Binding of Charged Ferritin to Alveolar Wall Components and Charge Selectivity of Macromolecular Transport in Permeability Pulmonary Edema in Rats

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SUMMARY. Rat lungs were inflated and incubated in either anionic or cationic ferritin, and alveolar and capillary basement membranes were examined by electron microscopy. Cationic ferritin bound to heparan sulfate proteoglycans on the external surface of the alveolar basement membrane, whereas cationic ferritin bound to the lamina densa of the capillary basement membranes. Anionic and cationic ferritin was also perfused through the pulmonary circulation of lungs isolated from control rats and rats previously injected with α-naphthylthiourea, which produces permeability pulmonary edema. Neither anionic nor cationic ferritin leaked from the pulmonary capillaries in perfused controls; cationic, but not anionic, ferritin adhered to endothelial cell surfaces. In lungs with α-naphthylthiourea pulmonary edema, perfused for 2-15 minutes, anionic ferritin leaked from pulmonary capillaries into the alveolar interstitium and alveolar space, while cationic ferritin remained within the capillary lumen. Five times as much anionic ferritin appeared in the capillary basement membranes on the thick side of the alveolar wall, as in the alveolar basement membranes on the thin side of the alveolar wall. In α-naphthylthiourea lungs perfused for 45-60 minutes, cationic ferritin also leaked through the injured endothelium and bound twice as much to the alveolar as the capillary basement membranes. The negatively charged pulmonary capillary endothelium, the positively charged capillary basement membranes, and the negatively charged alveolar basement membranes may influence the transport of macromolecules from the pulmonary circulation in permeability pulmonary edema. (Circ Res 55: 155-167, 1984)

PULMONARY edema has been separated pathophysiologicaly into two broad categories: that due to elevated capillary hydrostatic pressure, most commonly associated with cardiac failure, and that due to the increased pulmonary vascular permeability, associated with a wide variety of lung injuries (Staub, 1978). Permeability pulmonary edema has been characterized by leak of fluid and plasma protein out of the pulmonary capillaries into the lung interstitium and, ultimately, into the alveolar space, resulting in impaired gas exchange. Despite the frequency of permeability pulmonary edema, the mechanisms by which the vascular leak of macromolecules occurs are unclear. Neither the site of lung injury nor the pathway of macromolecular traffic has been elucidated (Staub, 1978). Even in massive pulmonary edema, ultrastructural evidence of endothelial injury is rare (Bachofen and Weibel, 1977). Molecular sieving, which characterizes normal vascular permeability, continues in permeability pulmonary edema, although pore sizes change slightly, as does the proportion of flow through large and small pores (Brigham and Owen, 1975; Rutili et al., 1982). Recent studies in normal lungs have shown preferential appearance of anionic over neutral or cationic molecules in lung lymph, which represents drainage from the alveolar interstitium (Pietra et al., 1983; Weaver et al., 1982; Fairman et al., 1982), suggesting that charge, as well as size, may influence normal vascular permeability, and raising the question of whether charge might influence permeability in pulmonary edema.

Several studies of renal vascular permeability have shown that charge may have an important effect on macromolecular traffic in the kidney (Rennke et al., 1975, Rennke and Venkatachlam, 1977; Brenner et al., 1978; Fairquhar, 1981). The barrier between glomerular capillary and urinary space is negatively charged and, under normal circumstances, contributes to the repulsion of negatively charged molecules such as plasma proteins, thus preventing the appearance of protein in the urine. In certain disease states, or when this charge barrier is eliminated experimentally, negatively charged molecules appear in the urine (Caulfield and Farquhar, 1978; Kelley and Cavallo, 1980; Kan-
Heparan sulfate proteoglycans, associated with the glomerular basement membrane, have been implicated by some authors as being an important component of the renal charge barrier (Farquhar, 1981).

We have recently described the ultrastructural characteristics of the alveolar (epithelial) and capillary (endothelial) basement membranes* of the lung, hereafter referred to as ABM and CBM, respectively (Vaccaro and Brody, 1981). The ABM is a dense amorphous structure containing a linear array of heparan sulfate proteoglycans, much as does the glomerular basement membrane, making it a negatively charged barrier. In contrast, the CBM is a loosely organized fibrillar structure containing relatively few proteoglycans. The negative charge of the ABM has recently been confirmed by Simionescu and Simionescu (1983), who found that it bound cationic ferritin. The differences in these two transport barriers prompted us to explore their relative charges and the potential role of charge in regulating transport of molecules in permeability pulmonary edema.

Our studies, in which anionic ferritin, cationic ferritin, and ruthenium red were used as electron dense markers, have shown that the ABM binds cationic ferritin and ruthenium red, acting as if it had a negative charge, whereas the CBM binds anionic ferritin and little ruthenium red, acting as if it had a more positive charge. During vascular perfusion of the normal lung, both anionic and cationic ferritin remain within the capillaries. In α-nylthiophioura (ANTU)-induced permeability pulmonary edema, there is distinct charge selectivity; negatively charged ferritin moves out of the pulmonary vasculature, whereas positively charged ferritin does not. There appear to be several different stages at which charge may influence transport of macromolecules in the lung, including the endothelium cell, the CBM, and the ABM.

**Methods**

**Experimental Design**

To demonstrate the anionic or cationic nature of alveolar wall basement membranes, we inflated adult male rat lungs, via the trachea, with the cationic dye ruthenium red, as previously described (Vaccaro and Brody, 1979, 1981), or with 25 mg/ml anionic or cationic ferritin in Krebs-Henseleit solution. The lungs then were minced and incubated in a solution similar to that used in the trachea for 1 hour prior to fixation and processing for ultrastructural analysis, as described below. One rat lung was used for each incubation with sections from several blocks sampled from each lung.

In order to determine the influence of different charged structures of the alveolar wall on transit of anionic and cationic macromolecules from the pulmonary circulation, adult rats were given an intraperitoneal injection of either 15 mg/kg body weight ANTU, dissolved in Tween 80, or the Tween vehicle alone. Four hours after the injections, rats were anesthetized with 40 mg of intraperitoneal pentobarbital. At this time, permeability pulmonary edema was evident in ANTU rats, as demonstrated by a 23% increase in wet-to-dry lung weight ratios, and an increase in lung lavage protein concentration, as compared to the Tween controls (Hill and Rounds, 1983). The lungs were isolated as previously described (Hill and Rounds, 1983) and ventilated through a tracheal cannula with 95% air, 5% CO₂ at 10 cm H₂O inspiratory and 2.5 cm H₂O end expiratory pressure, 60 times/min. The lungs were perfused at a constant flow rate of 0.04 ml/min per g body weight with Krebs-Henseleit solution containing 10 mm glucose at pH 7.35-7.40.

After the perfusate effluent was free of visible blood (about 3 minutes), the perfusate was changed to one containing 5-10 mg/ml of either anionic or cationic ferritin. The lungs from two ANTU animals and one control animal were perfused with ferritin-containing solutions for each of the following time periods: 1.5-2, 5, 15, 45, or 60 minutes. One control rat was perfused with cationic ferritin for 10 minutes and then perfused for 10 minutes with perfusate containing no ferritin to flush unbound ferritin from the pulmonary circulation. In one instance, a lung from an ANTU-treated rat was perfused with blood containing anionic ferritin for a period of 15 minutes. Thus, a total of 11 control and 21 ANTU rat lungs were perfused. Lung tissue was then fixed and processed for ultrastructural analysis, as described below.

**Materials**

Pathogen-free, male, Sprague-Dawley rats, 250-300 g, were obtained from Charles River Breeding Laboratories. Horse-spleen ferritin, twice crystallized, cadmium free, was obtained from Calbiochem or Polysciences. N.N-di-methyl-1-3(3-diethyl-1-3-propanediamine (DMPA) was obtained from Fisher Scientific, and 1-ethyl-3(3-diethylamino propyl) carbodimide hydrochloride (EDC) was from Calbiochem. DMPA was not redistilled before use. Ruthenium red was purchased from Toussimis Research Corporation.

**Ferritin and Cationized Derivatives**

The cationization reaction utilized the method of Danon et al. (1972), with the modification of Kanwar and Farquhar (1979a) and Rennke et al. (1975), which involves keeping the pH constant and varying the amount of carboxyl group activator EDC. Small amounts of highly concentrated, cationic ferritin were prepared as follows: 14.0 ml of 2.0 M DMPA (pH 7.0) were mixed with 3.5 ml (350 mg) of native ferritin, followed by the addition of 425 mg of EDC. The reaction was carried out at room temperature for approximately 2 hours with constant stirring. This mixture remained clear if pH was kept constant by addition of 1 N NaOH. After modification, the ferritin was dialyzed twice against sterile 0.15 M NaCl, over a period of 24 hours at 4°C. Solutions were concentrated in a Diaflow unit (Amicon Corp), using a PM-10 membrane,
FIGURE 1. Anionic (panel a) and cationic ferritin (panel b) viewed unstained on a formvar-coated grid. Both tracers are monodispersed and equal in size. 272,000 X; bar = 0.01 μm.

and stored at 4°C. The isoelectric point (pI) of anionic and cationic ferritin derivatives were determined by electro-focusing and isoelectric titration (Rennke et al., 1978). The pI of anionic ferritin was 4.1-4.5 and cationic derivatives were slightly greater than 8.8. Molecular weight has been reported as 480,000 daltons and Stoke’s radius of 6.1 nm (Rennke and Venkatachalam, 1977). Figure 1 (a and b) demonstrates the respective uniformity of anionic (a) and cationic (b) ferritin dispersal on a formvar-coated grid, which was examined unstained by electron microscopy. These tracers appear monodispersed and equal in size.

Tannic Acid Fixation

At the end of each perfusion, the lungs were clamped at the trachea and at the cannula to the heart, and fixed in the expanded state for 5–10 minutes by dripping 2% tannic acid and 2% glutaraldehyde in 0.075 M phosphate buffer (pH 7.2–7.4) over the surface of the lung. The trachea then was opened and the lungs inflated at 20–25 cm H2O pressure with fresh fixative for an additional 5 minutes. Samples of each lobe were excised and cut into 1 mm³ blocks and immersed in fresh fixative for 3 hours at room temperature. After tannic acid/glutaraldehyde fixation, tissue blocks were washed four times over 10 minutes with 0.075 M phosphate buffer containing 0.18 M sucrose. The tissue was postfixed for 1–2 hour in osmium tetroxide in phosphate buffer with sucrose at room temperature. After osmication, the tissue was rinsed in two changes of phosphate buffer and rapidly processed through ascending concentrations of ethanol or acetone.

FIGURE 2. Panel a: ruthenium red stain of a portion of ABM from normal rat lung. Ruthenium red positive anionic sites (arrows) organize in a linear fashion on the lamina rara externa of ABM and have been shown to represent heparan sulfate proteoglycans. 102,144 X; bar = 0.1 μm. Panel b: ruthenium red stain of a portion of CBM. Note that there are fewer anionic sites which appear randomly distributed within the CBM lamina densa. 159,600 X; bar = 1.0 μm.
Figure 3. Panel a: distribution of cationic ferritin (Fe⁺) on the ABM after intratracheal instillation. Aggregates (arrows) form along the lamina rara external region and are similar to ruthenium red binding sites. 103,700 ×; bar = 0.10 μm. Panel b: localization of cationic ferritin on the CBM forms random aggregates immersed within the lamina densa. 103,700 ×; bar = 0.10 μm.

Blocks were infiltrated in a Polybed 8122-Araldite 502-DDSA-DMP-30 mixture split 50:50 with acetone for 20 minutes on a standard rotor. Rapid processing involved two additional changes with 100% resin; then the plastic was polymerized at 60°C for 24 hours. Before thin sectioning, the plastic was further polymerized for 1 hour at 100°C.

Preparation of Sections and Analysis

Sections (1 μm thick) were cut, mounted on glass slides, and stained with methylene blue in order to identify edematous areas within lung parenchyma. These areas were then included in thin sections, 60–80 nm, which were mounted on grids, viewed unstained, and photographed using a Phillips EM 300 electron microscope. At least two sections from each of three or four blocks were examined for each experimental animal.

To quantify the extracapillary distribution of anionic ferritin in ANTU pulmonary edema, tissue samples from two ANTU lungs that had been perfused with anionic ferritin for 5 minutes and from two ANTU lungs perfused with cationic ferritin for 45 minutes, were fixed and processed for electron microscopy as described above. Twenty pictures providing a cross-section of a capillary and complete alveolar wall were taken at random from unstained grids prepared from four different blocks. The prints were enlarged to a standard magnification, and the number of electron dense ferritin particles in the CBM on the thick side of the alveolar wall and in the ABM on the thin side of the alveolar wall were counted. The length of basement membrane analyzed was measured, and the linear density of particles in each basement membrane calculated. In addition, ABM-associated anionic ferritin particles were divided into those on the inside of the ABM (i.e., facing the endothelium) and those in the remainder of the ABM.

Results

Normal Lung Minces: Distribution of Ruthenium Red and Ferritin

Ruthenium red, a cationic dye, stains anionic sites which appear in a linear array along the external or epithelial side of the ABM (Fig. 2a). These sites, which we have previously shown to represent heparan sulfate proteoglycans (Vaccaro and Brody, 1981), appear in the ABM which lines both the thick and thin side of the alveolar wall. There is no staining of the lamina densa of the ABM. In contrast, a few ruthenium red-stained sites are randomly distributed within the loosely organized CBM. There was no ruthenium red staining of either the internal or external surface of the CBM lamina densa (Fig. 2b). The distribution of cationic ferritin on ABM and CBM (Fig. 3, a and b) was similar to that of ruthenium red. Anionic ferritin preferentially bound to the CBM lamina densa (Fig. 4b), and was only irregularly associated with the ABM (Fig. 4a).

Control Lung Perfusion: Distribution of Ferritin

Both anionic and cationic ferritin remained within the pulmonary capillaries in normal lungs perfused for periods up to 15 minutes. Anionic ferritin tended to remain free within the capillary lumen, while cationic ferritin appeared adjacent to the endothelial plasmalemma. The intraluminal distribution of anionic and cationic ferritin was similar to that illustrated for ANTU lungs in Figures 7 and 8. Some cationic ferritin appeared in endothelial vesicles. Flushing the circulation with ferritin-free perfusate failed to remove all of the cationic ferritin associated
FIGURE 4. Panel a: thin side of alveolar wall from rat lung instilled with anionic ferritin (Fe\(^-\)). Epithelium (Ep) is damaged and Fe\(^-\) has penetrated from the alveolus (Alv). A small amount of Fe\(^-\) is deposited in area of ABM, predominantly in lamina rara interna (arrowheads). Panel b: Fe\(^-\) deposits in lamina densa of CBM in a fashion similar to that seen in ANTU Fe\(^-\) perfused lungs. Panels a and b: 79,800 ×; bar = 0.1 μm.

with the endothelial cells, suggesting that it was adherent to the cell surface.

Control lungs perfused for 45 to 60 minutes displayed some evidence of focal endothelial damage with leak of anionic or cationic ferritin in areas of damage (see section on severe injury).

Permeability Pulmonary Edema—General Comments

ANTU in the dose used in this study, in perfusions of 15 minutes or less, produced irregular patches of edema recognized at a light microscopic level (Fig.

FIGURE 5. Light microscopic view of control lung (panel a) and ANTU lung (panel b) fixed intratracheally. Note that in alveoli (Alv) of the ANTU lung, the edema (arrowheads) is mild and patchy. 290 ×; bar = 1.0 μm.
Clusters of alveoli which contain amorphous edema material are surrounded by alveoli which appear to be normal. Even in areas of edema formation, there is little ultrastructural evidence of endothelial cell injury (Fig. 6). Although cytoplasmic blebs along the luminal surface, intracellular swelling, and cell separations from the CBM were occasionally noted, these types of endothelial cell abnormalities were neither prominent nor consistently observed in areas of edema. There were no areas of endothelial cell loss, nor were abnormalities of endothelial cell junctions noted. Structural damage to endothelial cells was noted focally in controls, and, to a greater extent, in ANTU lungs which were perfused for 45 or 60 minutes. Interstitial edema in ANTU lungs occurred on the thick side of the alveolar wall, with the thin side of the alveolar wall remaining free of edema fluid (Fig. 6). Thus, the epithelial-ABM-endothelial barrier across which alveolar-capillary gas exchange occurs remained thin, even in severe pulmonary edema.

**ANTU Lung Perfusion: Distribution of Ferritin**

Anionic ferritin leaked from the pulmonary capillaries of ANTU lungs perfused for 2–15 minutes. Although the structure of the endothelium and intercellular junctions appeared normal following ANTU, large amounts of the anionic tracer leaked from the capillaries and preferentially accumulated in the capillary basement membrane and within the interstitium along the thick side of the alveolar wall (Fig. 7b). Fewer ferritin particles penetrated the endothelium along the thin side of the alveolar wall. The majority of the ferritin particles that penetrated the thin side and that appear along the thick side ABM are restricted to the internal region of the ABM (Fig. 7a), although some anionic ferritin does appear in the alveolar lumen. Morphometric analysis from the two ANTU lungs perfused for 5 minutes with anionic ferritin shows that there is almost five times as much anionic ferritin on the CBM facing the thick side of the alveolar wall than is associated with the ABM along the thin side of the alveolus (Table 1). Furthermore, 77% of the anionic ferritin associated with the thin side ABM lies on the internal side of the ABM.

Unlike its negatively charged counterpart, ultrastructurally recognizable cationic ferritin does not penetrate the endothelium in large amounts in ANTU pulmonary edema. These positively charged
FIGURE 7. Panel a: alveolar wall from ANTLU rat perfused with Fe⁺ for 15 minutes. Type 1 alveolar epithelium covers both sides of alveolar wall. Fe⁺ leaks from capillary (Cap) and adheres to capillary basement membrane (CBM) lamina densa, while small amounts of Fe⁺ appear irregularly in the vicinity of lamina rara externa (arrowheads) of the alveolar basement membrane (ABM). Most Fe⁺ appears on the thick rather than thin side of the alveolar wall. 71,820 X; bar = 0.1 µm. Panel b: magnified view of CBM adherent Fe⁺. Note white blood cell (WBC) in capillary lumen (Cap) 111,720 X; bar = 0.1 µm.
TABLE 1
Distribution of Ferritin in Basement Membranes of ANTU
Lungs

<table>
<thead>
<tr>
<th></th>
<th>Anionic ferritin (perfused for 5 min)</th>
<th>Cationic ferritin (perfused for 45 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABM (Fe/nm)</td>
<td>17.0 ± 1.4</td>
<td>169.2 ± 11.2</td>
</tr>
<tr>
<td>CBM (Fe/nm)</td>
<td>78.8 ± 6.3</td>
<td>84.2 ± 8.0</td>
</tr>
<tr>
<td>ABM/CBM</td>
<td>0.2</td>
<td>2.0</td>
</tr>
<tr>
<td>Internal-ABM</td>
<td>0.77 ± 0.09</td>
<td>0.09 ± 0.01</td>
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Mean values ± 1 SEM from 21 capillaries in two lungs perfused with anionic ferritin and 26 capillaries in two lungs perfused with cationic ferritin. Cationic ferritin perfusions were carried out for longer periods, until severe damage occurred, since no cationic ferritin traversed the capillaries at 5 minutes. Internal-ABM indicates the proportion of ABM associated ferritin particles on the internal side of the ABM. No data are presented for control lungs, since neither anionic nor cationic ferritin leaked from the capillaries in a consistent fashion in these lungs.

Severe Lung Injury: Distribution of Ferritin

Perfusion of ANTU-treated lungs for 45 or 60 minutes produced massive endothelial cell damage which allowed both anionic and cationic ferritin to leak from the capillaries and appear in the interstitium and alveoli. In these instances, the capillary endothelium is swollen, many intracellular components have precipitated, and luminal and abluminal areas of plasma membrane have been disrupted (Fig. 9). Anionic ferritin easily traversed these damaged areas, diffusely binding to the CBM lamina densa and concentrating in the internal side of the ABM. Figure 10 demonstrates an area of endothelial damage in a control lung perfused for 45 minutes which allowed anionic ferritin to leak from the capillary on the thin side of the alveolar wall and accumulate on the internal side of the ABM.

Cationic ferritin also penetrated severely damaged apparent vesicles open to the lumen or enclosed within the cytoplasm.

**Figure 8.** ANTU rat lung perfused with Fe⁺ for 15 minutes. Fe⁺ (arrowheads) appears in capillary (Cap), along endothelial cell surfaces (En), and within apparent vesicles (V), but does not penetrate endothelial cell junctions (arrow) or appear in CBM or interstitium (In). 87,780 X; bar = 0.1 μm.
FIGURE 9. ANTU rat lung perfused with Fe⁺ for 60 minutes. Severe damage is present, with swelling and disruption of endothelial cells (En). Fe⁺ appears bound to CBM and accumulates in the lamina rara interna (arrows) of the ABM. Alv, alveolus; Cap, capillary. 87,780 ×; bar = 0.1 μm.

Endothelial cells, but appears in the interstitium and along the ABM in a pattern that differs from that of anionic ferritin (Fig. 11a). Cationic ferritin surrounds interstitial collagen, has crossed the ABM lamina densa, and accumulates in the external side of the ABM beneath the basilar surface of the alveolar epithelium. The distribution of cationic ferritin on the ABM (Fig. 11b) and on the CBM (Fig. 11c) in this setting is similar to that of ruthenium red stained lung (Fig. 2). In the instance of severe injury and capillary leak of cationic ferritin, two to three times as much cationic ferritin bound to the ABM than to the CBM (Table 1). In contrast to anionic ferritin, most cationic ferritin appeared on the external, rather than the internal, side of the ABM.

Discussion

These studies have shown that there is a distinct difference in the pattern of binding of anionic, as compared to cationic, ferritin to CBM and ABM. Capillary basement membrane is a loosely organized fibrillar structure which appears to have a more positive charge, binding anionic ferritin in its lamina densa. In contrast, the ABM is a compact structure which appears to have a more negative charge, binding cationic ferritin and the cationic dye ruthenium red in its external region adjacent to the alveolar epithelium. The charge nature of these structures has been shown in lungs inflated and then incubated with either cationic or anionic ferritin.

Our studies have also shown that charge may be a determinant of the movement of macromolecules out of the pulmonary capillaries in permeability pulmonary edema. Neither cationic nor anionic species of ferritin move across the pulmonary capillaries in sufficient quantities to be recognized ultrastructurally in normal lungs perfused for up to 15 minutes. When lungs from rats with ANTU pulmonary edema were perfused for periods ranging from 2 to
FIGURE 10. Control rat lung perfused with Fe⁺ for 45 minutes. Area of endothelial damage (En) with Fe⁺ appearing along lamina rara interna (arrowheads) of ABM but not adhering to ABM lamina densa or crossing to lamina rara externa. Alv, alveolus. 102,600 X; bar = 0.1 μm.

15 minutes, anionic ferritin moved across capillary endothelium into the pulmonary interstitium and, ultimately, into the alveolar space, while cationic ferritin remained within the pulmonary vasculature. Most of the cationic ferritin in the latter experiments appeared adjacent to endothelial cell surfaces or in endothelial vesicles. When anionic ferritin traversed the capillary endothelium, it appeared in the interstitium adherent to the CBM lamina densa, lying free within the interstitium, and adjacent to the internal or interstitial side of the ABM, with some ferritin appearing in the alveolus.

The finding that anionic ferritin escaped more readily from the capillary lumen suggests that capillaries of injured lungs are more permeable to anionic, compared to cationic, ferritin. These results are consistent with those reported from other laboratories which suggest that lung capillaries are more permeable to anionic molecules (Weaver et al., 1982; Fairman et al., 1982; Pietra et al., 1983). It is possible that the binding of cationic ferritin to the endothelial cell lumenal surface prevented or slowed leakage of the cationic macromolecule from the vascular space.

The pattern of appearance of anionic ferritin was similar, although more dramatic, in ANTU lungs which were severely injured by perfusion for 45-60 minutes; i.e., anionic ferritin adhered to the CBM and appeared on the interstitial side of the ABM. When cationic ferritin left the vasculature in severely injured vessels, it surrounded collagen fibers and was found on the external or epithelial side of the ABM.

These findings are different from studies of renal glomerular permeability. Several groups of investigators have shown that the normal glomerular capillary is more permeable to cationic than anionic macromolecules. These studies have been done with electron dense markers whose molecular radii varied from 3.0 nm horseradish peroxidase (Kanwar and Farquhar, 1979a), to 6.1 nm ferritin (Rennke and Venkatachalam, 1977; Farquhar, 1981), and have also been done with dextrans which cover a broad range of sizes from 1.0 to 4.5 nm (Brenner et al., 1978). Our finding that, in the normal lung, ultrastructurally recognizable cationic and anionic ferritin do not traverse the capillaries is probably due to the fact that, in contrast to the glomerulus, pulmonary capillaries are lined by continuous endothelium with tight, intercellular junctions and no fenestrae.

Our results are compatible with there being several different charged surfaces across which macromolecules traverse in permeability pulmonary edema. The endothelial cell surface appears to be highly negatively charged, binding cationic and repelling anionic ferritin. The CBM appears to have a positive charge and the ABM appears to have a negative charge.

The first charged barrier in the pulmonary microvessels is the lumenal surface of the endothelial cell. This surface appears to be negatively charged, since ultrastructural studies of various vascular beds and of cultured endothelial cells have revealed binding of positively charged substances, such as ruthenium red and cationic ferritin (Danon et al., 1972; Pelikan et al., 1979; Cavallo et al., 1980; Simionescu and Simionescu, 1981). The nature of the negatively charged substances located on this surface have not been completely identified, but biosynthetic studies and differential enzyme digestion have suggested that sialoproteins and glycosaminoglycans, particularly heparan sulfate, are present on the lumenal plasmalemma (Buorassiss and Root, 1975; Gramse et al., 1978; Shimada et al., 1981). Recently, Pietra and co-workers have shown that cationic ferritin binds in an uneven fashion to the pulmonary capillary endothelium, with specialized binding to some lu-
FIGURE 11. Panel a: ANTLI rat lung perfused with Fe⁺ for 60 minutes. Severe damage is present with swelling and disruption (arrows) of endothelial cells (En). Fe⁺ leaks from capillary and accumulates in lamina rara externa (arrowheads) of the ABM and around collagen (Col). Alveolus (Alv) 87,780 X; bar = 0.1 µm. Panels b and c: higher magnification of the distribution of cationic ferritin on the ABM (panel b) and CBM (panel c) in lungs perfused with Fe⁺ for 60 minutes. Panels b and c: 108,500 X; bar = 0.01 µm.
minal transport vesicles (Pietra et al., 1983). This binding is due to the presence of highly anionic sites on the endothelial surface. A similar distribution of cationic ferritin has also been demonstrated by Siminescu and Siminescu (1983).

The second and third major charged structure which we have described are the CBM and ABM. Basement membranes are complex structures which consist of type IV and perhaps type V collagen, the glycoproteins, laminin and fibronectin, and proteoglycans (Farquhar, 1981). The mechanisms by which these components interact to form the lamina densa and lamina rarae of the basement membrane are not clear. Of the various basement membrane components, proteoglycans have been studied most extensively as a contributor to basement membrane charge. These large hydrophilic molecules are highly anionic as a result of their sulfate groups (Lindahl and Hook, 1978). Proteoglycans have been shown to be present in all basement membranes studied to date. Most vascular basement membranes contain heparan sulfate. This proteoglycan has been identified by use of cationic dyes before and after selective enzyme degradation in a number of tissues (Ausprunk et al., 1981; Wright and Ross, 1975), by isolation from glomerular basement membranes (Kanwar and Farquhar, 1979b; Cohen, 1980; Lemkin and Farquhar, 1981), and by immunofluorescent studies using purified anti-heparan sulfate antibodies (Hassel et al., 1980). We have previously noted that, in the adult rat lung, heparan sulfate proteoglycans are present on the external side of the ABM facing epithelial cells, but that the CBM contained few proteoglycans (Vaccaro and Brody, 1981). The ferritin studies here confirm these observations, since perfusion of the severely edematous lungs or instillation via the trachea, followed by incubation, demonstrate cationic ferritin accumulation in the negatively charged external side of the ABM, but showed minimal adhesion to any elements of the CBM. Anionic ferritin does not adhere to ABM lamina densa; neither does it appear on the external side of the ABM. Rather, it accumulates on the internal side of the ABM, which is consistent with ABM having a negative charge which would impede movement of anionic ferritin into the alveolus. Anionic ferritin does, however, adhere to the lamina densa of the CBM, suggesting that CBM acts as if it has a positive charge. Although the reason for this CBM positive charge is not clear, it may relate to the paucity of CBM-associated proteoglycans, which are in themselves highly anionic, or which may serve to promote organization of the basement membrane and, thereby, alter its charge.

The mechanisms involved in the production of increased permeability pulmonary edema and the process by which macromolecules leak from capillaries are not known. Morphological studies have failed to show ultrastructural evidence of severe endothelial cell injury (Bachofen and Weibel, 1977). Possible routes for macromolecule leak include altered endothelial cell junctions or increased transendothelial vesicular transport, although there is considerable controversy concerning these potential pathways (Staub, 1978). Despite review of many sections, we failed to find evidence of ferritin passing through endothelial or epithelial cell junctions, and we do not know by what route ferritin leaked from the capillary lumen.

Regardless of the mechanism of injury or the route of leakage, injury is sufficient to allow a large molecule such as ferritin to leak from seemingly intact pulmonary capillaries. The fact that only anionic ferritin leaves the circulation suggests that lung capillaries retain the ability to differentiate between cationic and anionic molecules. This differential permeability may be due to binding of positively charged molecules to the endothelial cell surface or to endothelial vesicles. In addition, the CBM might influence macromolecular flow by attracting negatively charged species and impeding the flow of positively charged species. This could explain the preferential movement of anionic ferritin toward the CBM and thick side of the alveolar wall, as compared to the ABM and thin side of the wall.

The changes in the interstitium exclusive of the CBM appear to be predominantly negative. Connective tissue elements are invested in proteoglycans to which cationic ferritin or ruthenium red bind (Vaccaro and Brody, 1979). The ABM lamina densa does not itself bind either ferritin molecule, but contains anionic heparan sulfate proteoglycan on its external side. We speculate that there may be a tendency for negatively charged macromolecules, such as plasma proteins, to be repelled from interstitial structures and from the ABM and to move into lymph channels which drain the interstitium of the alveolar wall. The fact that, in permeability pulmonary edema, anionic ferritin accumulates on the internal side of the ABM lamina densa supports this concept. This process would tend to protect the lung from flooding of alveoli by edema fluid and would preserve the gas exchange function of the lung.

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Binding of charged ferritin to alveolar wall components and charge selectivity of macromolecular transport in permeability pulmonary edema in rats.

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