

ULTRASTRUCTURAL IMMUNOPEROXIDASE
DEMONSTRATION OF AUTOLOGOUS ALBUMIN IN THE
ALVEOLAR CAPILLARY MEMBRANE AND IN THE ALVEOLAR
LINING MATERIAL IN NORMAL RATS

JEAN BIGNON, PHILIPPE CHAHINIAN, GERARD FELDMANN, and CATHERINE SAPIN.
From the Laboratoire d'Histologie et Cytologie Pulmonaire, Hôpital Laënnec, 75007 Paris,
France; Unité de Recherche de Physiopathologie Hépatique, Institut National de la
Santé et la Recherche Médicale (INSERM), Hôpital Beaujon, 92110 Clichy, France;
and Laboratoire de Morphologie et d'Immunopathologie Rénale, INSERM,
Hôpital Broussais, 75014 Paris, France

The permeability of the alveolar-capillary membrane to protein macromolecules has been previously investigated using biochemical or morphological methods. The former have provided consistent data demonstrating that normal transfer of plasma proteins takes place in the lung from the blood vessels to the lymph channels (1, 2), probably through the interstitial space, and that albumin crosses the alveolar membrane in both directions, from the capillary to the alveolus (3) and from the alveolus to the capillary (4-6); moreover, several investigations have stressed the presence of serum proteins, particularly albumin, in surface-active alveolar lining material (7-10). The morphological studies, using foreign tracers of different molecular weights (ferritin, mol wt 500,000, molecular diameter [md] 110 Å [11]; catalase, mol wt 240,000 [11]; hemoglobin, mol wt 64,500, md 60 Å [12]; and horseradish peroxidase [HRP], mol wt 40,000, md 40-50 Å [11, 13, 14]), in-

jected intravenously, have shown that the endothelial membrane is permeable to these macromolecules; however, the injection of some of these heterologous substances can induce an increase in pulmonary arterial pressure (11, 14) or cellular lesions (15).

By contrast, a method for the detection of autologous plasma proteins *in situ* could represent a control for studies carried out with foreign substances. The albumin molecule appears to be a valid indicator for morphological assessment of plasma protein transfer in the lung, because of its abundance in the plasma and because of its molecular size (mol wt 69,000, md 70 Å), which is intermediate between that of HRP and that of hemoglobin.

The aim of this work was to confirm the presence of autologous albumin in the alveolar wall and in the alveolar lining material (ALM) of rat lung under normal physiological conditions, and to visualize this macromolecule at the ultrastructural level. For this purpose the location of albumin has been demonstrated *in situ* by immunoelectron microscopy using antibodies labeled with HRP.

MATERIALS AND METHODS

Normal adult male rats (CD, COBS, Charles River) weighing 250–300 g were used.

Preparation of Antirat Albumin Antibodies and Fab Fragments Labeled with HRP

Albumin was isolated from normal rat serum by precipitation with 10% trichloroacetic acid. The precipitate was dissolved in ethanol (16), and the solution obtained was tested by acrylamide agarose electrophoresis. The darkest and fastest moving band was assumed to be rat albumin. Samples of this band containing a total of 5 mg of protein were homogenized in phosphate-buffered saline and mixed with an equal volume of complete Freund's adjuvant. A sheep was immunized by intramuscular injection of this mixture. Booster injections were repeated every month in the same way. After 3 mo, the serum was tested by immunoelectrophoresis against normal rat serum. Only one line corresponding to albumin was observed. Pure antirat albumin antibodies were obtained using normal rat serum insolubilized with glutaraldehyde (17). Fab fragments were obtained by digestion with 1% papain (18) and purified by DEAE-cellulose chromatography, equilibrated with sodium phosphate buffer 0.01 M, pH 8. Fragments were concentrated to 2.5 mg/ml and labeled with HRP (RZ 3.0, Sigma

Chemical Co., St. Louis, Mo.) according to Avrameas (19) (specific conjugate).

Preparation of Fab Fragments from Normal Sheep IgG

Sheep IgG was isolated from normal sheep serum by ammonium sulfate precipitation at 40% saturation, followed by DEAE-cellulose chromatography with a sodium phosphate buffer 0.017 M, pH 6.9. Fab fragments were then prepared and labeled with HRP as described above for antirat albumin antibodies (nonspecific conjugate).

Fixation Procedures

Rats were anesthetized with 4.5 mg/100 g body weight of sodium thiopental (Abbot, Paris, France) administered intraperitoneally. Lung specimens were fixed using three different methods.

AIRWAY INFUSION: The fixative was infused into the trachea at a pressure of 20 cm H₂O. Thereafter the heart-lung block was removed and immersed in the corresponding fixative for 12 h at +4°C. The lungs were then sliced into 1-mm thick sections and washed for 24–48 h in the corresponding cacodylate buffer at +4°C.

IMMERSION: After excision of the heart-lung block, the lung tissue was cut into small blocks which were immediately immersed in the fixative, left for 4 h at +4°C, and subsequently washed for 24 h in the corresponding cacodylate buffer.

VASCULAR PERFUSION: While a tracheotomy allowed artificial ventilation, the thorax was opened and a catheter tip inserted into the pulmonary artery (20). Pulmonary vessels were washed at 37°C for 5 min with a Ringer's solution containing heparin (Fournier, Paris, France), 5000 IU/100 ml, and isoproterenol (Winthrop, Paris, France), 0.1 µg/min/kg body weight. The fixative was then perfused for 30 min. A constant perfusion flow was ensured by a pressure head of 30 cm height, with a constant alveolar pressure of 4 cm H₂O. The heart-lung block was excised and processed as after airway infusion.

Three fixatives of different osmolarity were used in these experiments: 2.3% glutaraldehyde (TAAB, Reading, England) in 0.045 M cacodylate buffer, pH 7.4 (380 mosM). In some experiments using vascular fixation, glutaraldehyde was added to 1.5% dextran (Dextran 60, mol wt 60,000–90,000; Serva Fein Biochemical, Heidelberg, W. Germany) (21); 2.7% glutaraldehyde (TAAB) in 0.14 M cacodylate buffer, pH 7.4 (550 mosM); 4% paraformaldehyde (Prolabo, Paris, France) in 0.2 M cacodylate buffer, pH 7.4 containing 8.5% sucrose (1,300 mosM). For rinsing after fixation, the osmolarity of the buffer was adjusted to the fixative osmolarity by the addition of sucrose.

Immunoelectron Microscopy

After fixation and washing, 30–40-µm thick cryostat sections were made and incubated for 2 h with the

specific conjugate diluted to 1:40 in 0.1 M phosphate-buffered saline (PBS), pH 7.4, at room temperature and washed with PBS for 60 min (three washings of 20 min each). Peroxidase was demonstrated by Graham-Karnovsky's method which was used either as described by the authors (22) or modified for immunohistochemical reaction. Sections were initially incubated for 15 min at 37°C in 10 ml of 0.05 M Tris HCl buffer, pH 8.6, containing 10 mg of 3',3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co., St. Louis, Mo.), and then 0.2 ml of 1% H₂O₂ was added for a further incubation of 15 min. Sections were then rinsed in 0.05 M Tris HCl buffer, pH 8.6, and postfixed with 2.5% osmium tetroxide in 0.07 M cacodylate buffer, pH 7.4, for 1 h at room temperature, dehydrated in a graded series of ethanol at +4°C and embedded in Epon. Sections were cut to a thickness of 0.5–1 μm using a Reichert OM U2 ultramicrotome (C. Reichert, sold by American Optical Corp., Buffalo, N. Y.) and studied under a light microscope without additional staining. Ultrathin sections from selected areas of parenchyma were examined with a Siemens Elmiskop 101 electron microscope at 60 kV without further staining.

For *control reactions*, sections were incubated with: (a) nonspecific conjugate using the same procedure as above; (b) Graham-Karnovsky medium for the demonstration of endogenous peroxidase activity; and (c) a solution of HRP (RZ 3.0 Sigma, 1 mg/ml in PBS) and then with Graham-Karnovsky medium. Furthermore, as an additional control, in some experiments postfixation with osmium tetroxide was not used.

RESULTS

The presence of albumin was demonstrated in the alveolar-capillary membrane by the immunoperoxidase reaction, which resulted in electron-dense deposits. No obvious difference in the intensity of the reaction could be found with fixatives of different types or of different osmolarities, although tissue preservation was more satisfactory with 2.3% and 2.7% glutaraldehyde than with 4% paraformaldehyde. The addition of dextran to glutaraldehyde during vascular fixation did not obviously change the immunocytochemical results. Specimens studied without osmium tetroxide postfixation gave similar results, but with a poor contrast. The results concerning the location of the electron-dense deposits were different according to whether the lung was fixed by airway infusion, immersion, or vascular perfusion.

Interstitialium

Whatever fixation procedure was used, the reaction product was strongly positive in the interstitial space, at the level of distinct (Fig. 1) or fused

(Figs. 2 and 3) endothelial and epithelial basement membranes and also at the level of thick portions of the alveolar wall, where collagen bundles were occasionally positive while elastic fibers were constantly negative.

Endothelial and Epithelial Cells

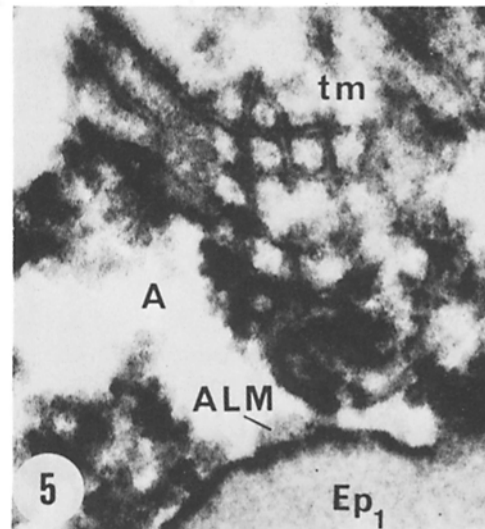
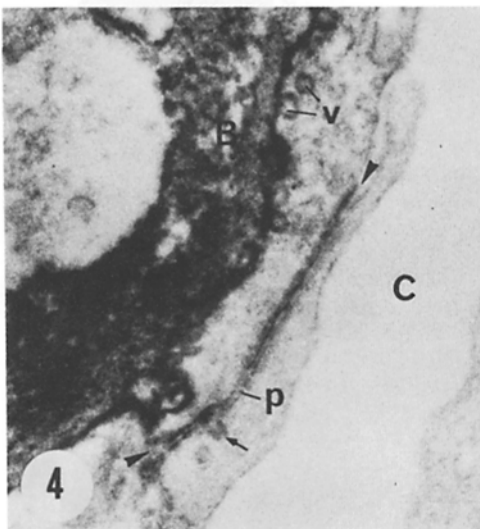
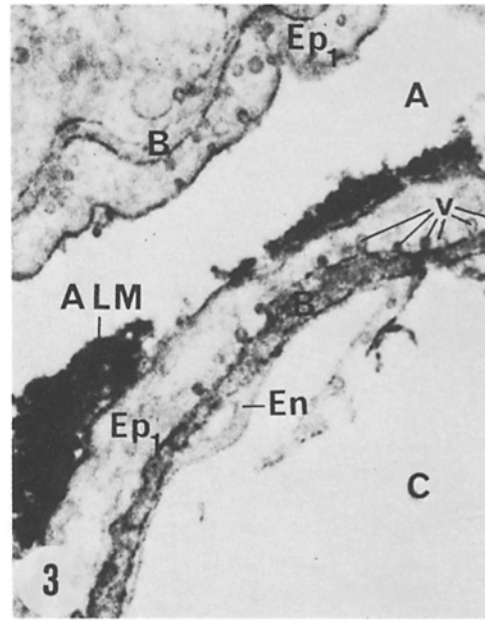
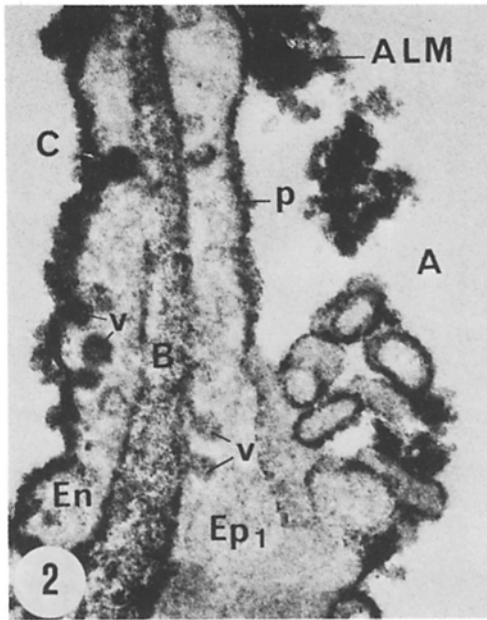
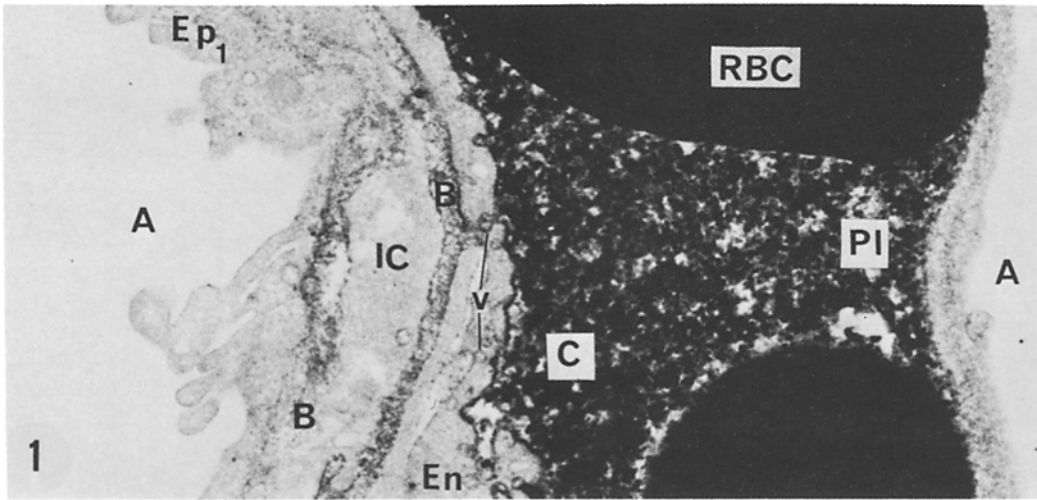
Both endothelial and type I alveolar cells (type I cells) contained numerous vesicles, with a mean external diameter of 630 Å, looking like pinocytotic vesicles (Figs. 1, 2, and 3). Some of the vesicles were completely electron dense, as if full of albumin (Fig. 2); others appeared lightly stained or even unstained, except at the level of their limiting membrane (Figs. 2 and 3). Vesicles were either free within the cytoplasm of both cells or open as an invagination of the cell membrane, their electron-dense content being continuous with the positive material on the endothelial or alveolar surface and within the basement membrane (Figs. 2 and 3). Occasionally, two to four vesicles were seen to be connected together but apparently not forming a continuous channel between the two fronts of the cell. Elsewhere, there was no staining of any other organelle or of the hyaloplasm of endothelial and type I cells. Type II alveolar epithelial cells (type II cells) were negative.

Plasmalemmal Membranes

After fixation by immersion, the plasmalemmal membranes limiting the endothelial and epithelial cells were heavily delineated by the reaction product, either on the basement membrane side or on the luminal sides. On the endothelial (Fig. 2) and alveolar surfaces, they were coated by a positive material. After fixation by airway infusion, the alveolar epithelial membrane was not stained (Fig. 1), or stained only slightly (Fig. 2). After these fixation procedures, the plasma was heavily stained in the capillary lumen (Fig. 1). By contrast, after vascular perfusion fixation, the plasma was washed out and the plasmalemmal membranes towards the capillary lumen appeared badly stained or unstained (Figs. 3 and 4).

Intercellular Spaces

Whatever fixation procedure was used, no interepithelial space was seen to be positive with the immunoreaction at alveolar epithelium level. By contrast, some interendothelial spaces were more or less positive, but more frequently after vascular perfusion fixation than after airway infusion or



immersion. Most often, plasmalemmal membranes appeared delineated by a positive reaction (Fig. 4), but usually with some discontinuity of the staining. However, with these preparations not stained by lead citrate, plasmalemmal membranes were not accurately delineated, so that it was not possible to ascertain the location of dense positive material within interendothelial spaces. The reaction product was rarely found in the intercellular spaces, but mainly in the adluminal portion and sometimes also in the abluminal portion, whereas the intermediate portion was badly stained or even unstained. Some positive plasmalemmal vesicles opened into the interendothelial space (Fig. 4).

Alveolar Lining Material

Usually, after fixation by airway infusion, the ALM was not preserved (Fig. 1), except in a few alveoli (Fig. 2). By contrast, after fixation by

vascular perfusion (Fig. 3) or by immersion (Fig. 5), a thick, granular, strongly positive ALM was observed. Positive intra-alveolar tubular myelin figures were also seen, appearing either as a regular square lattice (Fig. 5) or as parallel bands, with a regular spacing of a $473\text{-}\text{\AA}$ ($\pm 26\text{ }\text{\AA}$) period.

Control reactions carried out on sections incubated with nonspecific conjugate, with peroxidase alone, or with Graham-Karnovsky medium alone, gave negative results except for endogenous peroxidase activity (peroxisomes and red blood cells); in particular, the membranes of both endothelium and epithelium, of vesicles and the ALM were always negative.

DISCUSSION

The advantage of the method used in this work is that it enables one to locate an autologous serum protein, namely albumin, in the alveolar wall of the

All the figures represent frozen sections treated with HRP conjugate antirat albumin, postfixed in 2.5% OsO_4 , without further staining by uranyl acetate and lead citrate.

FIGURE 1 Pulmonary capillary of a normal adult rat fixed by airway infusion with 2.7% (550 mosM) glutaraldehyde. In the capillary lumen (C), the plasma (P) shows a diffuse, intensively electron-dense immunoperoxidase staining indicating the presence of albumin. The black staining of red blood cells (RBC) is due to the peroxidatic activity of hemoglobin. Some plasmalemmal vesicles (v) are seen open on the blood front of the endothelium (En). The endothelial and epithelial basement membranes (B) are markedly positive. With this method of fixation, there is no obvious positive reaction at the surface of the type I alveolar epithelial cell (Ep_1) (A: alveolus, IC: interstitial cell). $\times 28,200$.

FIGURE 2 Alveolar-capillary membrane of rat lung fixed by airway infusion with 2.3% (380 mosM) glutaraldehyde. There is a heavily positive material coating the surface of the endothelial cell (En) forming an endocapillary lining material. The reaction product has the same density at the level of numerous vesicles (v) (open or closed) as that seen on the capillary lumen (C) side of the endothelium. Fused endothelial and epithelial basement membranes (B) are positive. There are also some slightly positive open plasmalemmal vesicles (v) in the type I alveolar epithelial cell (Ep_1), communicating with the basement membrane (B). At the alveolar surface, the plasmalemma (p) of the type I epithelial cell (Ep_1) is positive and there is an accumulation of a heavily positive alveolar lining material (ALM) (A: alveolus). $\times 50,000$.

FIGURE 3 Rat lung fixed by vascular perfusion with 4% paraformaldehyde under 30 cm H_2O pressure. The capillary lumen (C) is free of plasma. The basement membranes (B) show an albumin-staining. Vesicles (v), more or less stained, are seen in endothelial (En) and epithelial (Ep_1) cells. There is abundant positive alveolar lining material (ALM) at the surface of a collapsed alveolus (A). $\times 24,600$.

FIGURE 4 Capillary endothelium from rat lung fixed by vascular perfusion with 4% paraformaldehyde under 30 cm H_2O pressure showing an interendothelial space (between the two arrowheads) with positive labeling of the plasmalemma (p). The albumin-positivity within the intercellular space is slight or even difficult to appreciate. Some pinocytic vesicles (v) are positive. The arrow indicates a positive vesicle opening into the interendothelial space. The basement membrane (B) is heavily stained (C, capillary lumen). $\times 32,000$.

FIGURE 5 Rat lung fixed by immersion with 2.3% (380 mosM) glutaraldehyde. The alveolar surface of a type I cell (Ep_1) is covered by an albumin-positive alveolar lining material (ALM). Positive tubular myelin figures (im) are seen in the alveolus (A) showing a square lattice of $480\text{-}\text{\AA}$ spacing, with two electron-dense albumin-positive lines separated by a gray line of $56\text{-}\text{\AA}$ width. $\times 114,800$.

lung of live rats under physiological conditions without intravenous injection of exogenous substances. The following points will be discussed: fixation procedures, presence of albumin in the alveolar wall and on the alveolar surface, and mechanisms of albumin transfer through the blood-air barrier.

Role of Fixation Procedures

In order to insure preservation of protein molecules during their movement across the alveolar-capillary membrane, several fixation procedures have been compared, the fixative acting from the alveolus, from the blood vessels, and from both. The best fixation could be the immersion method; but the very fact of cutting into fresh tissue before fixation entails the risk of displacing some of the plasma proteins. However, as was demonstrated in preliminary experiments, a prefixation by immersion of the lung in toto gave similar results as the immersion of freshly cut blocks of tissue. The airway infusion method presents the disadvantage of washing out the alveolar surface (20). During vascular fixation, the washing away of the vascular content by Ringer's solution changes the concentration gradient for albumin at the time of fixation, and the perfusion pressure, being difficult to control, could modify the capillary permeability (20).

Albumin in the Alveolar Wall

This immunoelectron microscope study brings an ultrastructural morphological demonstration of the presence of plasma albumin in the alveolar wall. This agrees with and illustrates the results of authors who have stressed the lymphatic clearance of plasma proteins from the pulmonary vascular bed (1, 2) or who have shown that radioactive albumin injected into the pulmonary circulation could be recovered in alveolar washings (3). However, the interstitial clearance pathway of albumin cannot be deduced from the present morphological study.

The presence of proteins, mainly serum albumin, in the ALM has been shown by numerous authors using various isolation methods, either homogenization of lung tissue (7), or alveolar lavage (8-10, 23), or alveolar micropuncture (24); but in all these studies to make a distinction between natural constituents of the surfactant and contaminants was difficult (24). Here, with the use of labeled antibodies, albumin was visualized *in situ* in the ALM. It must be stressed that lamellar

bodies of type II cells were never stained by the immunoreaction. This seems to indicate that the chemical components of the ALM are in part different from those of the lamellar bodies.

That the content of some vesicles within endothelial or type II cells was negative was probably not due to an uneven distribution of the immunological reagents because vesicles immediately contiguous were either dark (black) or clear (gray or positive only at the level of plasmalemma), but is more likely due to a different albumin concentration in vesicles or due to some vesicles containing substances antigenically different from albumin.

Considering the discontinuous labeling of most interendothelial spaces, the following interpretations are possible: either the method was not sensitive enough to detect a low or inhomogeneous concentration of albumin, or there were, between endothelial cells, junctions impermeable to albumin, as suggested by recent studies with tracers (25).

Mechanism of Albumin Transfer

The mechanism of albumin transfer through the blood-air barrier cannot be deduced from this static study showing only the distribution of autologous albumin at the time of fixation. The presence of albumin in numerous vesicles, apparently related to pinocytosis (26), in endothelial and type I cells suggests the importance of this mechanism in albumin transfer. By contrast, at the endothelial level the part played by interendothelial junctions cannot be finally established by this study, whereas at the alveolar epithelium level the absence of staining of the interepithelial cleft agrees with the notion of tight junctions between epithelial cells (13, 25) totally sealed to the passage of macromolecules of the size of albumin (27, 28).

SUMMARY

The location of autologous serum albumin within the alveolar-capillary membrane was studied in the rat under physiological conditions using anti-albumin antibodies labeled with peroxidase. Albumin was detected in the lung interstitium, and in numerous pinocytic vesicles within endothelial cells and type I alveolar epithelial cells. The immunoreaction was also positive at the level of plasmalemmal membranes of both cell types and in the alveolar lining material.

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