NEPHROTOXIC SERUM NEPHRITIS IN THE RAT

Electron and Light Microscopic Studies

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Anti-kidney serum nephritis was first produced in rats by Masugi.[1] Subsequent investigators[2,3] pointed out that the experimental disorder caused relatively mild "nephritic" symptoms (hematuria, hypertension, azotemia), and much more prominent "nephrotic" manifestations (proteinuria, hypoproteinemia, lipemia, edema). During the nephrotic stage, the glomerular lesions were generally mild and often inconspicuous as seen in conventional histologic preparations. The disease in the rat has therefore been compared with so-called lipoid nephrosis in man.[2] The pathologic features of lipoid nephrosis and of nephrotic glomerulonephritis in man are now under intensive study by a number of investigators, including electron microscopists. Various alterations have been observed; among these are disappearance of the epithelial foot processes in the glomeruli,[4,5] thickening and mottling of the capillary basement membranes,[4,5] splitting of the basement membranes,[6,7] and deposition of "hyalin" (protein) between the basement membranes and the epithelium.[7-9] Electron microscopic studies[10-18] indicate that similar lesions may also be found in experimental nephritis. We have examined the glomerular changes in nephrotoxic serum nephritis in the rat by light and electron microscopy. The present report deals with the observations during the first 6 weeks of the experiment.

Material and Methods

Anti-rat kidney serum was prepared by immunizing rabbits according to the procedure recommended by Heymann.[19] When first collected, the serum exhibited considerable "primary" toxicity. After the usual method of partial detoxification for ½ hour at 56° C., the serum was immediately subdivided into small vials and kept in the freezing compartment of a household refrigerator for 1 to 2 months. By that time toxicity was considerably reduced. The serum potency was then tested and if satisfactory, the whole batch was transferred into a freezer at −20° C. Under these conditions the serum kept well for as long as 3 years.

Male Sprague-Dawley albino rats, weighing 100 to 150 gm., were given a single

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intravenous dose of a potent serum, 0.7 to 1.8 ml. per hundred gm. of body weight. The animals were kept in individual metabolic cages and offered food (Purina Chow) and water *ad libitum*. Urinary volume, total protein excretion, and body weight were measured daily. The normal urine volume for the strain used was 5 to 7 ml. in 24 hours; total protein excretion was up to 10 mg. in 24 hours. The latter was measured by a modification of the biuret method of Kingsley.[20] Blood and urine were also collected for paper electrophoresis, and nonprotein nitrogen (N.P.N.) was determined at intervals or at the time of sacrifice, using a modification of the standard nesslerization procedure of Folin and Wu.

Experimental nephritis was induced in over 100 animals. They were sacrificed by exsanguination or died spontaneously between 2 hours and 6 weeks after the injection. In all of these, the tissues were examined using conventional histologic procedures after fixation in 4 per cent, cold (5 to 10° C.) neutral buffered formaldehyde. In 25 of the animals, kidney tissue was also fixed immediately in cold buffered (pH 7.4) 2 per cent osmium tetroxide for electron microscopy, and thin (0.5 μ) sections were prepared for light microscopy. Sections intended for light microscopy, whether standard or thin, were stained with hematoxylin and eosin, periodic acid-Schiff (PAS) reagent, Jones's periodic acid-silver methenamine (PA-SM) and a modification of Mallory's aniline blue, chromotrope-aniline blue (CAB), as used in previous studies.[21] Osmium-fixed tissue was embedded in butyl methacrylate and sectioned with a Porter-Blum microtome. Electron microscopy was performed with the Phillips EM 100 B microscope, equipped with a 35-mm. camera. All micrographs were taken on Eastman Kodak Spectroscopic film (#6490).

Results

Clinical Data

As might be expected, the course of the experimental illness and the severity of the anatomic changes varied considerably with the dose of the anti-kidney serum. A large dose induced an almost immediate oliguria with the urinary output dropping down to 2 ml. or even less than 0.5 ml. in 24 hours. Even complete initial anuria has occurred in some instances.[12] Some of the animals died during the first 3 days, apparently in uremia. The oliguria persisted in varying degree for 4 to 5 days and was followed by diuresis, up to 25 ml. per 24 hours, and general clinical improvement. Thereafter, persistent polyuria (10 to 12 ml. per 24 hours) developed in some animals.

Oliguria went hand in hand with rapidly increasing subcutaneous edema and ascites. Weight gain became apparent in the first 24 hours of illness, reaching a maximum during the first week. The largest gain was 100 gm., nearly equal to the initial body weight; the average gain was 50 to 60 gm., 40 to 50 per cent of body weight. This was caused almost entirely by accumulation of water, because the animals ate very little but continued to drink. If diuresis occurred, edema disappeared in the course of 2 to 4 days. If diuresis did not occur, edema increased and the animal died apparently in cardiac failure, but with only moderate nitrogen retention (N.P.N., 75 to 85 mg. per hundred cc.).

Proteinuria appeared almost immediately after injection of the serum. Specimens collected for the first 3 hours contained several gm. per hun-

dred ml. Protein loss was accompanied by a rapidly developing hypoproteinemia with serum levels dropping below 2 gm. per hundred ml. by the third or fourth day. During the ensuing week serum proteins showed partial recovery despite continued or even increasing proteinuria. This gradually declined after the second week, but most animals alive at 6 weeks still showed appreciable loss of protein and some degree of hypoproteinemia.

Nitrogen retention was apparent 6 hours after injection of the serum, increasing rapidly up to 100 to 200 mg. of N.P.N. by the end of the third day. If the rat did not die at that time, the level of N.P.N. began to decrease, reaching normal or even subnormal levels in some animals in 2 to 4 weeks. This drop occurred even in the rats that did not develop diuresis. However, in the animals that showed persistent proteinuria, elevated N.P.N. was seen even at 6 weeks.

Microscopic hematuria and casts appeared during the first week in most of the animals. None showed gross hematuria.

Anatomic Lesions

The histologic description is based upon the maximal damage observed and represents a composite of the standard sections, thin sections and electron micrographs. The standard sections, particularly those stained with PAS, provided an over-all view of the renal alterations and their distribution in the glomeruli, the cortical tubules and the medulla. The thin sections were cut from the same blocks utilized for electron microscopy; this again allowed us to judge the uniformity of lesions within the glomeruli and to correlate the light microscopic appearance with the electron microscopic pattern.

Anatomic alterations in the glomeruli appeared very soon after the injection of serum. Piel[17] observed them within the first hour. In our experiments, noticeable swelling of the cytoplasm of the epithelial and the endothelial cells, particularly of the attenuated layer (*membrana attenuata*) and partial disappearance of the endothelial pores was present 2 hours after the injection. The capillary lumens contained many neutrophils and also mononuclear blood cells, the former often adherent to the capillary walls (Fig. 5). The tubules showed no significant changes at this time. At 6 hours, cellular edema became much more pronounced. The epithelial foot processes were swollen and distorted and in places completely absent. There was also swelling of the endothelial cells lining the capillary plexus at the junction of the cortex and the medulla.[22] The proximal convoluted tubules contained a small number of hyaline droplets. In one animal, the inner zone of the medulla and the papilla were almost completely filled with pale hyaline casts.

At 24 hours the glomeruli appeared quite bloodless with but a few red

cells, neutrophils and mononuclear leukocytes in the capillaries. The lumens of the latter were almost completely obliterated by tremendously swollen vacuolated endothelial cells (Fig. 10). The urinary space was markedly narrowed by swollen epithelium (compare Figs. 1 and 2). The foot processes had almost completely disappeared, and the uniform, pale cytoplasm was directly applied to the basement membrane (Fig. 10). Even the mitochondria appeared swollen. Occasional red cells were seen in the urinary space between the epithelial cells. The capillary basement membranes appeared altered; the dense middle layer was irregularly thickened, and the paler layers were uneven (compare Figs. 9 and 10). Thickening of the basement membrane was observed by Piel[12] as early as 6 hours after the introduction of serum. Some capillaries contained "hyaline" thrombi which, under the electron microscope, had a fibrillar structure (Figs. 11 and 12). These fibrils exhibited a dominant periodicity of 230 A; there was also a suggestion of two shorter periods, at about 160 Å and the other less than 100 A. The endothelium and, to a lesser degree, the epithelium of the thrombosed capillaries were destroyed. The proximal convoluted tubules contained numerous hyaline droplets. A number of casts which varied in their affinity for PAS stain were seen in Henle's loops and convoluted tubules. Protein precipitate appeared in the corticomedullary capillary plexus.

By the third day the cellular changes were at a maximum. In the most severely affected animals, many glomeruli were filled with "hyaline" thrombi which almost completely replaced the endothelial cells (Fig. 2). Where the latter were preserved, they contained many vacuoles, while osmiophilic droplets lay within the cells or between them and the basement membranes. The epithelium was devoid of foot processes, but often showed long thin interlacing projections crossing the urinary space (Fig. 13). In addition to vacuoles the cells contained hyaline droplets and fat droplets. The basement membranes were distinctly thickened, with focal splitting of the dense layer. There were fewer hyaline droplets but more casts in the convoluted tubules. The peritubular capillaries contained a number of free-lying mononuclear cells, and there was an increased number of cells in the interstitial connective tissue.

In the animals which improved clinically, the glomerular cells began to recover after the third day. At 6 days the cytoplasmic swelling decreased and the epithelial foot processes re-appeared though they were distorted and focally fused. The capillary lumens became patent. Thickening and splitting of the basement membrane increased, and for the first time there was widening of the intercapillary spaces (Fig. 3) with increase in cells and basement membrane branches (Fig. 6). If, on the other hand, there was no diuresis and no reduction in edema, the glo-

merular changes continued unabated. In animals sacrificed in a terminal state on the eighth day, the foot processes were either markedly distorted or nearly totally absent. Deposition of a dark substance with the size and shape of the foot processes within the epithelial cytoplasm near the basement membrane was noted (Fig. 14). It could have represented "attempts" to reform the foot processes. This substance ("foot process material") was considerably denser than the basement membrane but was similar to the substance normally present in the foot processes. A few fibrin thrombi still filled the capillary lumens. Splitting of the basement membrane reached the stage where it could be recognized in thin sections by means of the light microscope (Fig. 7).

Animals sacrificed during the second to the fourth week showed only slight to moderate cellular edema and distortion of foot processes. There was progressive thickening of the basement membrane up to 5,000 Å (normal width, 800 to 1,200 A) and extensive splitting. In addition to cells and basement membrane branches (BMB), bundles of very fine fibrils appeared in the widened intercapillary spaces. Small crescents composed of the epithelium of Bowman's capsule were present in some instances.

At 6 weeks, at the termination of the experiment, the cell and foot process changes persisted to a degree (Figs. 4 and 15) while the basement membrane alterations showed further progression. The two split layers of the dense zone were now as much as 5,000 A apart at some points (Fig. 16), though often linked by connecting strands (Fig. 15). Three or 4 layers were seen occasionally. In addition, the outer split layer was often broken up into irregular fragments which lay perpendicularly to the capillary wall, while the epithelial cytoplasm in the form of thick trabeculas extended between these fragments to the inner split layer (Figs. 8 and 17).

Comment

There are only a few published reports of electron microscopic observations in anti-kidney serum nephritis; those of Simer[10,11] and Reid[13] in mice, those of Sakaguchi, Suzuki and Yamaguchi,[14] and Churg, Mautner and Grishman[16] in rabbits, and those of Piel and associates,[12,17] Miller and Bohle,[15] and Churg, Grishman and Mautner[18] in rats. These studies supplement each other and reveal basic similarity of the glomerular lesions in the several species. Cellular damage, inflammation, basement membrane changes and alterations in the intercapillary space are invariably present, differing only in degree and in the rate of development. In the rat, inflammation is mild and intercapillary

changes develop slowly while cellular and basement membrane lesions are prominent from the start.

Cellular Alterations

Swelling and vacuolation of the endothelium appeared in the rat almost immediately after the injection of serum, reaching a maximum between 1 and 3 days and declining thereafter. In severely affected animals these features were present in some degree at the end of 6 weeks. Osmiophilic lipid droplets were seen in the cytoplasm after the third or fourth day, perhaps a reflection of both the cell damage and the elevation of lipids in the blood.

Endothelial lesions have been observed in mice [10,11,13] and rats.[12,15] They have long been recognized by conventional microscopy [1-3] though their severity was not appreciated. At the height of damage, swollen endothelium almost completely fills the capillary lumen. This may well be responsible for the early oliguria and azotemia, though interference with passage of filtrate across the altered capillary wall could contribute significantly, or even predominantly. The initial vascular spasm [23] and the thrombi in the capillary lumens may also play a role. The appearance of thrombi in the capillaries has been related to the destruction of the endothelium.[1] The thrombi give a positive staining reaction for fibrin [1,24,25] and have a finely fibrillar structure with the typical periodicity of fibrin (230 A). Their similarity to the glomerular thrombi encountered in the generalized Shwartzman phenomenon has been commented upon by many observers.[12,26] Recent electron microscopic studies showed that the fibrillar substance observed in the Shwartzman phenomenon has a periodicity only about half as long (120 Å) as that of fibrin.[27]

Swelling and vacuolation of the visceral epithelium occurred simultaneously with similar alterations in the endothelium. Changes in the foot processes were first seen by Simer [10,11] and were confirmed many times in both experimental [17] and human [4,5] tissues. Swelling, distortion and disappearance of the foot processes in the rat were most evident 1 to 3 days after the injection of serum. The dark substance within the processes (foot process material) disappeared at the same time (Figs. 10 and 11). Simer [11] also noted a decrease in electron density of the pedicels in nephritic mice. Restoration of foot processes was accompanied by reaccumulation of the dark substance; however, in some of the more severely damaged animals, the processes might not be completely restored; yet the dark substance accumulated within the cytoplasm close to the cell membrane, in the form of bands and masses. It is possible that

this substance has functional significance.[4] In severe but nonfatal disease some foot process change was still seen at 6 weeks.

An interesting finding was the formation of long slender cytoplasmic protrusions which coursed the urinary space and formed an intricate network. They were first seen about the third day of illness when the foot process changes and cellular edema were at the maximum. Similar protrusions were observed by Sakaguchi and co-workers in nephritic rabbits.[14] Hyaline droplets appeared in the glomerular epithelium on about the third day; at the same time they began to disappear from the tubules.

Basement Membrane

Thickening and splitting of the basement membrane in experimental as well as in human nephritis has been recognized by both light [6,7,25,28,29] and electron microscopists.[5,10–12,14] Sakaguchi and co-workers [14] stated that in rabbits, thickening was accompanied by a decrease in electron density of the membrane. Splitting of the dense middle layer was a more advanced lesion. It has been observed in rats and rabbits [16–18] and in man in the course of the nephrotic syndrome.[30] Subdivision of the dark zone into two layers was seen faintly in electron micrographs of normal glomeruli and could be very well demonstrated by maceration and silver impregnation in a mild alkaline solution (Gomori's silver methenamine).[31] The process of splitting suggests laminated construction of the glomerular basement membrane, similar to that of the tubular basement membrane.[5,7] It has been postulated that the basement membrane of glomerular capillaries consisted of two fused elements, endothelial and epithelial.[32–36] The generic relationship of these elements to the corresponding cells is not known, nor is it yet clear whether the splitting seen by light microscopy (Fig. 7) and that observed by electron microscopy (Fig. 15) represent the same phenomenon.

Fragmentation of the outer split layer of the basement membrane was a late feature. The tendency of the fragments to arrange themselves perpendicularly to the surface of the capillary, between protrusions of the epithelial cytoplasm, produced an appearance that was not unlike that seen in so-called "membranous transformation" in the nephrotic syndrome of man.[8,37] The mechanism of this phenomenon and its possible significance in human nephrosis are currently under investigation.

Intercapillary Space

The existence of the intercapillary space has recently received support from several electron microscopic studies of the normal glomerulus.[36,38,39] The pathologic lesions encountered by us first became

apparent toward the end of the initial week, and progressed slowly for the next 5 weeks. Cells and branches of the basement membrane became somewhat more numerous, and bundles of fine fibrils were seen between the cells. The basement membrane branches corresponded to the "basement membrane-like material" of Farquhar, Vernier and Good.[4,5] Electron microscopy of silver-impregnated tissue showed that they originated from the basement membranes and ran through the intercapillary spaces in the manner of tortuous cross-beams.[31] They separated, though incompletely, the intercapillary cells from each other and from the endothelial cells. The fibrils were very delicate; they sometimes revealed a suggestion of periodic structure, but further data are needed to establish their nature and their relation to the basement membrane branches. These will be gathered as part of the study, now in progress, of the chronic stage of experimental nephritis.

Relation of Anatomic Alterations to Edema and Proteinuria

Farquhar and co-workers [4,5] called attention to the association of foot process changes with the nephrotic syndrome in man. In the rat, edema appeared almost simultaneously with the alterations of the foot processes and began to clear when foot process recovery became evident. Failure of recovery was associated with further retention of water. Though the edema was undoubtedly caused by hypoproteinemia, general capillary damage and also injury of the glomerular capillary walls could be the contributing factors. It has been claimed that the foot processes assist in glomerular filtration,[40] and it is possible that their injury, manifested by the disappearance of the dark foot process substance, interferes with transfer of water and sodium across the capillary basement membrane. The onset of edema within the first 24 hours of illness and its eventual disappearance despite continued protein loss, was reminiscent of the "nephritic" rather than the "nephrotic" syndrome in man,[41] particularly since it was accompanied by reduced rather than increased or normal glomerular filtration.

It has not been definitely established whether the glomerular ultrafilter which separates water and crystalloids from the plasma proteins is represented by the cytoplasm of the endothelial and particularly the visceral epithelial cells or by the dense layer of the basement membrane,[4,5] though the weight of evidence appears to be in favor of the latter.[42] Proteinuria of the nephrotic syndrome in man is accompanied by changes in both structures,[4,5] by fusion of the foot processes and by thickening and mottling of the basement membrane. Spiro [42] has described actual defects in the basement membrane, which he has held to be responsible for the proteinuria. In the nephritic rat, the initial pro-

teinuria was also associated with loss of foot processes and with changes in the basement membrane. In the later stages, loss of protein persisted despite recovery of the foot processes and eventually abated despite progression of the basement membrane damage. It is possible that a certain combination of changes in both the epithelial cells and the basement membrane is necessary to cause proteinuria, or that the increased porosity of the capillary wall is the result of molecular rearrangement at a sub-electron-microscopic level.

Summary

Masugi-type nephritis was induced in rats by a single intravenous injection of anti-kidney serum derived from rabbits. The clinical behavior of the animals and the anatomic lesions in the kidneys, particularly in the glomeruli were followed for a period of 6 weeks.

The animals developed mild hematuria, transient nitrogen retention and pronounced proteinuria and edema. The glomeruli showed cellular damage with acute inflammation and fibrin thrombi. There were also thickening and splitting of the capillary basement membrane with fragmentation of the outer split layer, widening of the intercapillary spaces with mild cellular proliferation, increase in basement membrane branches and deposition of fine fibrils. Relation of the anatomic to the clinical features has been discussed briefly.

Review of the pertinent literature on anti-kidney serum nephritis indicates basic similarity of glomerular lesions in the animals of several species.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

All figures represent part or all of the rat glomerulus. Time given is that elapsed after the intravenous injection of anti-kidney serum.

Figures 1 to 4: Light photomicrographs, standard sections, periodic acid-Schiff (PAS) stain. × 450.

FIG. 1. Normal glomerulus.

FIG. 2. Three days. The bottom glomerulus shows swelling of cells and loss of capillary lumens; the top glomerulus exhibits obstruction of the lumens by "hyaline" thrombi and disappearance of most of the cells.

FIG. 3. Two weeks. There is focal thickening of the capillary walls and widening of the intercapillary spaces. A small epithelial crescent is seen on the right.

FIG. 4. Six weeks. Capillary lumens are for the most part patent; their walls are thickened. There is a capsular adhesion near the top of the glomerulus.

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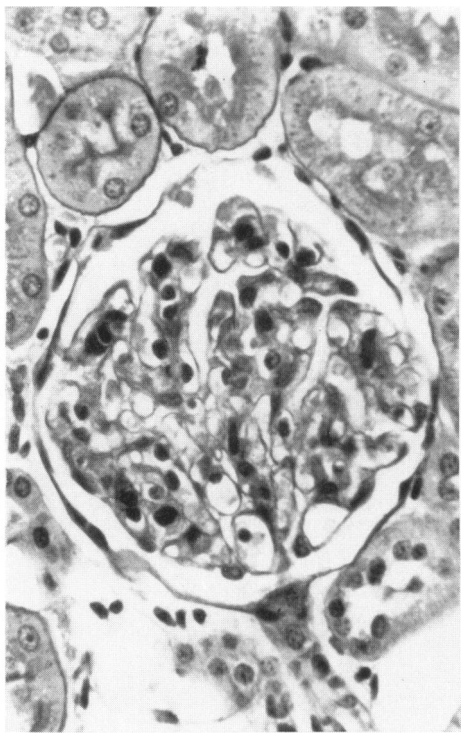

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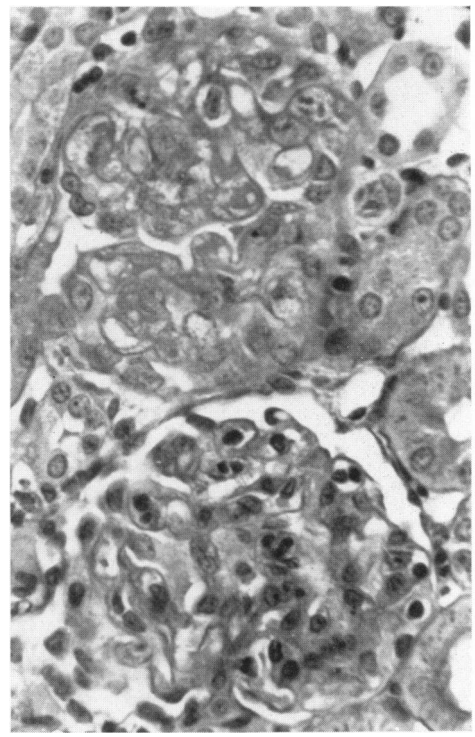

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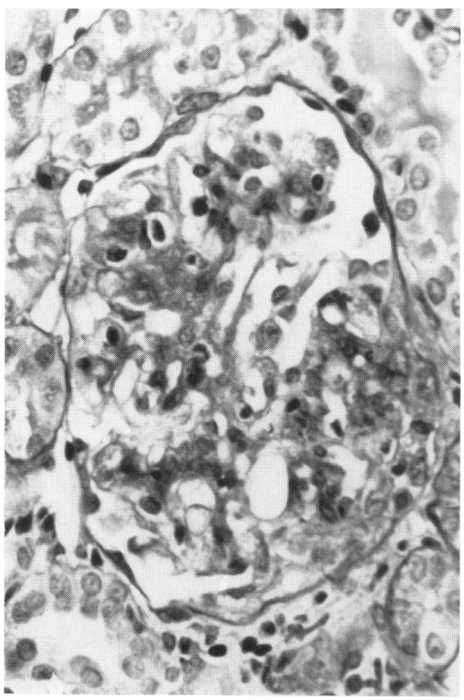

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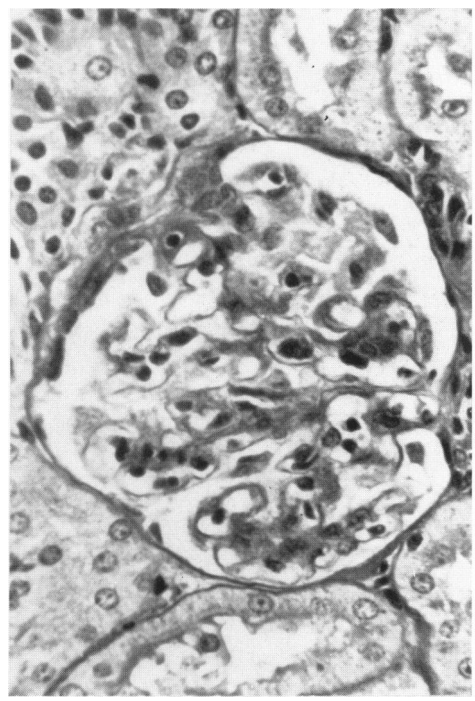


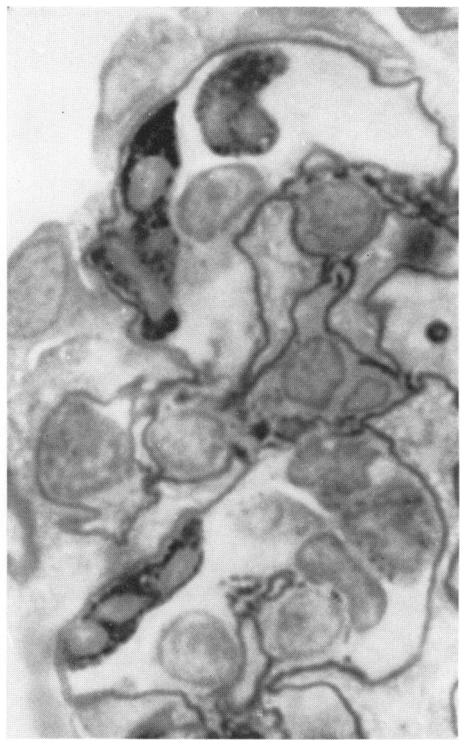
Figures 5 to 8: Light photomicrographs, thin (0.5 μ) sections, PAS stain.

FIG. 5. Two hours. Many neutrophils and mononuclear leukocytes are evident in the capillary lumens. The darkly granulated neutrophils adhere to the capillary wall. × 2200.

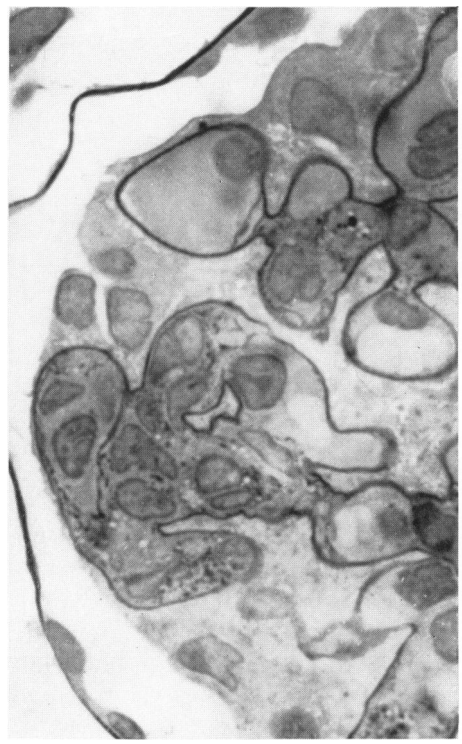
FIG. 6. Six days. Swelling of epithelial and endothelial cells is manifest, and there is an increased number of intercapillary cells. × 1600.

FIG. 7. Eight days. Splitting of the capillary basement membranes is shown. × 1600.

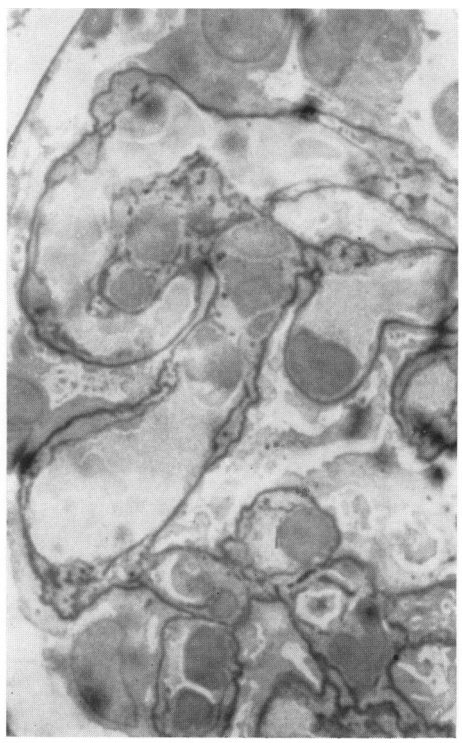
FIG. 8. Six weeks. There is splitting and fragmentation of the basement membrane of the lower capillary with formation of hair-like projections (arrow). × 1600



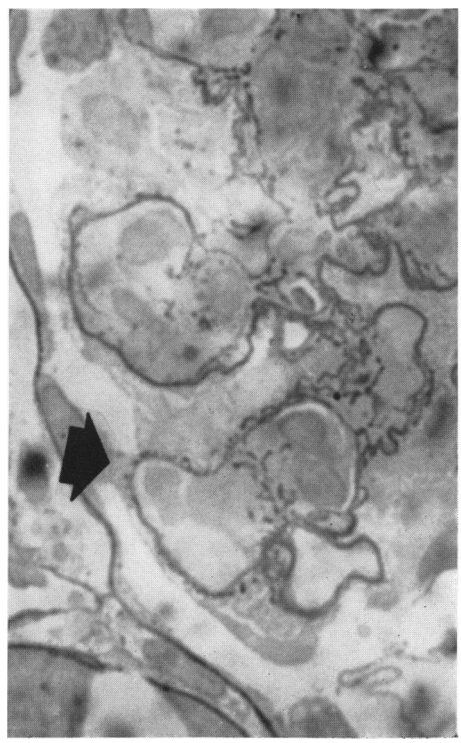
5



6



7

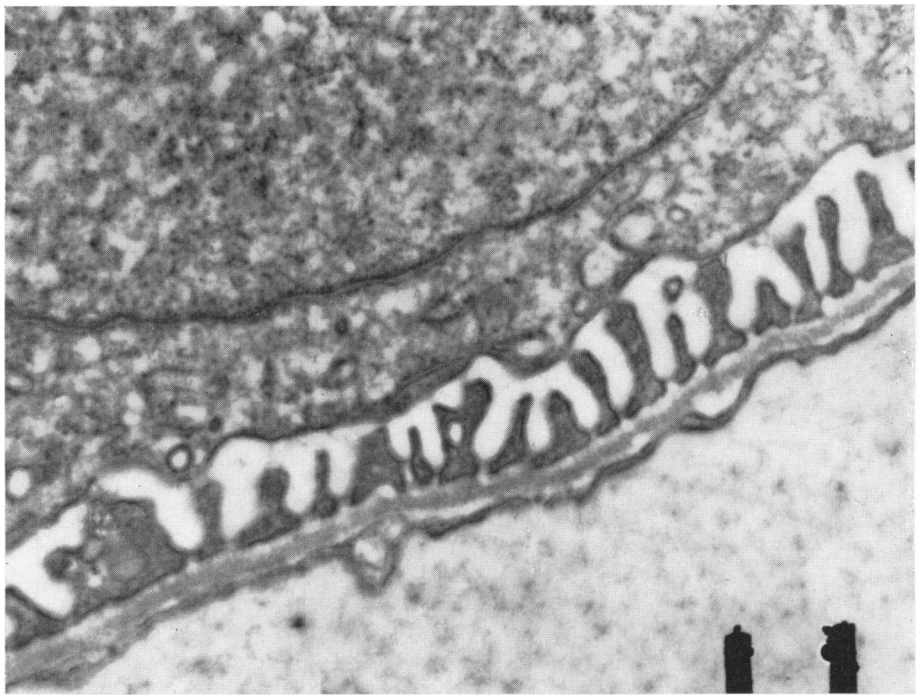


8

Figures 9 to 17: Electron micrographs.

FIG. 9. Capillary wall of a normal glomerulus. Shown are (from top to bottom): part of the nucleus and cytoplasm of an epithelial cell, foot processes, the 3 layers of the basement membrane and the attenuated layer of endothelial cytoplasm. × 12,500.

FIG. 10. Twenty-four hours. Swelling of the epithelial cytoplasm and complete loss of foot processes is evident in the upper right. There is swelling of endothelial cells with obstruction of the lumen (lower left) and irregular widening of the dense middle layer of the basement membrane. U, urinary space; B, basement membrane; C, capillary lumen. × 12,500.



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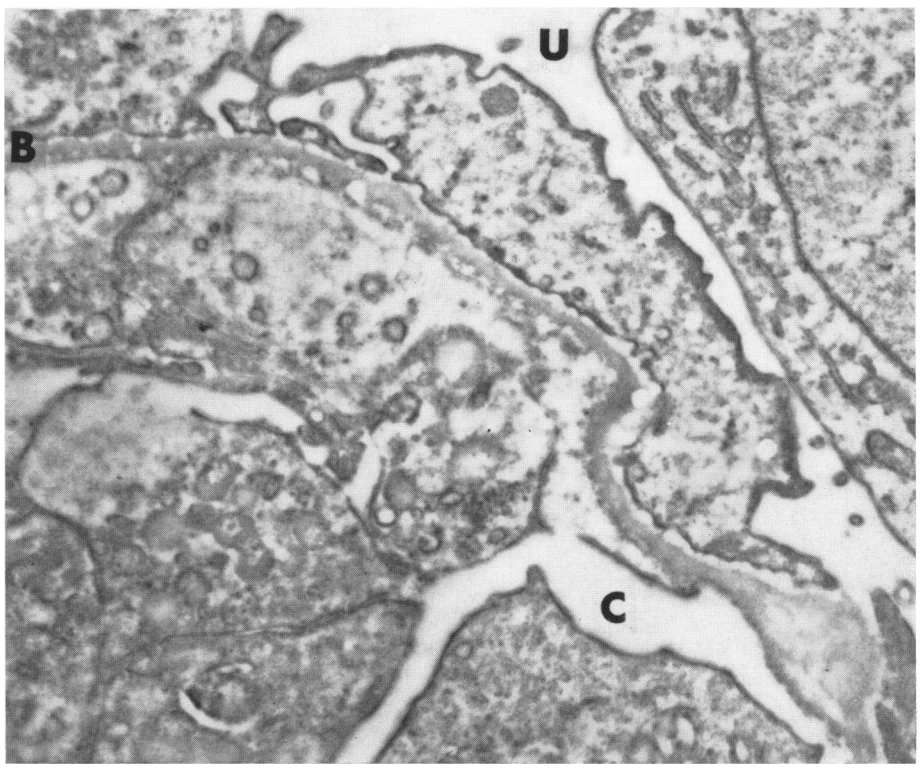

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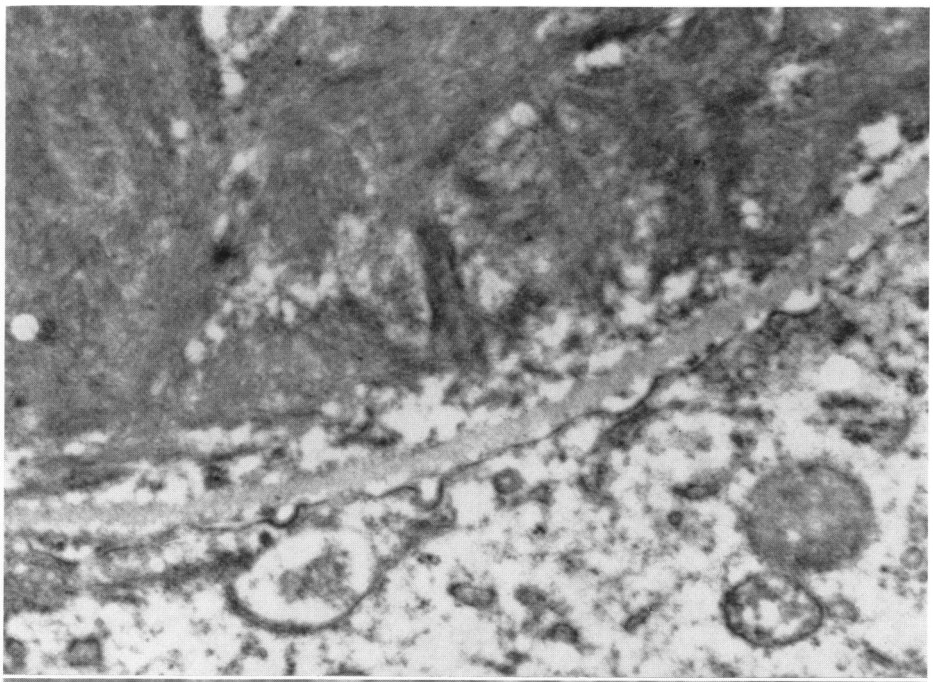


12

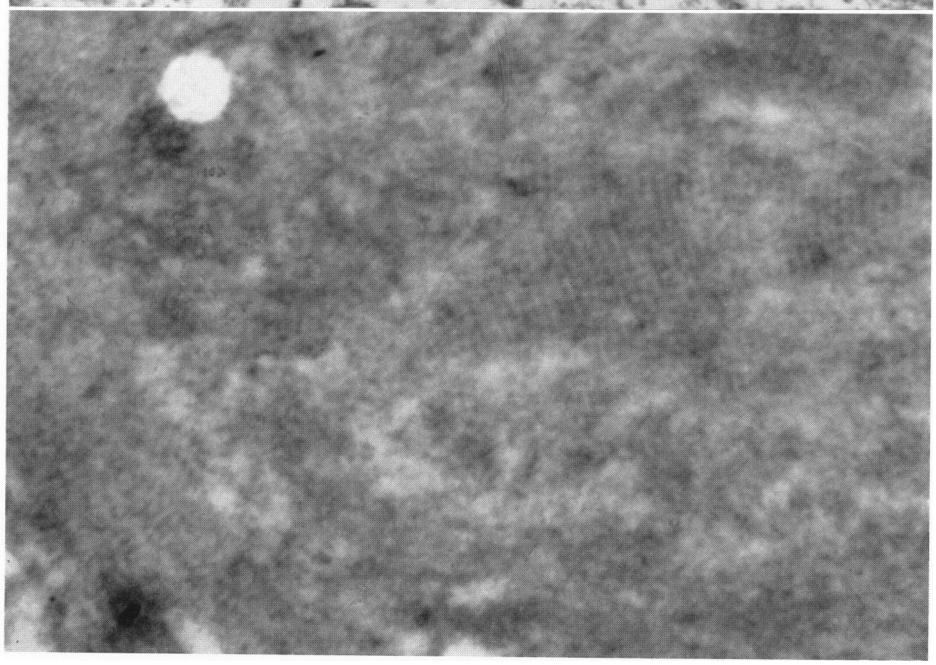
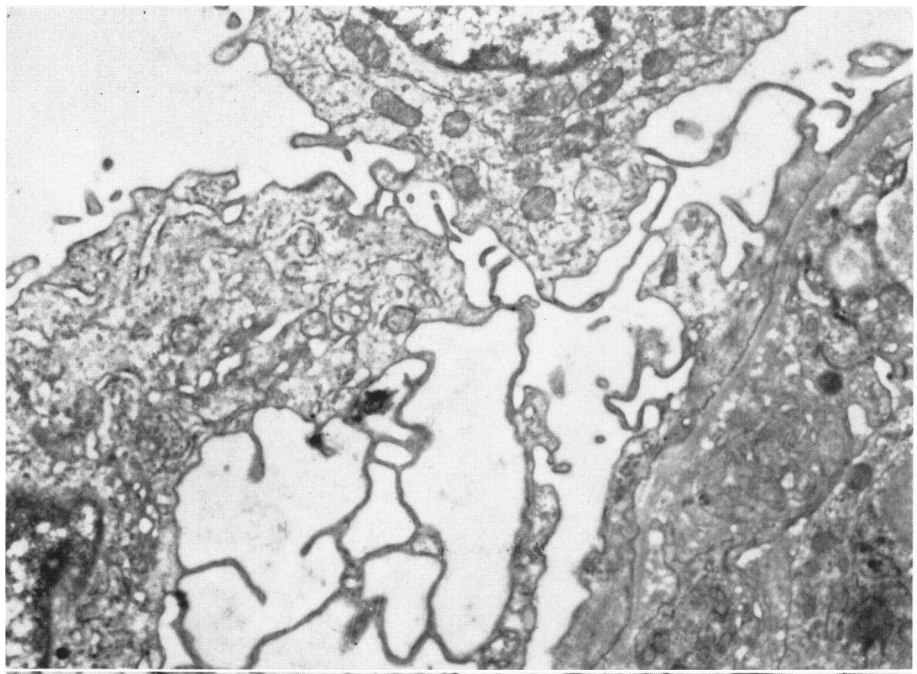


FIG. 11. Twenty-four hours. The capillary lumen is filled with a fibrillar substance, presumably fibrin. The endothelial cytoplasm is destroyed. × 25,000.

FIG. 12. Higher magnification of the previous figure, showing periodic structure of the fibrils (period equals 230 Å). × 62,500.



13

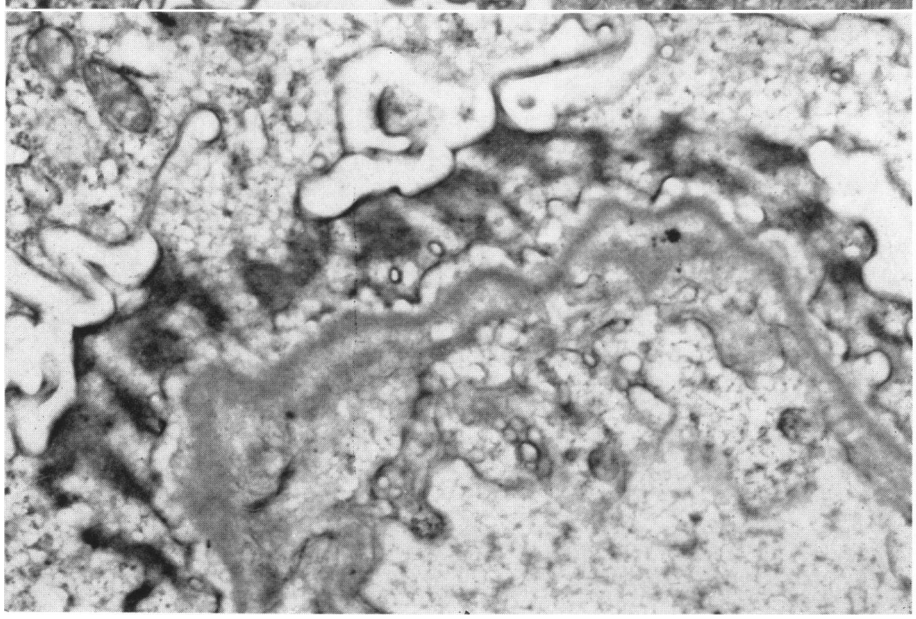


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FIG. 13. Three days. Slender interlacing projections of the visceral epithelial cells are evident. The capillary lumen in the right lower corner is filled with cells. Foot processes are absent. × 8500.

FIG. 14. Eight days. The cytoplasmic membrane of an epithelial cell is directly applied to the capillary basement membrane. Within the cytoplasm, close to the membrane, are deposits of dark substance (foot process material). × 12,500.

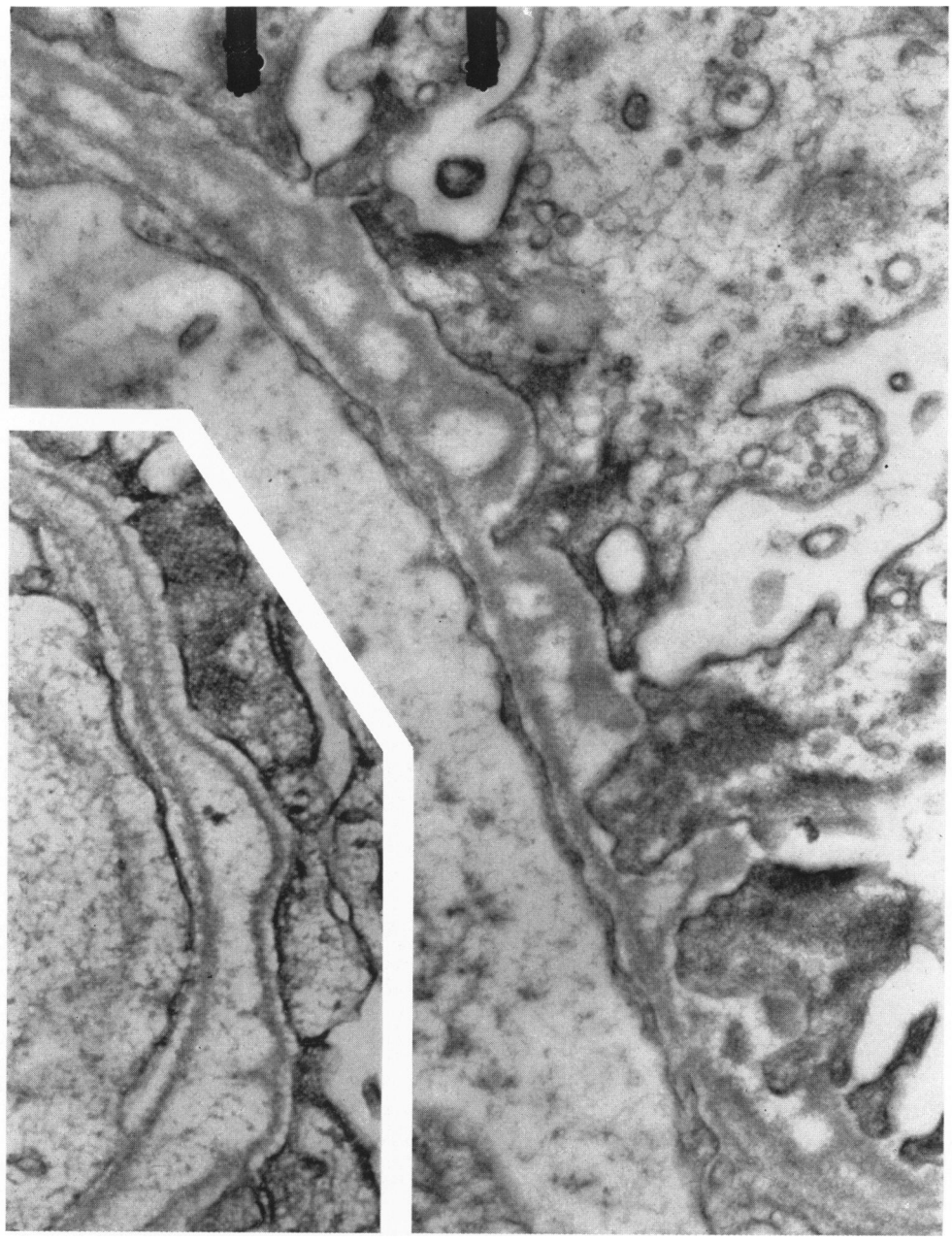
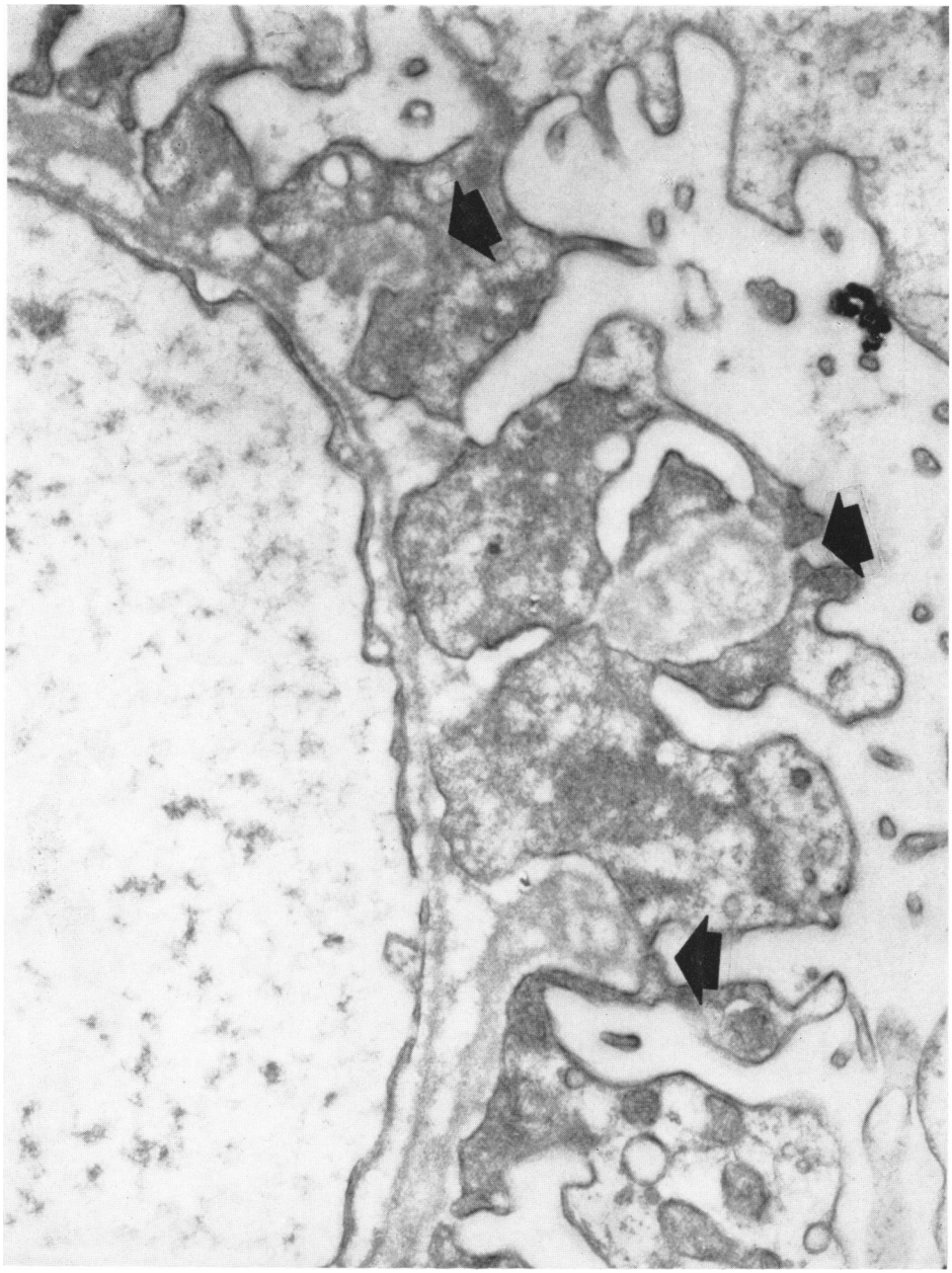


FIG. 15. Six weeks. Capillary basement membrane shows longitudinal splitting of the dense middle layer (top) and beginning fragmentation of the outer of the two split layers (bottom). × 30,000.

FIG. 16. Six weeks. Part of a capillary basement membrane exhibits wide separation of the split layers. × 30,000.



17

FIG. 17. Six weeks. Large epithelial trabeculas—abnormal foot processes (?)—lie between fragments of basement membrane (arrows). Two normal-sized foot processes are seen in the top left corner. × 30,000.