

# Role of Endothelial Cell Cytoskeleton in Control of Endothelial Permeability

D. Michael Shasby, Sandra S. Shasby, James M. Sullivan, and Michael J. Peach

*From the Departments of Medicine and Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia*

**SUMMARY.** Increased permeability of the pulmonary microvasculature is felt to cause acute noncardiogenic lung edema, and histological studies of edematous lungs show gaps between apparently healthy endothelial cells. To determine whether alterations in endothelial cell cytoskeletons would alter endothelial permeability, we exposed monolayers of pulmonary artery endothelial cells grown on micropore filters to cytochalasin B or D. Cytochalasin exposed monolayers demonstrated a 2- to 3-fold increase in endothelial permeability that was readily reversible by washing the monolayers free of cytochalasins. Parallel phase contrast and fluorescence microscopy demonstrated retraction of cell cytoplasm and disruption of bundles of microfilaments in cytochalasin exposed cells. These changes also were readily reversed after washing the cells free of cytochalasins. To test the relevance of these findings to an in situ microvasculature, we added cytochalasin B to the perfusate of isolated rabbit lungs and observed that cytochalasin B caused a high permeability lung edema. These studies suggest that endothelial cell cytoskeletons may be important determinants of endothelial permeability. (*Circ Res* 51: 657-661, 1982)

ACUTE noncardiogenic lung edema is believed to result from increased permeability of the pulmonary microvasculature (Staub, 1978). Although many animal and ex vivo models of noncardiogenic lung edema have been studied, the mechanism of increased endothelial permeability remains unknown. Several studies of the systemic circulation have suggested that increased endothelial permeability induced by histamine is associated with endothelial cells pulling apart and gaps forming between cells (Majno and Palade, 1961; Majno et al., 1967). Hurley (1982) has provided evidence that similar gaps in the continuity of the endothelium may be important in high permeability lung edema.

The permeability of epithelial surfaces has been studied more thoroughly than that of endothelial surfaces, and several studies have suggested a role of microfilaments of the cytoskeleton in regulating epithelial permeability (Meza et al., 1980; Bentzel et al., 1980; Duffey et al., 1981). We hypothesized that the cytoskeleton might also contribute to regulation of endothelial permeability. To test this, we added the microfilament disrupting agents, cytochalasin B and D, to monolayers of endothelial cells grown on micropore filters, and we observed that cytochalasins caused a readily reversible increase in endothelial permeability. The increased permeability was associated with disruption of the microfilament apparatus and formation of gaps between adjacent endothelial cells, whereas the recovery was associated initially with the localization of actin to the junction of adjacent cells and later reorganization into the normal pattern of microfilaments for endothelial cells. To support the relevance of these in vitro findings to an intact microvasculature, we added cytochalasin B to

the perfusate of isolated lungs, and we found that cytochalasin B increased the permeability of the lungs to fluid and protein.

## Methods

### Experiments with Endothelial Monolayers

#### A. Preparation of Cytochalasin B and D

Cytochalasin B and D (Sigma Chemical Co.) were dissolved at 1 mg/ml in dimethylsulfoxide and frozen at  $-70^{\circ}\text{C}$ . until use. Individual aliquots then were thawed and diluted in medium 199 with 2% bovine serum albumin (BSA) for addition to the monolayers.

#### B. Preparation of Filters

Polycarbonate filters (13 mm in diameter, 5- $\mu\text{m}$  pore size, Nucleopore Corp.) were gelatin-impregnated by the technique described by Postlethwaite (1976) and modified by Taylor et al. (1981).

#### C. Cell Culture

Pulmonary artery endothelial cells were obtained from porcine pulmonary artery using standard techniques with minor modifications (Ryan, 1978). Briefly, porcine pulmonary arteries were sterilely resected, rinsed with phosphate-buffered saline, and the lumen exposed to 0.1% collagenase (Worthington Biochemical) for 25 minutes. The lumen then was swabbed with a sterile cotton-tipped applicator and the adherent endothelial cells released into medium 199 enriched with 20% fetal calf serum. Cells then were cultured in medium 199 with 10% fetal calf serum and subculturing was done, using standard techniques. Cultures were determined to be pure by morphological criteria and by demonstrating positive immunofluorescence for factor VIII. Cells from passes 5-9 were plated onto the treated filters at a density of  $6 \times 10^5$  cells/cm<sup>2</sup>. The filters then were incubated by standard techniques and all studies were done 6 days after plating.

For studies using phase contrast and fluorescence microscopy, monolayers grown on traditional tissue culture plates (HA-20, Corning Glass) were used since the polycarbonate filter did not allow adequate transmission of polarized light.

#### D. Microscopy and Photomicroscopy

All microscopy was done with a Zeiss Axiomat. Visualization of F-actin was accomplished with the use of NBD phalloidin (Barak et al., 1980). To observe and photograph the F-actin patterns, we excited the stained cells at 450–490 nm and viewed them at wave lengths of 520 nm or more. The labeled cells were observed and photographed with the aid of a Javelin three-stage, first-generation image intensifier (Javelin, Electronics, Inc.) and a 35-mm camera.

#### E. Experimental Protocol

Treated filters with adherent monolayers were mounted in modified chemotaxis chambers after the lower well of the chamber was filled with medium 199. The upper chamber then was filled with medium 199 with 2% BSA with or without 5  $\mu\text{g}/\text{ml}$  cytochalasin B or 1  $\mu\text{g}/\text{ml}$  cytochalasin D. After 1 hour, the medium from the upper chamber was aspirated and the lower chamber sampled through a side port. To determine the reversibility of the effects of the cytochalasins, we exposed some monolayers to 5  $\mu\text{g}/\text{ml}$  cytochalasin B or 1  $\mu\text{g}/\text{ml}$  cytochalasin D for 1 hour. Then they were washed three times and reincubated in medium 199 with 1% fetal calf serum for 4 hours. At the end of the 4 hours, these filters were similarly mounted in the chambers and the movement of albumin across the monolayer in one hour was measured.

The albumin concentrations in the upper and lower chambers were determined by measuring the change in absorbance at 630 nm following reaction of the albumin in the sample with bromocresol green (Sigma Technical Bulletin No. 630, revised October, 1976).

The gelatin-impregnated filters were not infinitely permeable to albumin. After 1 hour, the ratio of the albumin concentration in the lower chamber to that in the upper chamber for gelatin impregnated filters without a monolayer was  $0.254 \pm 0.002$  ( $\pm \text{SEM}$ ,  $n = 4$ ). The permeability of monolayers in the experiments is expressed as the ratio of the albumin concentration in the lower chamber to that in the upper chamber standardized for the transit across a gelatin-treated filter without cells and is called the fractional transit. Hence, fractional transit =

$$\frac{\text{albumin concentration in lower chamber}}{\text{albumin concentration in upper chamber}} \div 0.254.$$

Experiments were done with separate groups of 12 filters, each group plated at the same time from the same passage of endothelial cells. Each group served as its own control and the standard error for the controls was  $\pm 10.1\%$  of the mean ( $n = 11$ ).

For experiments involving phase contrast and fluorescence microscopy, monolayers in tissue culture plates were exposed to cytochalasin B and photographed live or fixed with NBD-phalloidin at specified time intervals.

#### Experiments with the Isolated Perfused Lung

##### A. Experimental Protocol

Isolated perfused rabbit lungs were prepared as previously described (Shasby et al., 1982). After 15 minutes of

stabilization, 5  $\mu\text{g}/\text{ml}$  cytochalasin B were added to the lung perfusate through a venous port, and the perfusion continued for another 90 minutes. Lung edema was assessed by the increase in lung weight during the course of the experiment and by the ratio of the concentration of albumin in lung lavage to that in the perfusate.

##### B. Statistical Analysis

When two experimental groups were compared, we used an unpaired *t*-test, and when more than two groups were compared, we used a one-way analysis of variance and tested intergroup differences using the Student-Newman-Keuls test. Data are presented as the mean  $\pm 1 \text{ SE}$ , and differences are significant when  $P < 0.05$ .

## Results

#### Experiments with Endothelial Monolayers

Addition of 5  $\mu\text{g}/\text{ml}$  cytochalasin B to the culture media acutely and reversibly increased the fractional transit of albumin across the endothelial cell monolayers. The fractional transit of albumin across control monolayers of pulmonary artery endothelial cells was  $0.072 \pm 0.015$  ( $n = 7$ ), whereas that across similar monolayers exposed to 5  $\mu\text{g}/\text{ml}$  cytochalasin B was  $0.225 \pm 0.041$  ( $n = 7$ ). The fractional transit of albumin across pulmonary artery endothelial monolayers exposed to 5  $\mu\text{g}/\text{ml}$  cytochalasin B for 1 hour, washed and reincubated in medium 199 for 4 hours, was  $0.109 \pm 0.022$  ( $n = 7$ ) (Fig. 1), not different from control. Addition of 1  $\mu\text{g}/\text{ml}$  cytochalasin D had a similar effect. The fractional transit of albumin across control monolayers was  $0.113 \pm 0.012$  ( $n = 3$ ), whereas that across similar monolayers exposed to 1  $\mu\text{g}/\text{ml}$  cytochalasin D was  $0.245 \pm 0.023$  ( $n = 3$ ). The fractional transit of albumin across pulmonary artery endothelial monolayers exposed to 1  $\mu\text{g}/\text{ml}$  cytochalasin D for 1 hour, washed, and reincubated in medium 199 for 4 hours, was  $0.135 \pm 0.010$  ( $n = 3$ ), not different from control (Fig. 1).

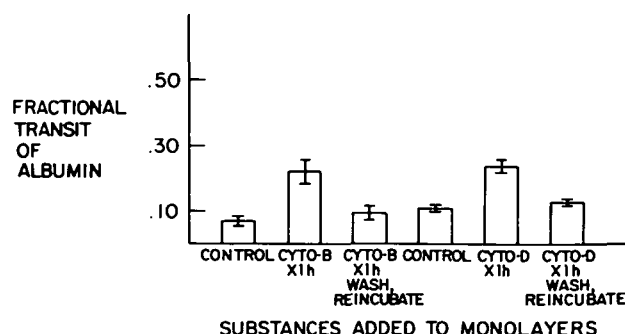
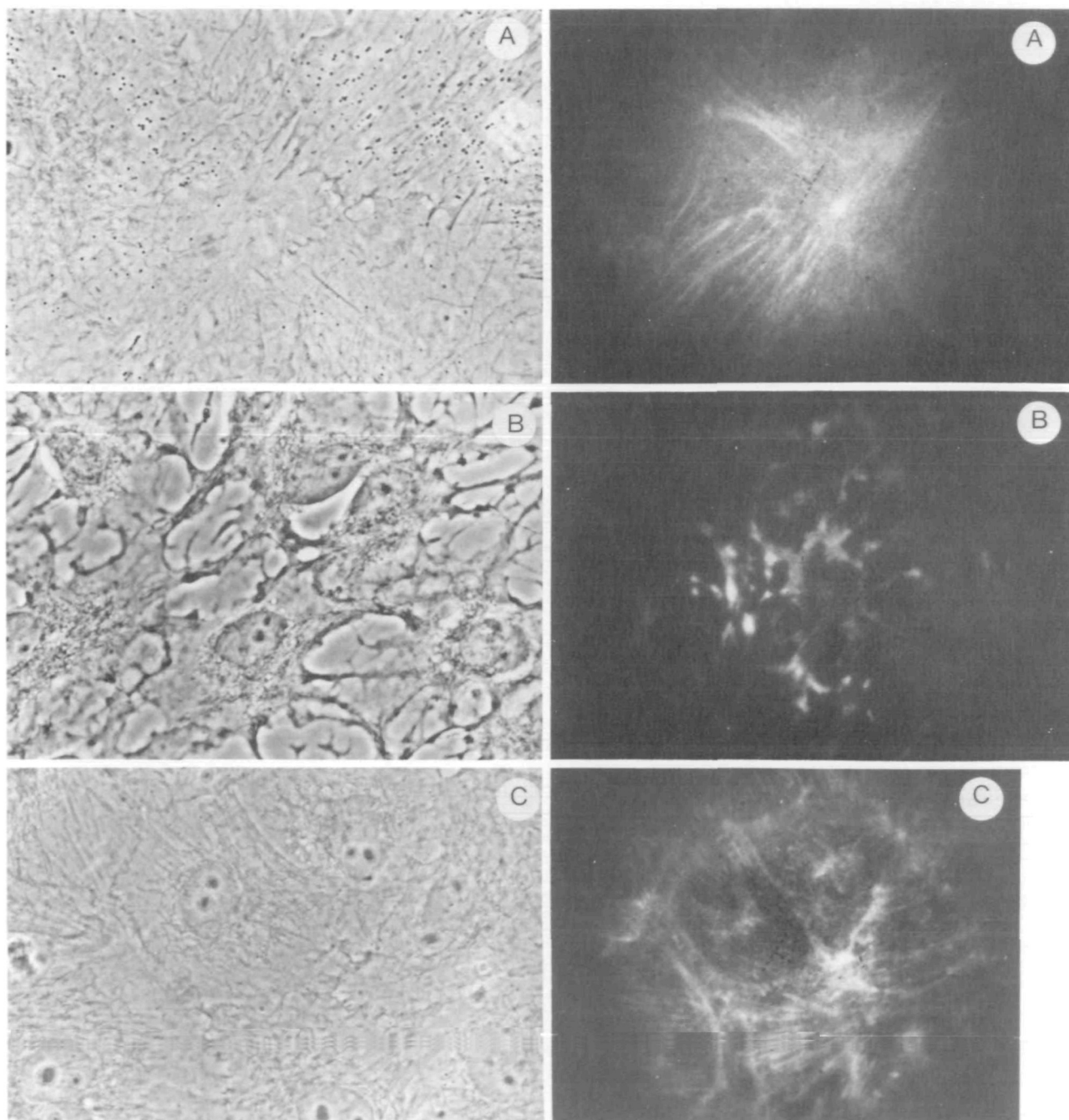


FIGURE 1. Fractional transit of albumin across pulmonary artery endothelial cell monolayers. The fractional transit of albumin across monolayers exposed to 5  $\mu\text{g}/\text{ml}$  cytochalasin B or to 1  $\mu\text{g}/\text{ml}$  cytochalasin D for 1 hour was increased above control. The fractional transit of albumin for monolayers exposed to the same concentrations of cytochalasins, washed, and reincubated for 4 hours was not different from control. For cytochalasin B,  $n = 7$  separate monolayers for controls and each exposure group. For cytochalasin D,  $n = 3$  separate monolayers for controls and each exposure group.

Phase contrast and fluorescence microscopy of control and cytochalasin-exposed monolayers demonstrated striking differences that paralleled the changes in permeability. Whereas control monolayers had the expected cobblestone appearance, with adjacent cells closely adherent to one another, monolayers exposed to cytochalasins showed gaps between adjacent cells. Fluorescence microscopy of control monolayers showed a delicate network of actin filaments surrounding the nuclei and extending to the cell margin.

In contrast, the actin of cytochalasin-treated cells appeared as fluorescent aggregates without any clear orientation. Monolayers exposed to cytochalasins for 1 hour, washed, and reincubated in medium 199 had a normal cobblestone appearance within one hour. Two hours after washing, these same monolayers showed deposition of actin along the cell periphery at the junction of adjacent cells and, after 4 hours of incubation, the fluorescent pattern of actin deposition had returned to normal (Fig. 2).



**FIGURE 2.** Phase contrast and fluorescence microscopy of endothelial cell monolayers. Panel A: control monolayer showing close apposition of adjacent endothelial cells and linearly oriented bundles of microfilaments (1200 $\times$ ). Panel B: Monolayer exposed to cytochalasin D For 1 hour demonstrating retraction of cell cytoplasm and disruption of microfilaments (1200 $\times$ ). Panel C: Monolayer exposed to cytochalasin B for 1 hour, washed, and reincubated for 4 hours, showing normal phase contrast appearance and restoration of linear orientation to microfilament bundles (1200 $\times$ ).

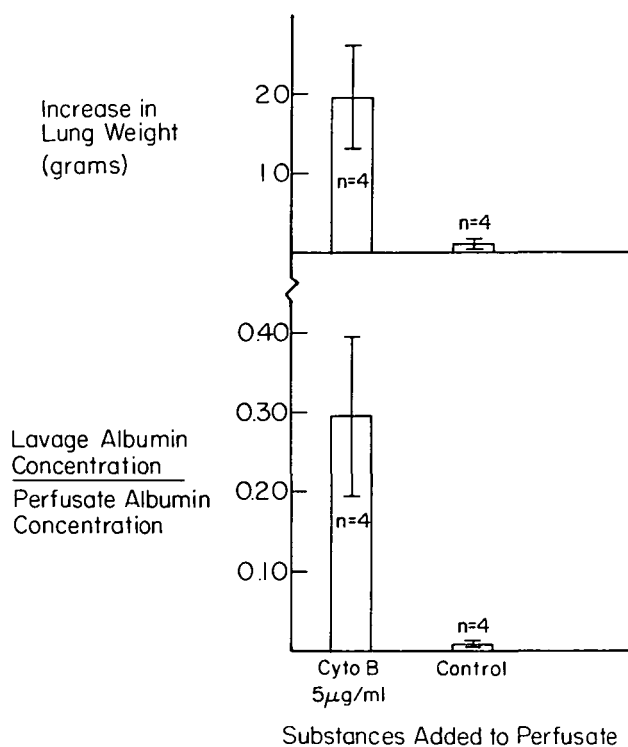


FIGURE 3. Isolated perfused lungs exposed to 5 µg/ml cytochalasin B showed increased lung weight and increased concentration of albumin in lung lavage compared to control lungs. *n* = number of individual lungs.

### Isolated Perfused Lung Experiments

To confirm the relevance of the above *in vitro* findings to an intact vasculature, we added 5 µg/ml cytochalasin B to the perfusates of isolated rabbit lungs. Lungs exposed to 5 µg/ml cytochalasin B increased  $19.5 \pm 6.6$  g in weight and had lavage albumin:perfusate albumin ratios of  $0.296 \pm 0.100$ , whereas lungs perfused for a similar time without cytochalasin B increased  $1.2 \pm 0.5$  g in weight and had lavage albumin:perfusate albumin ratios of  $0.009 \pm 0.002$  (Fig. 3).

### Discussion

Our data demonstrate that the microfilament-disrupting agents, cytochalasin B and D, cause an acute and rapidly reversible increase in the permeability of cultured endothelial monolayers to albumin. Similarly, addition of cytochalasin B to the perfusate of isolated lungs caused an acute increase in lung permeability to fluid and protein. Hence, primary alterations in endothelial cell cytoskeletons alter endothelial permeability and may be involved in regulating vascular permeability.

Ultrastructural studies utilizing histamine, bradykinin, serotonin, and other permeability-inducing agents have demonstrated loss of integrity of junctions between adjacent endothelial cells (Majno and Palade, 1961; Majno et al., 1967; Fantone, 1980). Whereas none of these studies directly addressed the role of endothelial cell cytoskeletons in determining endothelial permeability, a report by Heltianu et al.

(1982) noted the close proximity of venular histamine receptors and microfilaments of the venular endothelium. Savion et al. (1982) recently described the increased sophistication of endothelial cell cytoskeletons in confluent as compared to sparse endothelial cultures and have suggested that the development of bundles of microfilaments may "reflect their role in maintaining the flattened and closely apposed morphology . . . allowing the vascular endothelium to resist the high blood pressure and shear forces" to which it is exposed. Our own results support this contention that the endothelial cell cytoskeleton is important in maintaining the closely apposed morphology of endothelium and that this morphology is important to endothelial function. However, since our studies were done with pulmonary artery endothelium, it will be important to repeat them with endothelium from the microcirculation to confirm a role of endothelial cytoskeletons in alterations of microvascular permeability.

Studies of epithelial permeability have demonstrated that alterations in microfilaments are associated with changes in epithelial permeability. Bentzel et al. (1980) used cytokinins and phalloidin to alter microfilament structure and thereby altered permeability of gall bladder mucosa. In a similar study from the same group, Duffey et al. (1980) showed that cyclic AMP levels altered epithelial permeability and simultaneously caused changes in the microfilaments. Meza et al. (1980) used a cultured epithelium and demonstrated that microfilament disruption decreased epithelial electrical resistance and, by inference, increased epithelial ion permeability. Hence, there are precedents for participation of microfilaments in the regulation of permeability barriers, although not expressly for macromolecules.

In our studies with the isolated perfused lungs, the cytochalasin B may have affected epithelium of lung as well as endothelium. It is not possible for us to quantify the relative contributions of these two surfaces, both of which are involved in regulation of lung fluid and solute transport. However, microfilament alterations may be important in altering the barrier function of both these surfaces.

Acute high permeability lung edema is characterized by an increase of endothelial permeability to serum proteins (Staub, 1978), and most animal models of acute lung edema are characterized by rapid recovery of endothelial integrity (Heflin and Brigham, 1981; Flick et al., 1981; Till, 1982). In addition, acute high permeability lung edema can occur in the absence of severe endothelial cell injury (Hurley, 1982). Our data demonstrate that primary alterations of the endothelial cell cytoskeleton produce a rapidly reversible increase of endothelial permeability. Alterations of the endothelial cell cytoskeleton may contribute to changes in pulmonary vascular permeability.

Supported in part by Grant BRSG 5 S07 RR05431-19 from the National Institutes of Health.

Address for reprints: D. Michael Shasby, M.D., Pulmonary

Division, University of Iowa College of Medicine, Iowa City, Iowa 52242.

Received: June 11, 1982; accepted for Publication August 26, 1982.

## References

- Bentzel CJ, Hainau B, Ho S, Hui SW, Edelman A, Anagnostopoulos T, Benedetti EL (1980) Cytoplasmic regulation of tight-junction permeability: Effect of plant cytokinins. *Am J Physiol* **239**: C75-C89
- Borak LS, Yocum RR, Nothnagel EA, Webb WW (1980) Fluorescence staining of the actin cytoskeleton in living cells with F-nitrobenz-2-oxa-1,3-diazole phalloidin. *Proc Natl Acad Sci USA* **77**: 980-984
- Duffey ME, Hainau B, Ho S, Bentzel CJ (1981) Regulation of epithelial tight junction permeability by cyclic AMP. *Nature* **294**: 451-453
- Fantone JC, Kunkel SL, Ward PA, Zurier RB (1980) Suppression by prostaglandin E<sub>1</sub> of vascular permeability induced by vasoactive inflammatory mediators. *J Immunol* **125**: 2591-2596
- Flick MR, Perel A, Staub NC (1981) Leukocytes are required for increased lung microvascular permeability after microembolization in sheep. *Circ Res* **48**: 344-351
- Heflin AC, Brigham KC (1981) Prevention by granulocyte depletion of increased vascular permeability of sheep lung following endotoxemia. *J Clin Invest* **68**: 1253-1260
- Heltianu C, Simionescu M, Simionescu N (1982) Histamine receptors of the microvascular endothelium revealed in situ with a histamine-ferritin conjugate: Characteristic high affinity binding sites in venules. *J Cell Biol* **93**: 357-364
- Hurley JV (1982) Types of pulmonary microvascular injury. *Ann NY Acad Sci* **384**: 269-286
- Majno G, Palade GE (1961) Studies on inflammation. The effect of histamine and serotonin on vascular permeability: An electron microscopic study. *J Biophys Biochem Cytol* **11**: 571-605
- Majno G, Gilmore V, Leventhal M (1967) On the mechanism of vascular leakage caused by histamine-type mediators. *Circ Res* **21**: 833-847
- Meza I, Ibarra G, Sabanero M, Martinez-Paloma A, Cerejido M (1980) Occluding junctions and cytoskeletal components in a transporting epithelium. *J Cell Biol* **87**: 746-754
- Postlethwaite AE, Snyderman R, Kang AH (1976) The chemotactic attraction of human fibroblasts to a lymphocyte-derived factor. *J Exp Med* **144**: 188-1203
- Ryan US, Clements E, Habliston D, Ryan JW (1978) Isolation and culture of pulmonary artery endothelial cells. *Tissue Cell* **10**: 535-554
- Savion N, Vlodavsky I, Greenburg G, Gospodarowicz D (1982) Synthesis and distribution of cytoskeletal elements in endothelial cells as a function of cell growth and organization. *J Cell Physiol* **110**: 129-141
- Shasby DM, Van Berthuyzen KM, Tate RM, Shasby SS, McMurtry I, Repine JE (1982) Granulocytes mediate acute edematous lung injury in rabbits and in isolated rabbit lungs perfused with phorbol myristate acetate: Role of oxygen radicals. *Am Rev Respir Dis* **125**: 443-447
- Staub NC (1978) Pulmonary edema due to increased microvascular permeability to fluid and protein. *Circ Res* **43**: 143-151
- Taylor RF, Price TH, Schwartz SM, Dale DC (1981) Neutrophil-endothelial cell interactions on endothelial monolayers grown on micropore filters. *J Clin Invest* **67**: 584-587
- Till GO, Johnson KJ, Kunkel R, Ward PA (1982) Intravascular activation of complement and acute lung injury. Dependency on neutrophils and toxic oxygen metabolites. *J Clin Invest* **82**: 1126-1135

INDEX TERMS: Cytoskeleton • Cytochalasins • Permeability lung edema • Endothelium