PULMONARY HYALINE MEMBRANES STUDIED WITH THE ELECTRON MICROSCOPE*

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The "pulmonary hyaline membrane syndrome" has been well established as a clinical entity,[1-3] though its etiology is still debated. The pathologic changes in this disease are characterized by an eosinophilic membrane lining alveolar ducts and filling some alveoli as well as by atelectasis and hyperemia of the lungs.[1,4] The literature concerning this subject has been thoroughly reviewed by De and Anderson.[2]

The precise localization of the hyaline membrane has been in question. Gilmer and Hand[3] have stated that the hyaline membrane lies between an endothelial basement membrane and an epithelial basement membrane. However, most investigators believe that the hyaline membrane lies over the surface epithelium of the alveolar ducts and alveoli.

Histochemical methods have failed to specifically identify components of the hyaline membrane, until the recent work of Gitlin and Craig[5] who used a fluorescein-labeled antibody to demonstrate the presence of fibrin in the hyaline membrane.

The light and electron microscopic studies reported here were undertaken with the hope that the pulmonary hyaline membrane of human infants and experimental animals could be more definitely localized, that their fine structure could be demonstrated, that their content could be identified, and that some light might be shed on their etiology.

MATERIAL AND METHODS

Examinations were made of lung tissue from three sources, as follows: (1) from 37 premature human infants who died in the neonatal period, (2) from ten young adult guinea pigs in which pulmonary hyaline membranes had been experimentally produced, and (3) from five normal guinea pigs as controls for the second group. In about 50 per cent of the 37 human cases, lung specimens were essentially normal and served as controls for the pathologic material.

Immediately after death of infants in the neonatal period, specimens

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of lung were obtained by a technique devised by one of us (H.B.N.). A No. 4 Novak curette was passed into the trachea (under direct vision) and then into a major bronchus; as resistance was encountered, the curette was forced into the lung parenchyma and a piece of lung was removed which measured 2 to 3 mm. The tissue was immediately immersed in fixative. This method fulfilled requirements as follows: The specimens were obtained immediately after death without external mutilation of the body; adequate material was obtained; the procedure was simple and quick, insuring adequate preservation of the tissue for electron microscopy.

Young adult guinea pigs of both sexes were exposed to an atmosphere of about 98 per cent oxygen and about 98 per cent humidity at a pressure of 760 mm. of Hg. After exposure to these conditions for about 72 hours, the guinea pigs were sacrificed by a blow on the head, and pieces of lung were immediately cut and immersed in fixative.

Pulmonic tissue from each infant and from each guinea pig was cut into several pieces for fixation in 10 per cent formalin or in osmium tetroxide. The tissue fixed in formalin was processed routinely for light microscopy, sectioned at 5 μ, and stained with hematoxylin and eosin or with Gomori's trichrome stain. This was done for orientation and comparison, as well as for initial evaluation of the specimen to determine the presence or absence of hyaline membranes. For phase contrast microscopy and electron microscopy, tissue was fixed for 12 to 15 minutes in 1 per cent osmium tetroxide made up in 25 per cent Tyrode solution and adjusted to pH 7.4 to 7.6. After fixation, the tissue was washed in 70 per cent methanol, dehydrated in methanol over a period of about 1 hour, and embedded in n-butyl methacrylate, using dichlorobenzoyl peroxide as polymerizing catalyst.

In order to compare the structure of plasma clot and hyaline membrane, plasma clots were fixed and prepared in the same manner as the pulmonic tissues.

For direct light microscopy, the tissues embedded in plastic were sectioned at 2 μ with a Spencer rotary microtome modified with a wedge for thin sectioning. The sections were treated with toluene for 20 minutes and with xylene for 30 minutes to remove the plastic and were subsequently stained with hematoxylin and eosin; bleaching with hydrogen peroxide before staining produced brighter staining. For phase contrast microscopy, the tissues embedded in plastic were sectioned at 2 μ, treated with xylene for 10 to 15 minutes to remove part of the plastic, and then covered with a coverglass without staining. For electron microscopy, tissues embedded in plastic were sec-

tioned at 0.025 to 0.05 μ with a Sorvall cantilever microtome. As a substrate for mounting the sections, specimen holders for the electron microscope were covered with a very thin film of parlodion which was then covered with a film of carbon in a high vacuum evaporator; the parlodion film was not removed. A Philips electron microscope (EM-100A) was used. The Philips specimen holders have a viewing field 1 mm. long and 0.2 mm. wide, making it possible to study large areas of the sections. The carbon film stabilized the section in spite of this large area.

These four successive phases of study of pulmonic tissue, i.e., stained paraffin sections, stained plastic sections by bright field light microscopy, unstained plastic sections by phase contrast microscopy, and thin plastic sections by electron microscopy, yielded adequate data for orientation, comparison, and interpretation.

Observations

As illustrated by photomicrographs in Figures 1, 2, and 3, typical pulmonary hyaline membrane was found in about one fourth of the pulmonic specimens of the human infant and in about one half of those of the treated guinea pigs studied with the light microscope. Since the hyaline membrane seen in infant and guinea pig was almost identical in structure and in composition,[6] subsequent statements about hyaline membrane will refer to both groups.

When the specimens which contained hyaline membrane were studied with the electron microscope, the membrane was identified and detailed structure and relationships were observed (Figs. 4, 5, and 6). The hyaline membrane is apparently made up of cell débris, plasma proteins, and fibrin in various stages of polymerization. Tentative identification of these components of the hyaline membrane was made on a morphologic basis, as follows: The cell débris was compared to structures occurring in intact cells (structures such as nuclei, mitochondria, vacuoles, and endoplasmic reticulum) Figure 6; the finely granular material in the alveoli was compared to the finely granular plasma proteins in the capillaries (Fig. 5); and the matrix of the hyaline membrane, in which the constituent fibrils sometimes show a periodicity, was compared to the fibrillar structure and periodicity seen in a plasma clot (Figs. 7 and 8). A section of hyaline membrane may be compared directly to a section of a plasma clot, since both show the same component structures, as follows: cell débris, plasma remnants, incompletely polymerized fibrin, fibrin fibrils without periodicity, and fibrin fibrils with periodicity.

In sections studied with the electron microscope the hyaline membrane was found within the air spaces and overlying the epithelium lining the alveoli, alveolar ducts, and bronchioles. The epithelial lining was intact, as in Figure 4, except for scattered small breaks. At some sites, the epithelial cells were vacuolated and appeared to be partly degenerated (Fig. 9) and, more rarely, the basement membrane under the disrupted epithelium seemed to be partly disintegrated also. This was possibly the site of leakage of the plasma.

A complete break-through of the capillary-alveolar wall was rarely seen. At such sites, hemorrhage into the air space was evident. In general, the hyaline membrane contained few erythocytes, the cell débris being mostly from other cell types, probably from macrophages, white blood cells, and epithelial cells.

The presence of the hyaline membrane apparently stimulated phagocytic activity of macrophages, segmented neutrophils, and even epithelial cells. The number of macrophages and segmented neutrophils was increased, so that they often appeared in or near the hyaline membrane (Figs. 1, 2, 3, 9, and 10). Macrophages contained varying amounts of ingested hyaline membrane (Figs. 9 and 10), some cells apparently being full of ingested material. Figures 10 and 11 illustrate a common observation, a macrophage fixed in the process of phagocytosis and pinocytosis.

Discussion

Early during our electron microscopic studies of the structure of the hyaline membrane in lungs of premature human infants and treated guinea pigs, we became convinced that the matrix of the hyaline membrane was fibrin-like material. This led us to examine sections of a plasma clot, in which we found cell débris embedded in a matrix of fine fibrils of fibrin. In some parts of the sections of the clot a characteristic periodicity could be demonstrated in the fibrils (Fig. 7). In some specimens of lung, as in Figure 5, the relationship of un-polymerized plasma-like material and partially polymerized fibrillar matrix of the hyaline membrane was demonstrated. In hyaline membrane that was more completely polymerized or clotted, periodicity characteristic of fibrin fibrils was seen (Fig. 8). Fibrin clots studied with the electron microscope have shown variations with clotting duration, thrombin concentration, and pH.[7-9] When one compares results of such studies with our observations, the varied appearance of the matrix of the hyaline membrane from finely granular to fibrillar can be explained as successive stages of clotting. Because of the appearance of the fibrillar matrix and because we found sites of partial breakdown of the capillary-alveolar wall (Fig. 9), we are convinced, on a morpho-

logic basis, that the source of the matrix of hyaline membrane is the blood plasma which leaks from capillaries into air spaces and that the hyaline matrix is fibrin in various stages of clotting. [6]

While our morphologic studies were in progress, Gitlin and Craig[5] published results of their work with fluorescein-labeled fibrin antibody, demonstrating the abundance of fibrin in pulmonary hyaline membrane. Gitlin and Craig also concluded that hyaline membrane was formed from an effusion from the pulmonary circulation. They further stated that "conversion of fibrinogen to fibrin in pulmonary effiusions can and does take place *in vivo* without addition of any exogenous materials. Hence, amniotic fluid need not be an essential ingredient for the production of hyaline membranes alone as is apparent in the occurrence of these membranes in various disease states such as uremia (where thromboplastin may be supplied by tissue damage) or even in rats exposed to 100 per cent oxygen for prolonged periods; the membranes in these instances have also been demonstrated to be composed of fibrin." Our conclusions based on morphology are substantiated by the immunologic studies of Gitlin and Craig.

Gilmer and Hand[3] stated that the hyaline membrane may be found between an endothelial basement membrane and an epithelial basement membrane. They believed that this indicated the endogenous origin of the material, which they considered may be derived from the blood stream, may represent a change within the respiratory basement membrane, or may represent a change in the connective tissue substances between the two membranes. In disagreement with the statements of Gilmer and Hand, our studies show that the hyaline membrane lies in the air space over the surface of the epithelium lining the alveolar ducts and alveoli (Figs. 4 and 5). Furthermore, we have noted repeatedly that pulmonary capillary endothelium and alveolar epithelium may *share* a single basement membrane (Fig. 4). It is also true that endothelium and epithelium may be separated by fibrous connective tissue (Fig. 4); in this situation each cell layer has its own basement membrane. However, it is clear that neither the basement membrane nor the connective tissue space is the site of the hyaline membrane.

Various structures were found embedded in the matrix of the hyaline membrane (Fig. 6). In Figure 6 and in other material studied, the following components of the hyaline membrane were identified: a fibrin matrix; scattered remnants of erythrocytes or very few intact erythrocytes; cell débris such as endoplasmic reticulum, mitochondria, cytoplasmic vacuoles as found in macrophages and degenerating epithelial cells, and granules as found in neutrophilic leukocytes.

Our observations of an epithelial lining of the alveoli corroborate

those of Low.[10,11] An attenuated cytoplasmic layer extends from the perinuclear region of the "septal" cell and covers the neighboring capillaries, collagenous connective tissue, and fibroblasts (Fig. 4), forming a normally complete or nearly complete epithelial lining of the alveoli and alveolar ducts. Macrophages and white blood cells of various kinds may occasionally be found enclosed in the connective tissue of the septum. Alveolar cells—that is, free cells in the air space—are mostly macrophages and some are polymorphonuclear leukocytes. Both macrophages and polymorphonuclear leukocytes occur in greater number after irritation of the lung. We often have seen evidence of pinocytosis in the epithelial cells and what appeared to be evidence of phagocytosis in this cell.

As noted previously, broken capillary-alveolar walls were rarely found. The cause of such break-through and hemorrhage into the air spaces was uncertain. This could have been artifact produced by the stress of removal of tissue with the curette. However, such hemorrhage is a common pathologic finding in association with pulmonary hyaline membranes and is to be expected when capillaries are engorged as they are in this syndrome. The broken capillary walls that were observed in sections studied with the electron microscope could have been part of the pathologic picture and had all the appearance of being so. As for the sites of damage to, and partial "erosion" of, the epithelial lining of alveoli and alveolar ducts, these were more prevalent and were more obviously pathologic. The epithelial cells in these regions were vacuolated, loosened from the basement membrane, and disintegrated at some points. At the latter sites, the basement membrane and endothelium may have been sufficiently affected to increase their permeability and allow the passage of plasma proteins. After leakage of the plasma and formation of hyaline membrane, respiratory distress may be aggravated by shock, sludging of the blood, and engorgement of the pulmonary capillaries. Stickiness of the red blood cells, characteristic of sludging, was noted in the electron microscopic studies of much of the pathologic material.

In human infants, the pathogenesis of pulmonary hyaline membranes may depend upon a series of factors, such as "extrinsic," "predisposing," "precipitating," and "aggravating" factors suggested by Bruns and Shields.[12,13] Perhaps the last two factors would constitute the pathogenesis of hyaline membranes in guinea pigs. This report is primarily concerned with the results produced by the precipitating factor, that is, injury to the epithelium of the alveoli and alveolar ducts with resultant formation of hyaline membranes.

Summary and Conclusions

Light and electron microscopic studies were made of pulmonic tissue from premature human infants, young adult guinea pigs which were exposed to an atmosphere of high oxygen and high humidity, and normal guinea pigs. Light microscopy was used to locate specimens of lung which contained typical hyaline membrane. Then the electron microscope was used to study the detailed structure of the hyaline membrane, to identify its components, and to determine its location and relationships to septal structures.

The hyaline membrane was found in the air spaces, overlying the epithelial lining of the alveoli and alveolar ducts. It was made up largely of a finely fibrillar matrix, apparently fibrin, probably derived from plasma which leaked from the pulmonary capillaries. In the fibrin matrix was enmeshed cell débris, such as nuclei, mitochondria, endoplasmic reticulum, and vacuoles.

The presence of the hyaline membrane apparently stimulated phagocytic activity of the macrophages and occasionally of the alveolar epithelial cells. The number of macrophages and neutrophilic leukocytes was increased, so that they often appeared in or near the hyaline membrane.

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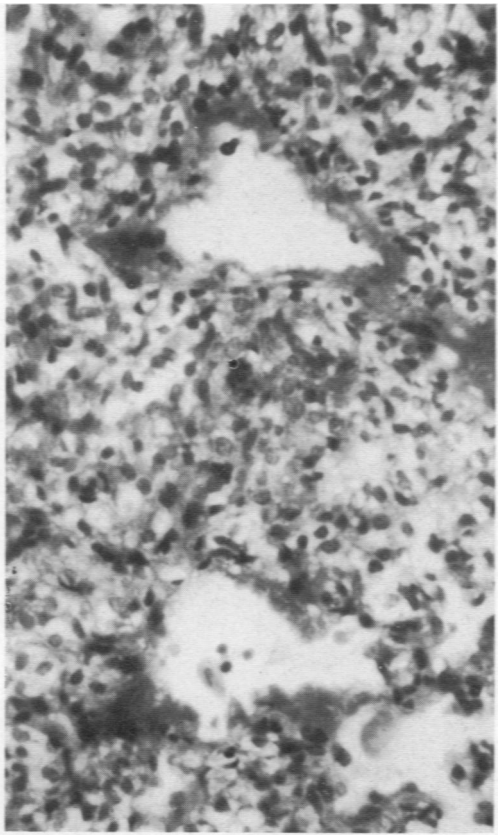
LEGENDS FOR FIGURES

FIG. 1. Premature human infant lung (case 29). The tissue was fixed in formalin, embedded in paraffin, cut at 5 μ, and stained with hematoxylin and eosin. Two alveolar ducts are lined with hyaline membrane and surrounded by atelectatic lung. Photomicrograph, × 155.

FIG. 2. Premature human infant lung (case 29). The tissue was fixed in osmium tetroxide, embedded in plastic, sectioned at 2 μ, and stained with hematoxylin and eosin. Better preservation of cells and tissue is very apparent. Alveolar ducts are lined with hyaline membrane and surrounded by partly atelectatic lung. An alveolus adjacent to the lower duct is filled by hyaline membrane. The lower duct and at least one alveolus contain plasma. Photomicrograph, × 155.

FIG. 3. Lung, guinea pig, exposed to about 98 per cent oxygen and about 98 per cent humidity. The tissue was fixed in osmium tetroxide, embedded in plastic, sectioned at 2 μ, and mounted unstained. In the upper half of the photomicrograph is a large macrophage and hyaline membrane in an alveolar duct. In the lower part of the picture (arrow) is an alveolus containing hyaline membrane and plasma; the alveolus is surrounded by collapsed lung. Phase contrast photomicrograph, × 630.



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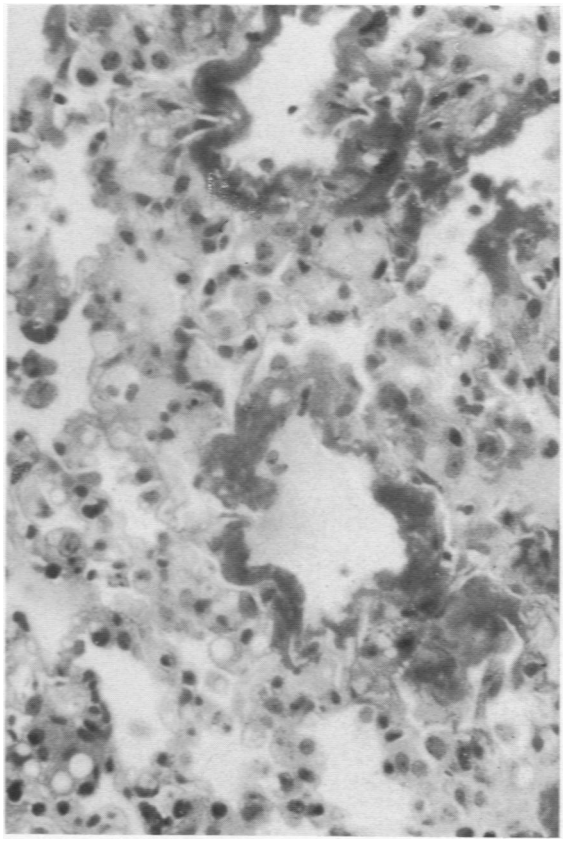

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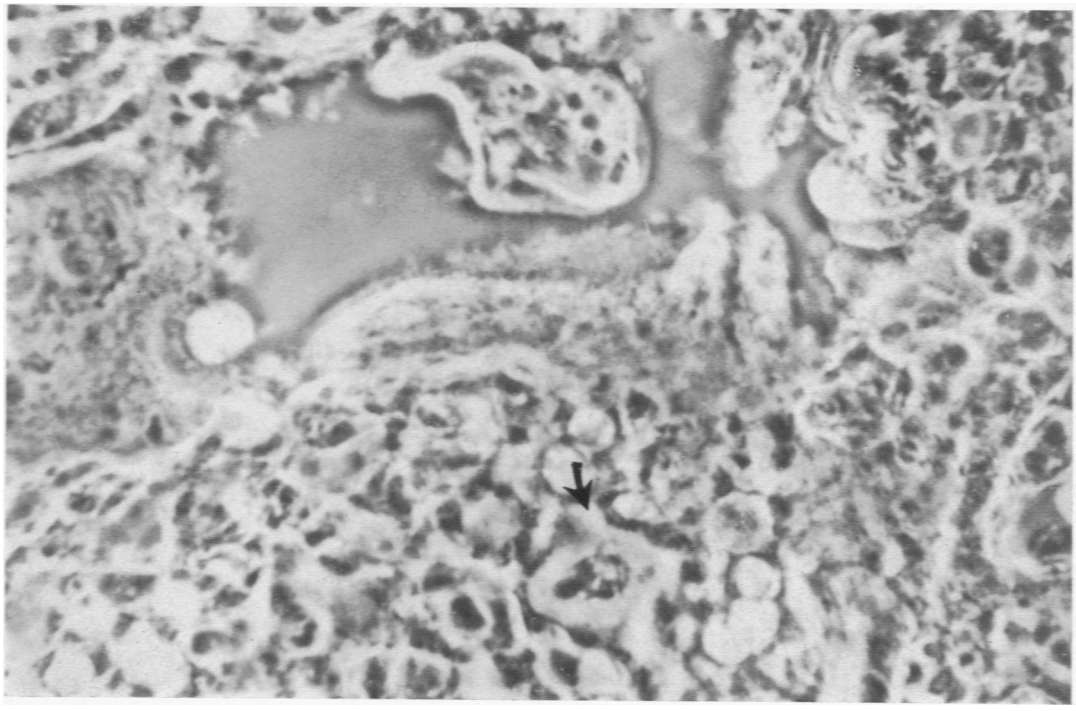

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FIG. 4. Premature human infant lung (case 29). Hyaline membrane is seen in the air space at the top of the figure. The large cells with round nuclei are epithelial cells, from one of which an attenuated cytoplasmic layer extends over the adjacent capillary (lower left). The epithelium is separated from the endothelium by a single basement membrane (a). At (b) is a subepithelial space containing some collagenous fibrils. In the lumen of the capillary are two red blood cells. Electron micrograph. × 4,620.

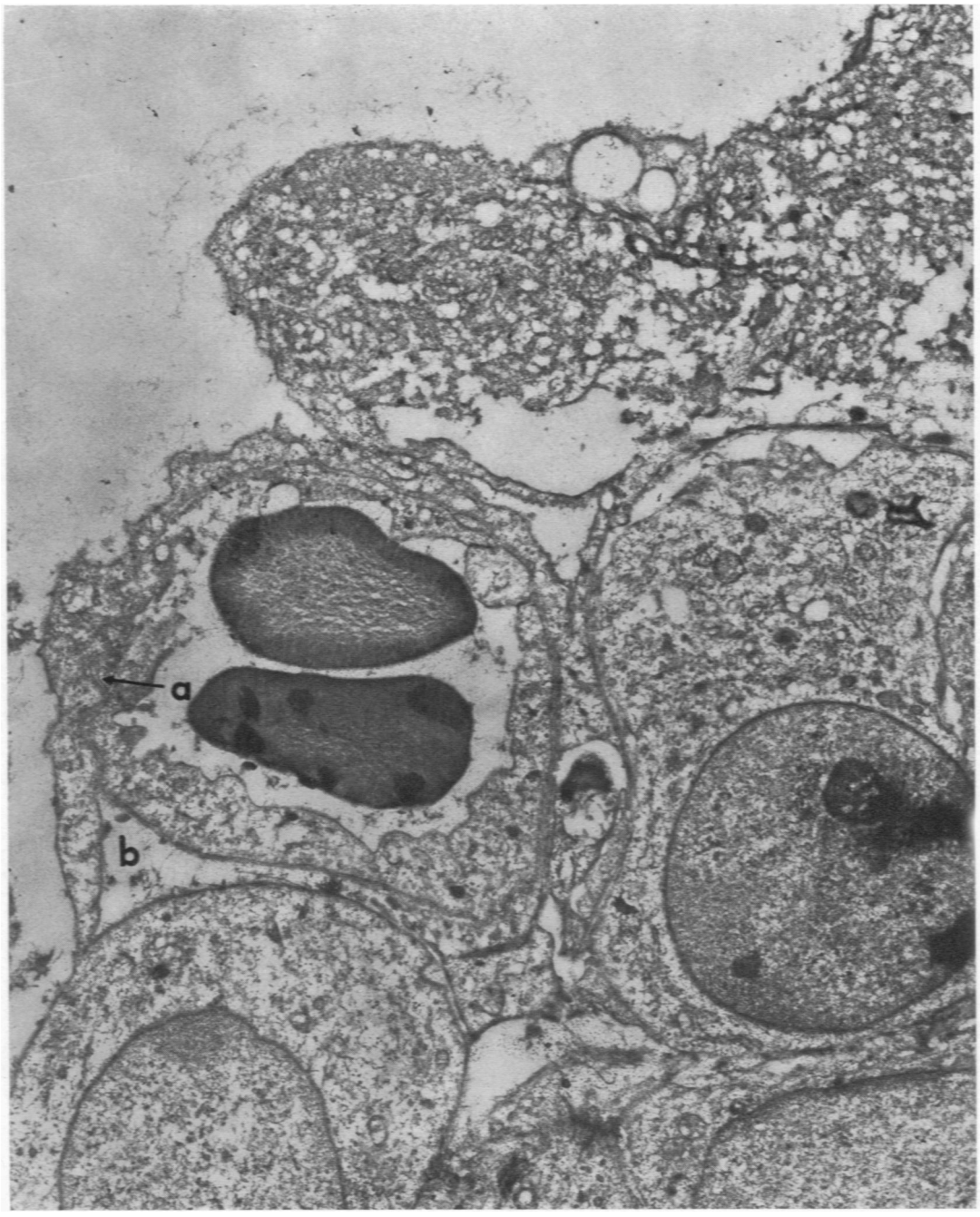

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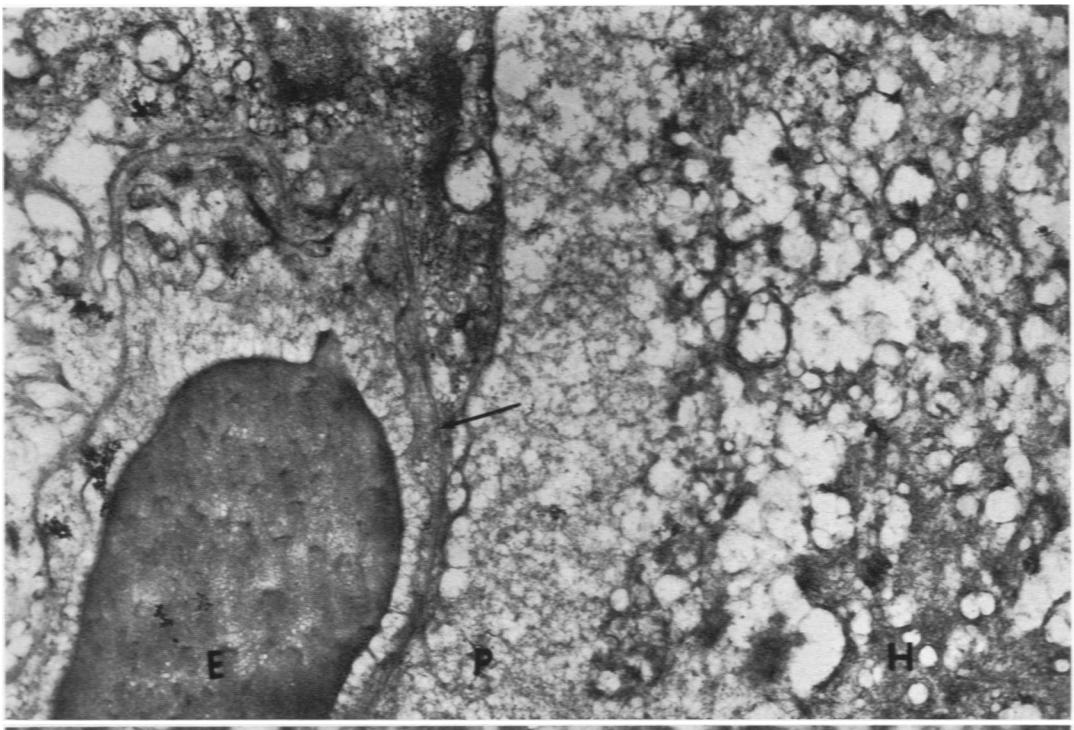
FIG. 5. Premature human infant lung (case 29). On the left side of the figure is a portion of a septal capillary. An erythrocyte (E) and plasma appear in the capillary. Endothelium and epithelium are separated by a basement membrane (arrow). In the air space overlying the epithelium is a layer of plasma (P), which, on the right side of the figure, extends into clotted hyaline membrane (H). The magnification is high enough to demonstrate the fibrous nature of the matrix of the hyaline membrane. Electron micrograph, × 6.875.

FIG. 6. Premature human infant lung (case 29). Higher magnification of hyaline membrane shows partially clotted fibrin matrix and cell débris. Electron micrograph, × 13,750.



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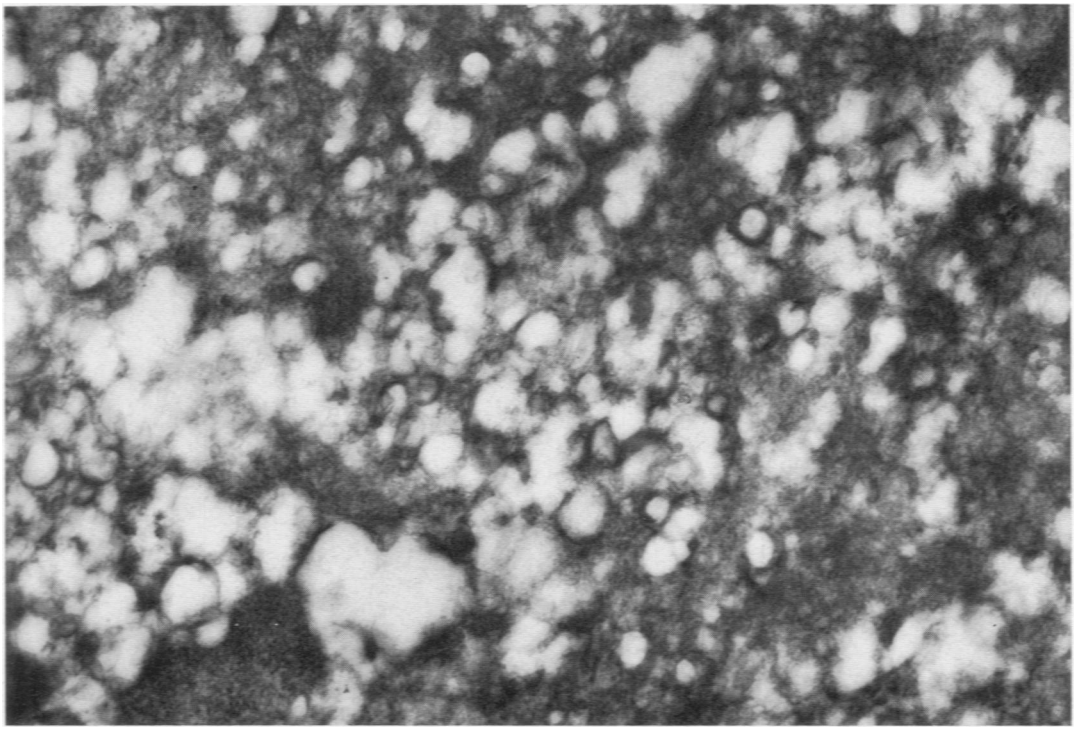

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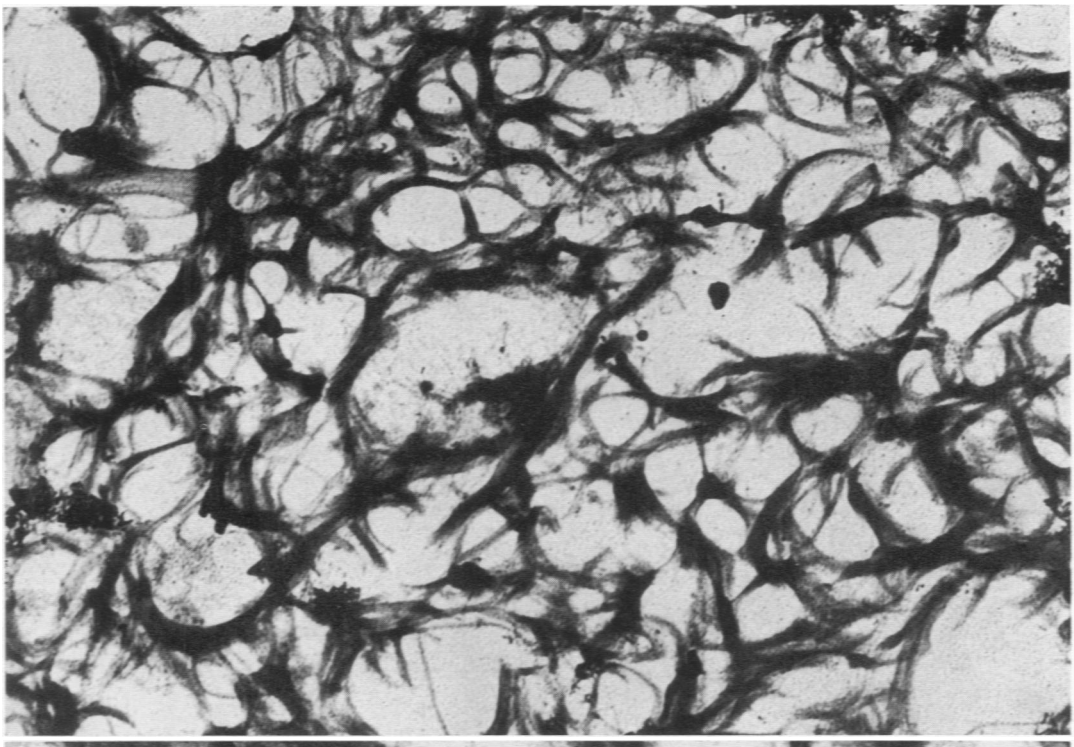
FIG. 7. Plasma clot. In this part of the section fibrin fibrils are the major constituent of the clot. Periodicity, or beading, is seen in many of the fibrils. Electron micrograph, × 15,170.

FIG. 8. Guinea pig lung. This figure shows the fibrillar matrix of the hyaline membrane at higher magnification, and demonstrates the periodicity, or beading, of the fibrils (example at arrow). Electron micrograph, × 22,760.



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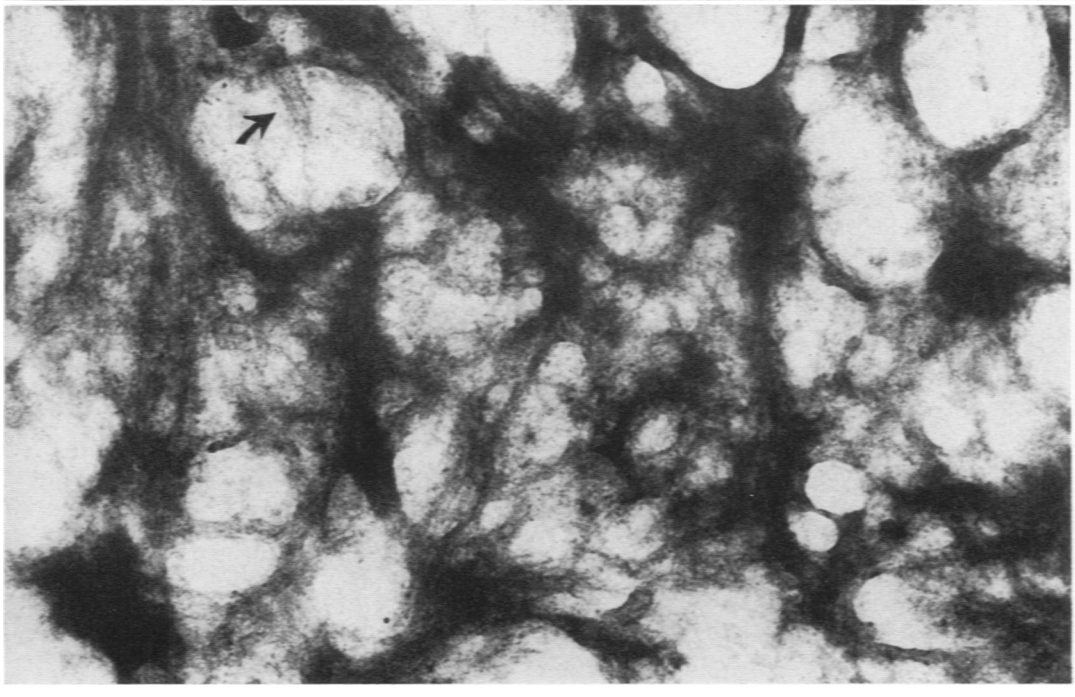

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FIG. 9. Human infant lung (case 29). Parts of three capillaries appear in this figure, one at the top and two at the bottom of the figure. The alveolar duct between the capillaries is filled with a macrophage and hyaline membrane; ingested hyaline membrane may be seen in the macrophage, in which no nucleus appears at the level of section. There are small processes on the surface of the macrophage. At the right of the alveolar duct is an opening into an alveolus, which is full of hyaline membrane. Electron micrograph. × 2.165.

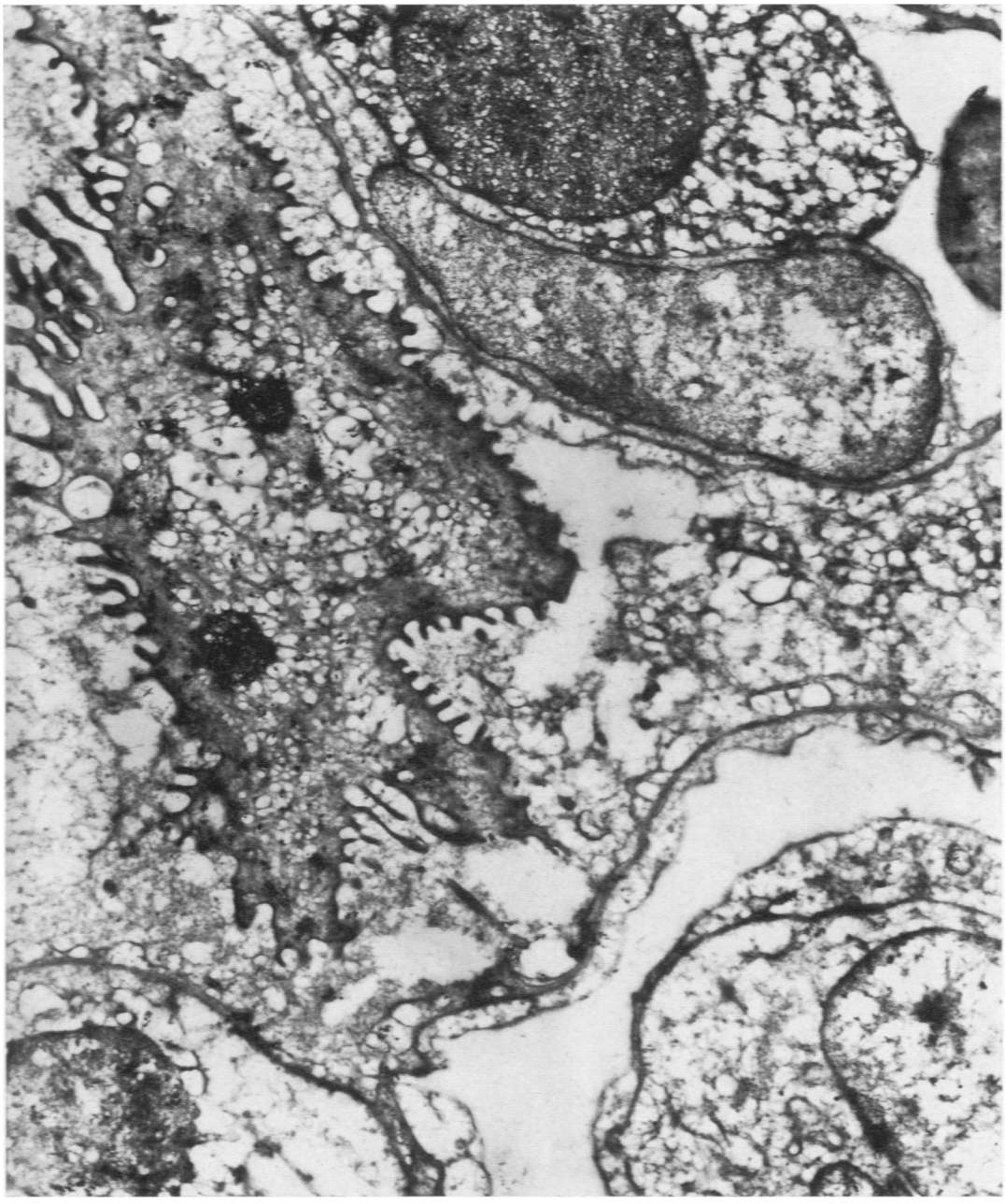

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FIG. 10. Human infant lung (case 29). A large macrophage seen in a bronchiole (ciliated epithelium may be noted). Ingested hyaline membrane may be seen in the macrophage. Electron micrograph, × 2,600.

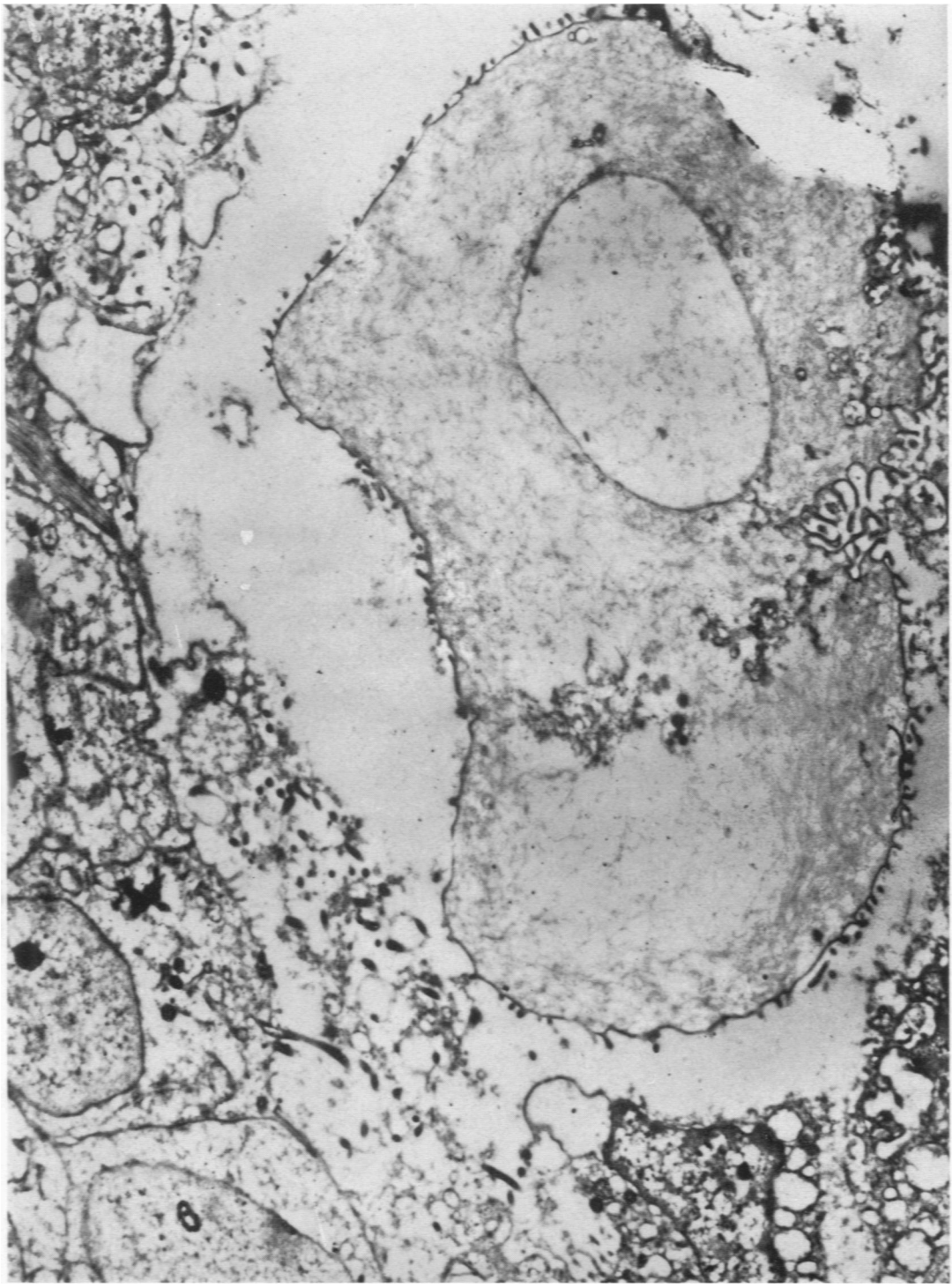

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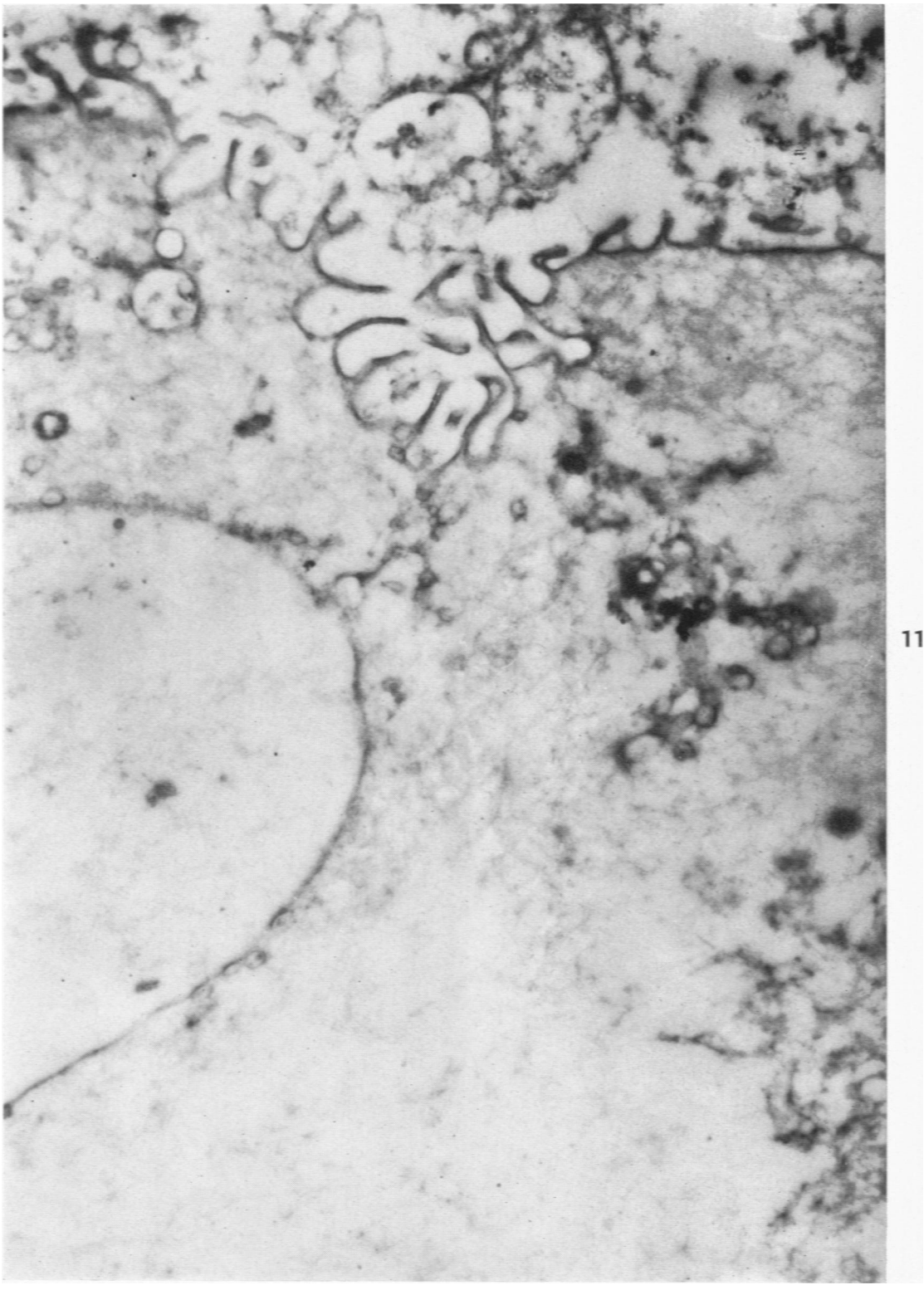
FIG. 11. Enlargement from Figure 10, showing the area of the microprojections at the cell surface, with evidence of ingestion of hyaline membrane and of pinocytosis. The very fine filaments seen in the cytoplasm of this macrophage are not from the hyaline membrane but are cytoplasmic components commonly found in cells, especially in macrophages and other ameboid cells. These submicroscopic filaments are believed to be part of the sol-gel mechanism of ameboid movement. Electron micrograph. × 8,500.



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