 10 Baboons @ \$550.00	\$ 5,500.00
b. Freight and handling on Baboons @ \$88.00	\$ 880.00
c. Per diem \$1.80 for each animal for 30 days	\$ 540.00
d. 100 Mice at \$0.71 each	\$ 71.00
e. Freight on mice @ \$25.00 each for 3 shipments	\$ 75.00
f. Per diem \$.0475 for each animal 10 days	\$ 47.50
	<u>\$ 7,113.50</u>

TOTAL \$ 29,382.50

Budget Justification:

1. The requested salary provides a 8.0% increase over last year's contract salary level. We feel that the salary level is competitive for the Chicago, Illinois job market.
2. Supplies and Reagents classification reflects the total supplies needed for the current research level.
3. Experimental Animals: We continue to believe that baboons and mice are the appropriate animals for these studies for both physiological and economic reasons.