ACUTE EXPERIMENTAL PNEUMOCOCCAL (TYPE I) PNEUMONIA IN THE MOUSE: THE MIGRATION OF LEUCOCYTES FROM THE PULMONARY CAPILLARIES INTO THE ALVEOLAR SPACES AS REVEALED BY THE ELECTRON MICROSCOPE

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LOS ANGELES

One of us (C.G.L.) several years ago studied the pathology and pathogenesis of pneumococcal pneumonia in man[1] and experimentally induced Type I pneumococcal pneumonia in the monkey and dog.[2, 3] Following introduction of pneumococci into the lungs with a tracheal catheter, the animals were sacrificed at short intervals of time throughout the course of the disease. The lungs were prepared for histological examination by intratracheal fixation immediately after death. In the sectioned lungs of animals sacrificed from one to five hours after onset of the infection, large numbers of leucocytes could be seen in the act of leaving the capillaries.[2, 3] Fixed in the process of migration, they appeared as star-like clusters at the sites where they were entering the alveolar spaces.[1, 2, 3]

In spite of the intense migration and filling of the air spaces with leucocytes, fibrin, serous fluid, etc., the alveolar walls remain quite intact so that following resolution of the inflammatory reaction their normal architecture is maintained. This is a characteristic feature of pneumonia produced by pneumococcus organisms.

Of course the actual avenues of exit of the constituents of the inflammatory exudate could not be determined by examination of the sectioned tissues with the ordinary light microscope. The electron microscope has provided the opportunity to study further the reaction of the lung tissue in experimental pneumococcal lung infections in the mouse. This report then represents preliminary observations concerning the manner and avenues by which the blood leucocytes leave the capillaries and enter the

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alveolar spaces in the early phases, from one to four hours, after onset of infection.

Materials and Methods

A 10 ml. broth culture of pneumococcus (A5 Type I) was incubated for 6 to 8 hours. It was then partially concentrated by centrifugation. The sediment was resuspended in 3 ml. of fresh broth.

White mice weighing from 10 to 12 grams and lightly etherized, were inoculated intratracheally with 0.03 and 0.05 ml of the suspended organisms. Sufficient numbers were employed to sacrifice groups of three every half hour beginning one hour after infection. The experiment was repeated on three occasions.

When a mouse was to be sacrificed it was given intraperitoneally a lethal dose of nembutal. When fully asleep, it was quickly pinned to the the board; its trachea exposed, and chest opened. A ligature was placed about the heart to keep the blood in the capillaries.

Before fixation a hurried inspection was made to determine the extent of gross involvement of the lungs. Then one percent buffered (veronal) osmium tetroxide was injected slowly, intratracheally, until the lungs were re-expanded to chest cavity size. A ligature was placed about the trachea to hold the fixative in the lungs. They were then removed in toto, and placed in one percent buffered osmium tetroxide for one hour. After fixation areas of lung approximately 1 $mm.^3$ in size noted in the fresh state to show an inflammatory reaction were excized with a razor blade and further fixed for one and one-half hours.

The fixed pieces of tissue were then rapidly dehydrated in graded percentages of alcohol. After absolute alcohol the samples were placed in 50-50 absolute ethanol-propylene oxide, then 100% propylene oxide, and finally Epon 812, one of the epoxy resins. Initiator and accelerator had been pre-mixed with the resin. After soaking for 6 hours in the Epon 812, No. 00 gelatin capsules were filled with Epon 812 and 1 piece of tissue put in each capsule. The Epon was polymerized in the oven at 60° centigrade for 24–48 hours.

After polymerization the blocks were trimmed by hand with a razor blade in such a manner that the block face to be cut on the microtome was of the order of 0.25 mm^2. Ultrathin sections 300 AU-600 AU in thickness were cut from these blocks on an LKB Ultratome, using glass knives. Sections were floated onto a distilled water surface and then picked up on collodion coated 200 mesh copper grids. Staining was done with lead according to the Karnovsky method,[4] whereby the grids with attached sections are floated section side down on a dilute lead hydroxide solution. Some sections were stained with uranyl acetate. Carbon was evaporated

onto the sections to stabilize them in the electron microscope. Micrographs were made on an RCA EMU-3F electron microscope at magnifications ranging from 4000 ×–28000 ×.

The left lung of each animal, previously osmium fixed, was placed in formalin for 24 hours and sectioned by the paraffin method, stained with haematoxylin eosin, and examined with the regular light microscope to determine the extent of the inflammatory response.

Observations

The lungs of animals killed after one hour already showed areas of gross involvement. Multiple and coalescing areas of consolidation could be seen usually in the right upper and middle lobes and the upper half of the left lobe. As an animal was placed on its back during intranasal inoculation, it would be expected that the superior and posterior portions of the lungs would be the loci receiving the major portion of the aspirated pneumococcus culture. The amount of lung involvement varied considerably in the different animals of the same group. Generally there was more consolidation in the animals sacrificed in the final hour than at one hour.

In the sectioned tissues of the left lobe examined with the regular light microscope, the inflammatory reaction was confined generally to the alveoli near the terminal bronchioles. In many sections of lungs of animals sacrificed from two to three and one-half hours after inoculation, alveoli were seen to be filled already wih exudate cells (white and a few red blood cells), fibrin, serous fluid and pneumococci. In these areas the capillaries were congested with leucocytes and some of the walls showed star-like groups of cells fixed in the process of leaving the capillaries and entering the alveolar spaces.

Because of the differences in degrees of magnification and thinness of sections, it has been difficult to locate the star-like clusters of migrating cells under the electron microscope. The sections for examination with the light microscope were cut 7 microns in thickness. To study a similar area serially in depth with the electron microscope requires examination of 140 sections. However, enough electron microscopic observations have been made to show the probable path leucocytes take in passing from the blood capillaries into the alveolar spaces. The avenues of exit thus far observed are at the junctions of the endothelial and epithelial cells.

The alveolar respiratory membrane as revealed by the electron microscope is composed of three layers. A thin cytoplasmic surface lining the alveolar spaces (EP), a basement membrane (BM) and an endothelial cell surface (E) lining the capillary. The basement membrane contains elastic and reticular fibers (CT) which give support to the alveolar walls. Figure 1 shows these alveolar wall constituents and a lymphocyte (L) in

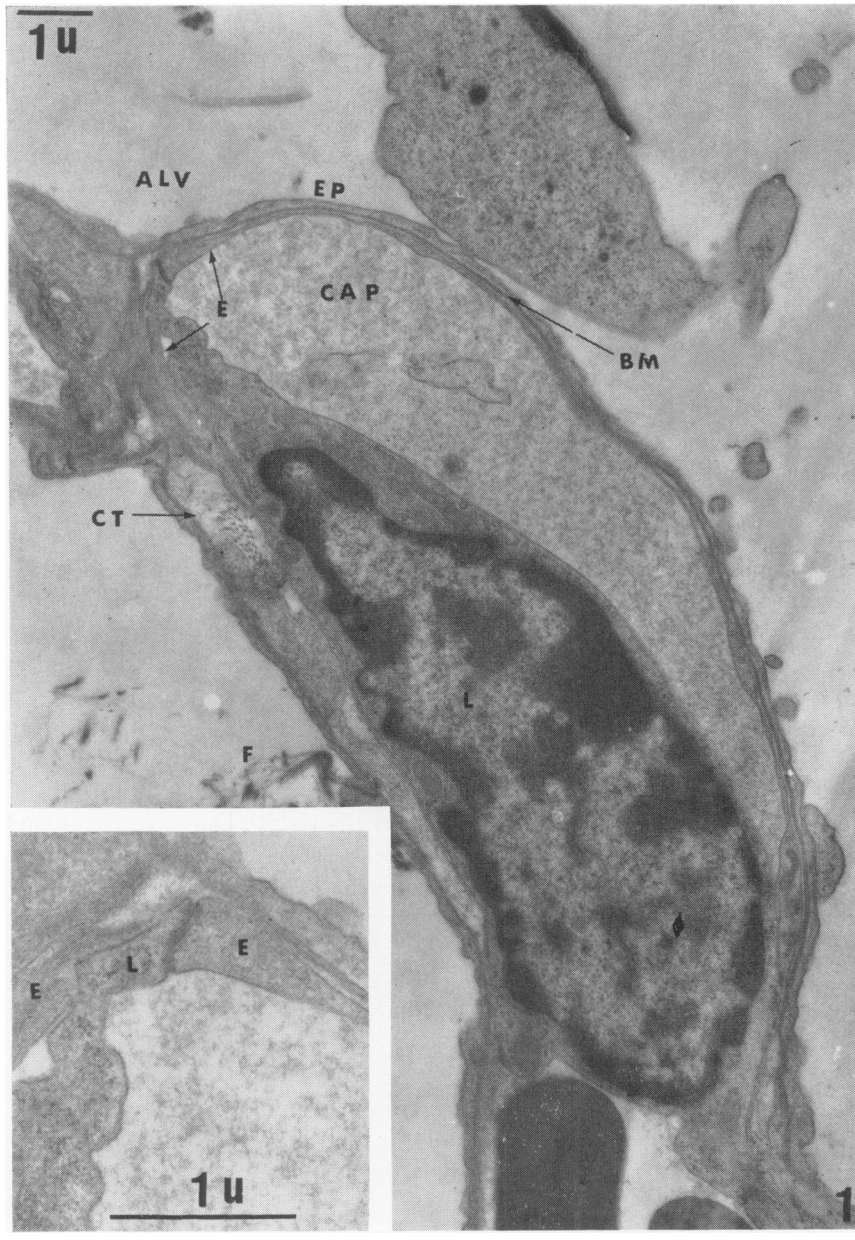

FIG. 1. This is a section of lung, three hours after infection, through the alveolar wall. It shows a leucocyte beginning to dissect its way (E, arrows) into the subendothelial space. The pseudopod of the lymphocyte (L) is shown in higher magnification in the lower left corner (insert). Connective tissue (CT) fibers can be seen in the basement membrane. The alveolar wall on the right with its epithelial cell (EP), basement membrane (BM) and endothelial cell (E) layers appears intact. Fibrin (F) can be seen in the alveolar space. × 18,000 and (insert) × 45,000 both reduced to plate size.

the capillary (CAP) with its cytoplasmic pseudopod penetrating the junction of two endothelial cells. The higher magnification (insert) in Figure 1 shows this clearly. The alveolar spaces contain fibrin (FB) and other cellular debris.

Cytoplasmic pseudopods of leucocytes are seen to extend considerable distances between the endothelial cells and the basement membrane as shown in Figure 2. During the process of emigration the leucocyte (POL) continues to "dissect" its way between the endothelium and basement membrane until it is completely enclosed by these boundaries as shown in Figure 3. In Figure 3 the polymorphonuclear leucocyte appears to be dissecting its way toward the epithelial cell junction (J) which appears as a dark condensation of cytoplasm.

Several cells have been seen in the process of leaving a capillary on the side where the connective tissue stroma is thickest. One such cell shown in Figure 4, probably a polymorphonuclear leucocyte, is shown clearly entering the space at the junction of two endothelial cells (arrows). The nucleus and the bulk of its cytoplasm are outside the capillary. The cytoplasm is in direct continuity with that in the capillary and separates the endothelial cells. Connective tissue fibrils (CT) can be faintly seen in the basement membrane (lower left). An epithelial cell (EP) with mitochondria can be identified. The respiratory membrane to the right shows a thickened edematous epithelial layer, a basement membrane and a thin endothelial layer.

Figure 5 shows clearly a polymorphonuclear leucocyte enclosed partly by the cytoplasmic layer of an endothelial cell and the basement membrane. It is near a thickened area of cytoplasmic epithelium which possibly represents a junction (J) where it may penetrate eventually and enter the alveolar space. The basement membrane appears to be swollen (ED).

It has been more difficult to identify the exact area where the leucocytes, after dissecting their way under the endothelium, penetrate the basement membrane and the epithelial membrane. Figure 5 shows a leucocyte near an epithelial junction. Figure 6 shows two leucocytes in the subendothelial spaces. The cytoplasm of the leucocyte in the upper part of Figure 6 has penetrated the basement membrane and the epithelial cell layer at or near the junction (J), and its cytoplasm protrudes into the alveolar space. Figure 6 also shows a leucocyte (POL) cut in such a way as to be completely surrounded by endothelial cell cytoplasm (E). On the opposite wall can be seen an epithelial cell and thickened endothelial cell cytoplasmic layer mitochondria. A mitochondrian (MI) can be seen in the condensed cytoplasm of the endothelial cell. On the right except where the leucocyte has penetrated the epithelial cell cytoplasm, the alveolar covering appears to be intact.

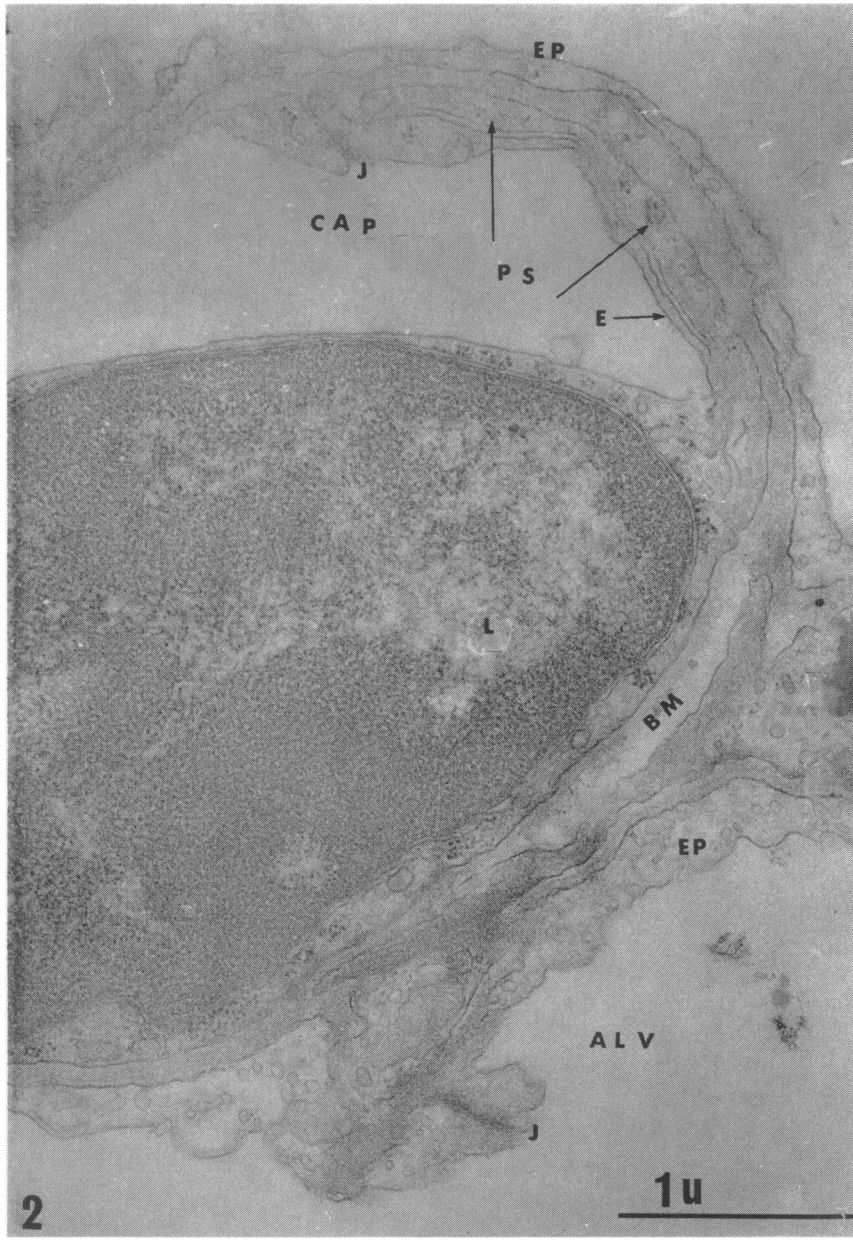

FIG. 2. This is a section of lung three hours after onset of infection. It shows a leucocyte (L) which has begun to dissect its way into the subendothelial space. Note the long pseudopod (PS) between the endothelium (E) and the basement membrane (BM). An endothelial cell cytoplasmic junction (J) can be seen on the capillary side (CAP) of the alveolar wall while an epithelial cell (EP) junction (J) can be seen on the air (ALV) space side of the alveolar wall. × 45,000 reduced to plate size.

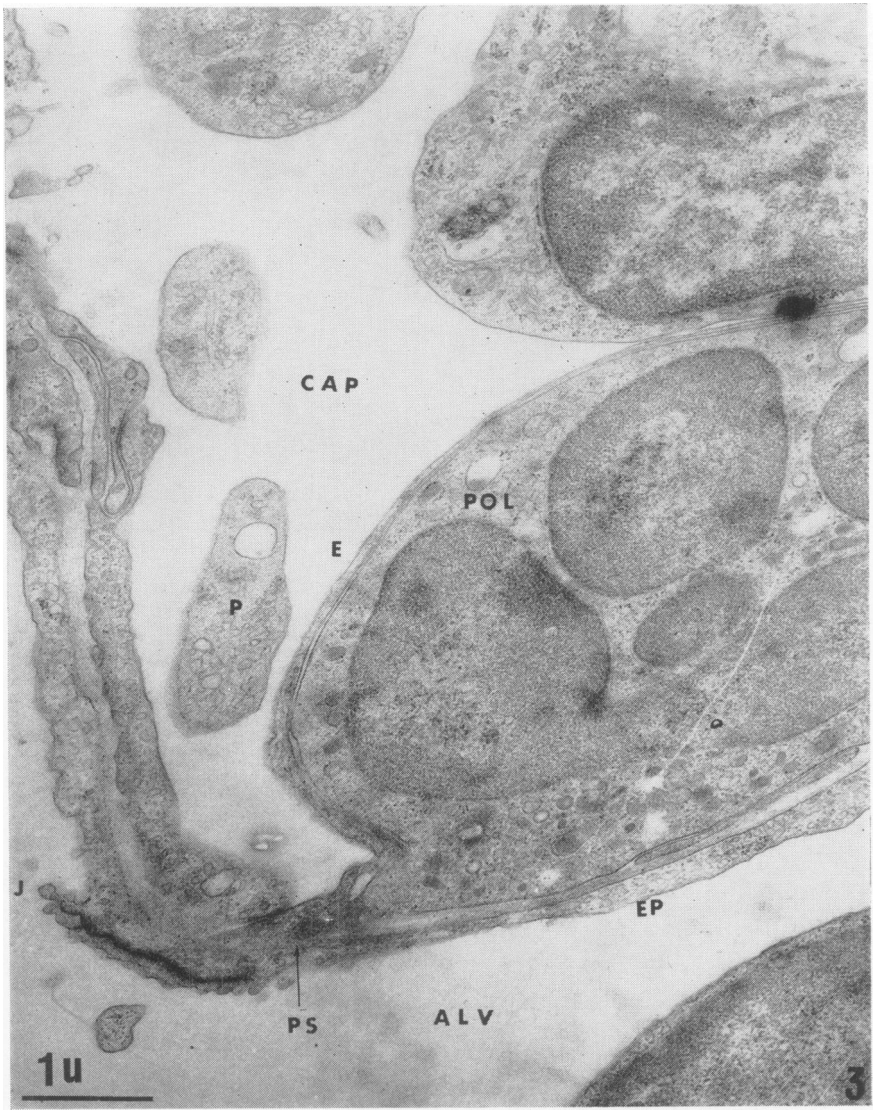

FIG. 3. This is a section of lung three hours after onset of infection taken through a leucocyte (POL) which is in the subendothelial space and is dissecting its way into the basement membrane (arrow) near the cytoplasmic junction (J) of two epithelial cells. A platelet (P) is seen in the capillary lumen. × 25,000 reduced to plate size.

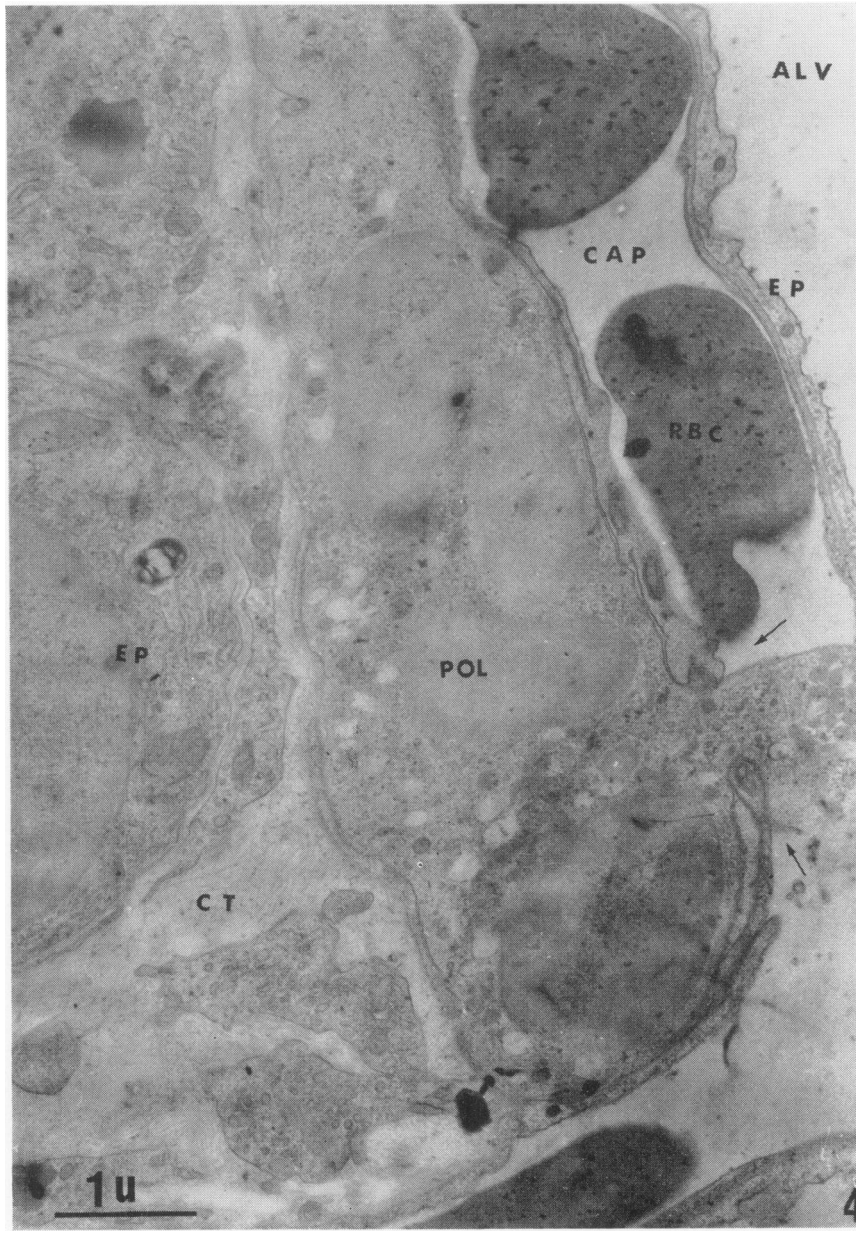

FIG. 4. This is a section of lung three hours after onset of infection. It shows a leucocyte (POL) in the process of leaving the capillary (CAP) at the endothelial cell junction. Some of its cytoplasm can clearly be seen in the capillary lumen. The epithelial cell (EP) can be seen on the left. The epithelial cell membrane (EP) on the right is somewhat thickened, but continuous. × 25000 reduced to plate size.

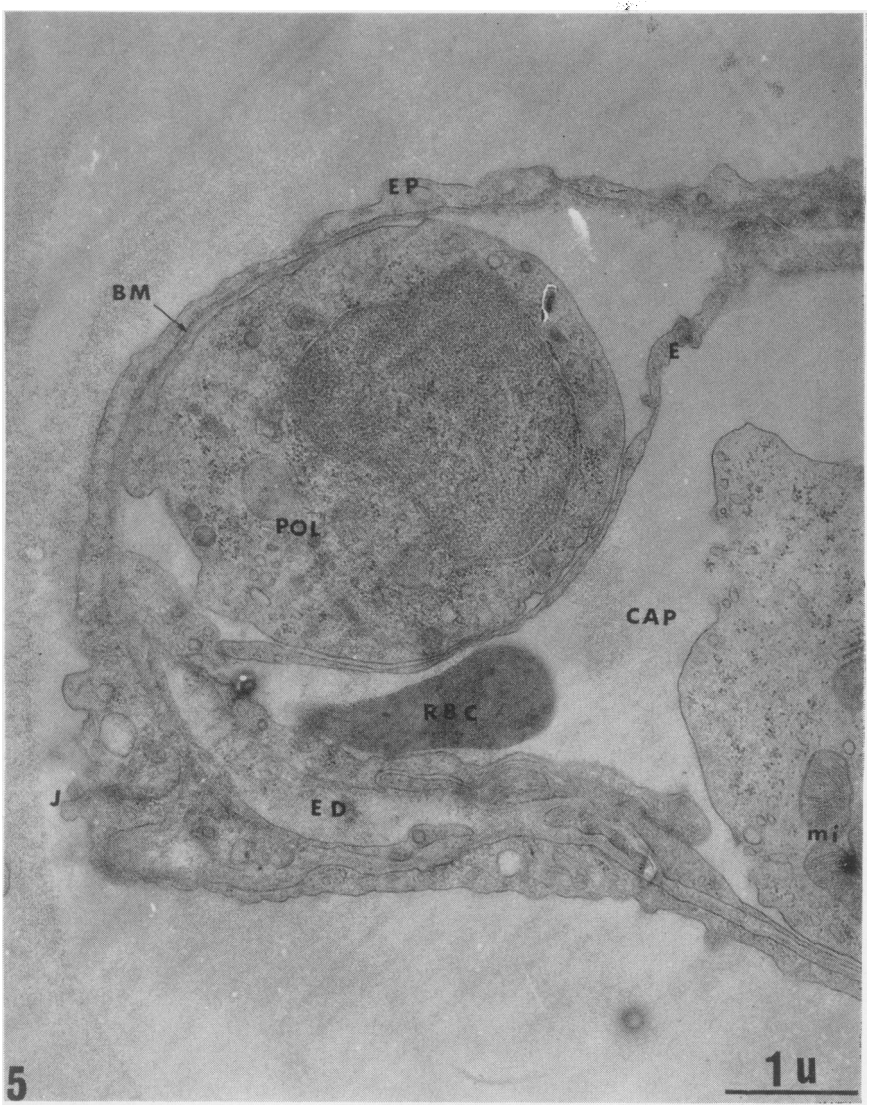

FIG. 5. This shows a leucocyte (POL) between the basement membrane (BM) and endothelial cell layer (E) outside the capillary lumen (CAP). There is edema (ED) in the wall area and a thickening of the epithelial cell layers (EP) at the junction (J) of two cells. Note the proximity of the leucocyte near the epithelial cell junction. × 25,000 reduced to plate size.

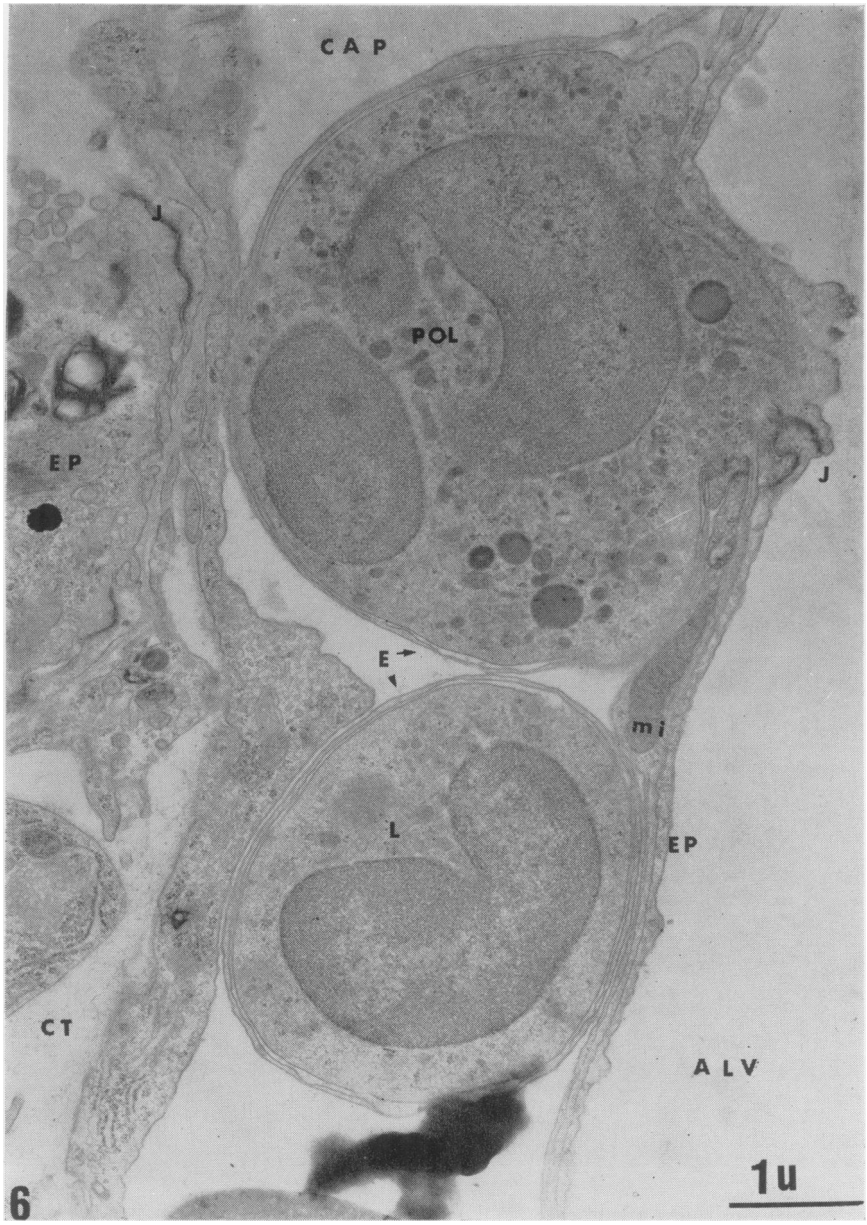

FIG. 6. This is a section of lung three hours after onset of infection. It shows a pulmonary capillary (CAP) cut longitudinally with two leucocytes in the subendothelial spaces. The lower cell (L) cut tangentially appears completely surrounded endothelial cell cytoplasm (E). The upper cell (POL) is breaking through the basement membrane and cytoplasm of epithelial cell (EP) at or near an epithelial cell junction (J). × 25,000 reduced to plate size.

In most of the lungs which showed an inflammatory reaction, pneumococci, both free and intracellular, were observed in varying numbers in the alveolar spaces. Figure 7 is the lung of an animal killed 1½ hours after onset of infection. It shows a polymorphonuclear leucocyte filled with phagocytized pneumococci. Others are seen free or in the process of being ingested. The leucocyte shown in Figure 7 appears to be lying in an alveolar pore. Other areas have been observed which show leucocytes ingesting fibrin debris.

The capillary in the upper right of the micrograph is filled with a leucocyte while red blood cells can be seen in the capillary in the lower left. In Figure 7 the cytoplasmic membranes covering the alveolar surfaces adjacent to the pore appear to be intact. However, other areas, not shown in this series of photographs, could be seen where the epithelial membranes showed "bleb" formation and actual rupture. These changes appeared to have no relation to the emigration of leucocytes from the capillaries. No fenestrations in either the endothelial or epithelial membranes were noted. In some areas one or all three layers comprising the alveolar walls were thickened due probably to edema fluid.

Discussion

This study must be considered a preliminary one. Attention has been paid only to the avenues of exit of leucocytes from the capillaries into the alveolar spaces in the acute phases of the pneumococcal infection. No other avenue than at the junctions between the endothelial and epithelial cells have been observed. Presumably all varieties of leucocytes emigrate from the pulmonary capillaries as observed in this study. How red blood cells, serous exudate penetrate the alveolar membranes to fill the air spaces as the inflammatory reaction develops is yet to be elucidated. No fenestrations in the endothelial or epithelial membrane have been noted so far.

Florey made a study of the endothelium of normal and inflamed small blood vessels of the mouse and rat colon.[5] He observed that "parts of the endothelium are tenuous and show fenestrations which are closed by a thin membrane which did not appear to be particularly permeable to ferritin or disrupted in inflamed vessels." Marchesi and Florey[6] have made electron microscopic observations on the emigration of leucocytes from the small vessels in the rats' mesentery after mild trauma which caused an inflammatory reaction. They observed in their first study that the leucocytes passed through the vessel walls at or near the endothelial junctions as well as directly through the cytoplasm. In a later study Marchesi presents several beautiful electron micrographs of cells passing through the intercellular junctions. Marchesi has more recently observed

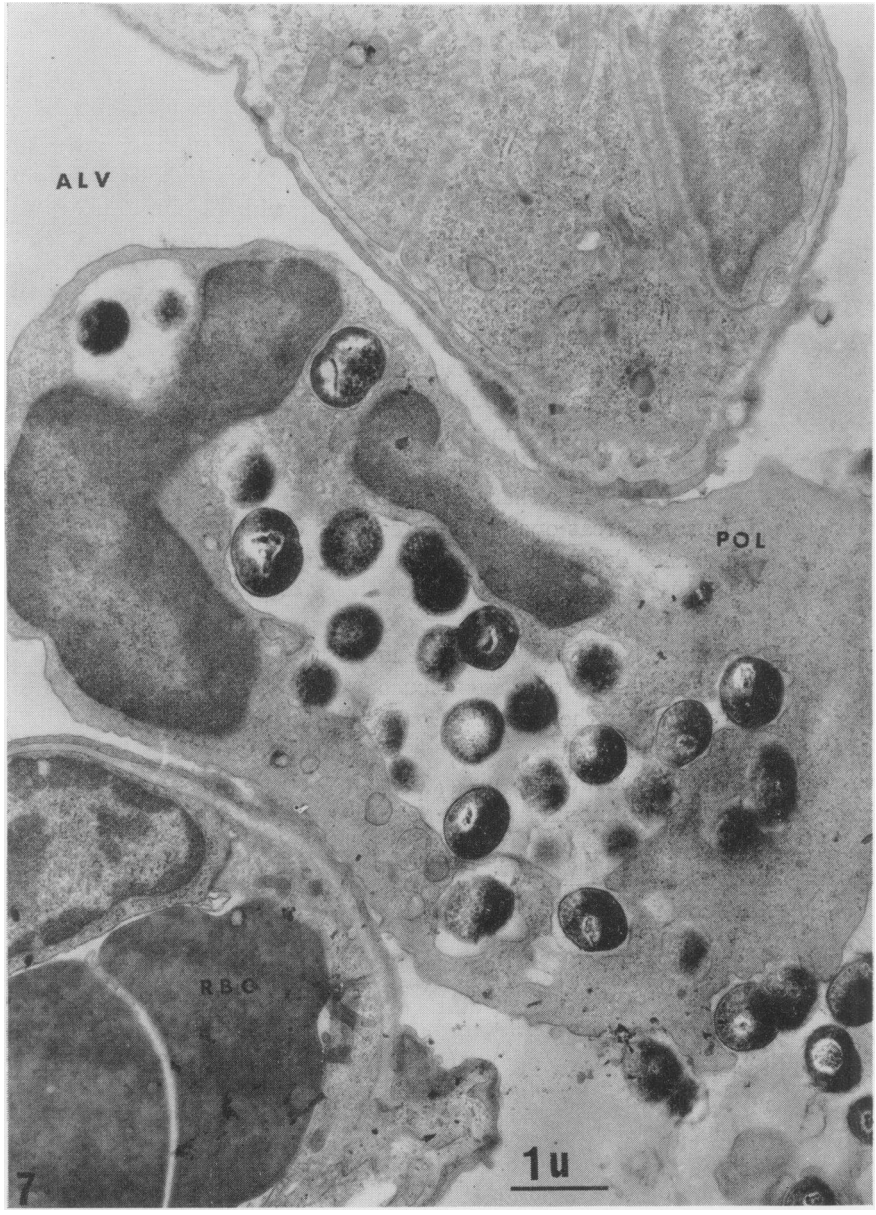

FIG. 7. A section of lung one and one half hours after inoculation shows a leucocyte filled with pneumococci. Other pneumococci are free and in the process of being ingested. The leucocyte is wedged in an alveolar wall pore with one capillary containing red blood cells (RBC) and the other a leucocyte. The alveolar membranes appear to be intact. × 18,000 reduced to plate size.

that colloidal carbon also passes chiefly through the intercellular junctions of the endothelium in the inflamed mesentery of the rat.[8] Our observations confirm those of Marchesi and Florey with respect to the avenues of exit of leucocytes from the lung capillaries into the air spaces.

The pulmonary respiratory membrane through which the leucocytes must pass is a living one and many factors must be considered in interpreting the above findings.[9]

Summary

In this preliminary study of experimental pneumococcal pulmonary pneumonia in the mouse the leucocytes were observed to pass from the capillaries into the interstitial tissue and eventually into the alveolar spaces through the intercellular junctions of the endothelial and epithelial cell membranes.

Explanation of Symbols on Figures One to Seven

ALV, alveolar space; CAP, capillary lumen; E, capillary endothelium; BM, basement membrane; CT, connective tissue; J, cell junction; PS, pseudopod; EP, epithelial cell; L, lymphocyte; POL, polymorphonuclear leucocyte; ED, edema; RBC, red blood cell; MI, mitochondria.

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DISCUSSION

Dr. Richard France (Nashville): I would like to ask a question concerning the relationship of the traveling leucocyte to the basement membrane. Does the base-

ment membrane offer a barrier to the leucocyte? Is there evidence that the leucocyte may pass through a fenestration in the membrane?

DR. ROBERT AUSTRIAN (Philadelphia): I suppose I should know but I do not. Does the mouse lung ever show red hepatization following inoculation of the type you have employed? And may I ask also whether or not you have given adrenocorticosteroids to any of these animals to see what effect such treatment would have on the behavior of their leukocytes?

DR. LEMUEL MCGEE (Wilmington): I wonder if Dr. Loosli has any evidence of the probable function on the cleft found in the epithelium.

DR. MORTON HAMBURGER (Cincinnati): Dr. Loosli, many years ago I believe you did some work demonstrating that the capillaries of the lungs were not surrounded by an epithelial lining, in certain species. There has been a good deal of controversy about this point over the years. Am I correct in interpreting this new work as indicating that the original thoughts were right and that the alveolar wall is in contact directly with the alveolar sacs?

DR. CLAYTON G. LOOSLI (Closing): With respect to the first question about the basement membrane, we are looking to see what happens to the basement membrane and we are finding that it stays pretty much intact. However, we have not yet observed points where a leucocyte goes through the membrane. One must recognize that sections are from 3/100's to 6/100's of a micron in thickness compared to 7 microns for the light microscope section. Therefore, one can see the problem we are up against in identifying a particular point in the wall where the leucocyte might have or be going through.

As far as I know, mouse pneumonia does not go through a stage we call lobar pneumonia. It does not go through a stage called red hepatization. However, red cells do appear in the alveolar spaces; whether they pass through the intercellular junctions or directly as a result of traumatic disruption of the capillaries has not been determined.

With respect to steroids, we have not tried steroids in this study to see if it affects the inflammatory process. As to the nature of the blebs, these were observed in very early stages after infection in mice killed from one and a half hours to three and a half hours. Therefore, I think the bleb represents a process caused by the serous fluid that pours out into the alveolar spaces and does not represent a reparative process.

With respect to the question about the nature of the epithelial lining raised by Dr. Hamburger, the electron microscope presents incontrovertible evidence that this is a living cytoplasmic membrane which lines the alveolar spaces. Whether these cells are epithelial or endothelial in origin remains yet to be settled.