Experimental Autoimmune Nephrosis in Rats

Morphogenesis of the Glomerular Lesion: Immunohistochemical and Electron Microscopic Studies

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HEYMANN *et al.*[1] originally described the induction of a chronic glomerular disease in rats by repeated injections of rat kidney homogenate emulsified with complete Freund's adjuvant and called it autoimmune nephrosis. Since that time a number of studies have defined the general histologic and electron microscopic changes observed,[2-6] described the pattern of immunoglobulin and complement deposition [7-9] and presented evidence to support a concept of pathogenesis.[5,6,10-13]

The studies cited above suggest that this disease is caused by antigenic stimulation by an autologous, relatively insoluble, large molecular weight protein(s). This protein(s) is normally found in the proximal renal tubule in or just below the brush border and is in low concentration in urine and serum. Why the rat can be induced to produce antibodies to autologous protein is not clear. Presumably, antibody production results in the formation of circulating antigen-antibody complexes which lodge in the kidney in a unique subepithelial position. Activation of the complement system of proteins by these complexes is considered to be a major factor in the ensuing functional and morphologic changes. The morphologic similarity between this disease and human membranous glomerulonephritis (which forms approximately 50% of adult significant proteinuria of unknown etiology) [14,15] was previously described.[16-18] A sequential description of the evolution of the glomerular lesion in the rat from the earliest discernible change may furnish useful information relative to the course of the human disease. In addition, the use of comparative thin section and histochemical, immunochemical, and electron microscopic studies may demonstrate more clearly that the deposits observed by the electron microscopist and the "lumps" seen by the im-

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munomorphologist are the same. Furthermore, the study will describe the ultrastructural changes seen in the glomerulus secondary to the deposits and their significance.

Materials and Methods

A total of 32 male rats (Sprague-Dawley or Lewis-Mai) received 5–10 intraperitoneal injections of rat kidney homogenate emulsified with complete Freund's adjuvant (A/K rats) at 2-week intervals, as previously described by Heymann *et al.*[1] Twelve rats received adjuvant injections alone (A rats) and 4 received no injections (C rats). Twenty-four-hour urine collections were performed weekly, and total protein content was determined by the Shevky and Stafford technique.[19] Persistent levels of more than 50 mg./24 hr. or a concentration over 0.3 gm./100 ml. were considered abnormal and significant. Total serum protein and lipids, creatinine, and blood urea nitrogen were also measured at the end of the experiment. Once significant proteinuria appeared, injections were discontinued. Four of the A/K rats were killed after the fifth injection before the development of proteinuria; 4 were killed 2 weeks after development of proteinuria, and thereafter animals were killed every 2 weeks. The longest period of observation was 6 months after the onset of proteinuria. The A rats and C rats were killed at intervals after the appearance of proteinuria in the A/K rats. The results are reported in four groups: Group I, preproteinuria; Group II, proteinuria of 2 weeks' duration; Group III, proteinuria of 4–8 weeks' duration; and Group IV, proteinuria of 12–24 weeks' duration.

Light and Immunofluorescence Microscopy

One-millimeter cubes of renal tissue were rapidly frozen in isopentane previously cooled to −160° C. in liquid nitrogen. Water was removed by placing the tissue in repeated changes of acetone at −65° C., and then the tissues were embedded in paraffin. Sections less than 0.5 μ thick were cut at −5° to −9° C. with a glass knife mounted on an International Minot ultrathin sectioning microtome installed in an International Harris cryostat, as previously described by Post.[20] These sections were deparaffinized in tetrahydrofuran for 2 min., washed in isotonic phosphate-buffered saline (pH 7.45), reacted with appropriate fluorescent conjugated antiserum for 3–16 hr., washed with buffered saline, and mounted in buffered glycerin. Portions of the glomeruli were photographed with an oil immersion (100 ×) objective. A stage micrometer and tissue map permitted the exact area photographed to be documented. The sections then were fixed on the slide with 4% glutaraldehyde, washed in phosphate buffer, and stained with a combination of periodic acid-Schiff (PAS) and iron hematoxylin stain [20] and the identical area rephotographed. The two photographs were enlarged approximately 9 times and the prints were compared. Antiserum to rat globulin was obtained commercially (rabbit anti-rat globulin fraction, Nutritional Biochemicals Corporation). Rabbit antiserum to rat complement was prepared as described by Linscott and Cochrane [21] and was absorbed with rat globulin. Specificity of both serums was tested by studying normal rat kidney (no glomerular fluorescence observed) and by diminishing or abolishing the reaction with fluoresceinated serum by previous reaction with unlabeled specific antiserum.

Electron Microscopy

Renal samples were fixed at 4° C. in 4% glutaraldehyde and phosphate buffer,

pH 7.2 to 7.3.[22] The tissues were washed with phosphate buffer and fixed at 4° C. in buffered 1% osmium tetroxide for 2 hr. After dehydration in graded acetone solution, the tissue was placed in a mixture of equal parts of Araldite 6005 and dodecenylsuccinic anhydride (DDSA) in a desiccator overnight. The tissues were embedded in a mixture of Araldite 6005, DDSA, and dimethylbenzylamine in a proportion of 5:5:0.1 [23] and incubated for 1–2 weeks at 50° C. Sections were cut with a Porter-Blum microtome and glass knives, stained with uranyl acetate and lead hydroxide, and examined with RCA-EMU-3F or 3G electron microscopes.

Results

Significant proteinuria appeared in the A/K rats as early as 2 weeks after the fifth injection and as late as 2 months after the tenth injection. Four to six weeks after the onset of proteinuria, a maximum excretion rate was observed which remained stationary until death. The maximum rate varied from one rat to another and values of 100–400 mg. of total protein per 24 hr. were usual. Occasionally much higher levels up to 1–2 gm./24 hr. were encountered.

Group I (Preproteinuria)

Light Microscopy. No differences between the controls and the experimental animals were observed. All were similar to noninjected controls.

Immunofluorescence. A/K rats and A rats revealed deposition of fluoroscein-labeled antibodies to gamma globulin. This fluorescence appeared as a diffuse intracytoplasmic mesangial deposition which tapered toward the periphery, where at times a linear pattern was evident beneath the glomerular basement membrane (GBM) (Fig. 1 and 2).

Electron Microscopy. In both A/K and A rats the mesangial areas were prominent, though this was more marked in the A/K rats. Rarely, two mesangial cells were present in the same area. Their nuclei were not remarkable. Many cytoplasmic projections extended an unusual distance from the central perinuclear cytoplasm. In many areas these projections reached the periphery of the capillary wall where they interdigitated the attenuated portion of the endothelial cell, and reached into the capillary lumen (Fig. 7, 9, and 10). The endothelial cells were not remarkable. The GBM was of the usual thickness and architecture. In the A/K rats, however, the continuity of the lamina rara externa was interrupted by very rare electron-dense deposits (Fig. 8). These were not seen in every glomerulus. The deposits stained darker than the lamina densa, were located under the broad portion of the foot processes, and were separated from the cell cytoplasm by a definite cell membrane. Less often, these deposits were also found between the foot processes and under the slit membrane. In some sections the slit membranes were

prominent and were seen in direct continuity with the outer layer of the epithelial cell membrane. In a single section many of them appeared to connect the epithelial foot processes in a continuous chain (Fig. 8). The epithelial cells demonstrated no significant ultrastructural changes but were swollen, as reflected by the narrowing of the urinary space into a slit-like zone containing many microvillus-like projections from the epithelial cell surface. The cytoplasm of the foot processes revealed an increased electron density adjacent to the basement membrane. Similar densities, although to a much lesser degree, were seen in the A and C rats.

Group II (Proteinuria, 2 Weeks)

Light Microscopy. The controls were similar to those previously described. The A/K rats at this stage revealed occasional PAS-positive projections of the GBM towards the epithelial cells. Some of these projections were seen adjacent to PAS negative subepithelial deposits (Fig. 3).

Immunofluorescence. In addition to the mesangial reaction observed in Group I A/K rats, irregular, nodular depositions outside the GBM were now observed at the periphery. These reacted with both the complement and globulin antiserums. The GBM itself did not react with the antiserums and separated the endothelial portions of the capillary wall either from a faint intracytoplasmic deposition in the epithelial cell or from the nodular deposits (Fig. 4). When the same section was stained with PAS iron and hematoxylin, the GBM and its projections were positive, whereas the deposits did not react with the Schiff reagent (Fig. 3 and 4). Not infrequently, swollen epithelial cells were observed to contain vacuoles which exhibited varying degrees of reactivity with the antiglobulin and complement serums.

Electron Microscopy. The mesangial areas were still prominent (Fig. 9 and 10). The epithelial cells showed more frequent membrane-limited vacuoles filled with dense homogeneous osmiophilic material (lipid droplets) or the vacuoles contained osmiophilic materials of various densities (cytolysomes). The GBM was still of the usual thickness and appearance; however, the lamina rara externa was interrupted by very frequent electron-dense deposits (Fig. 11). Most of these deposits were still in contact with the epithelial processes. The deposits were larger, and many of them encroached on the broad base of the epithelial foot processes which were now concave instead of flat. Rarely, the larger deposits were surrounded by fused epithelial foot processes.

Group III (Proteinuria, 4–8 Weeks)

Light and Immunofluorescence Microscopy. The findings were similar to those described for Group II. However, more frequent GBM projections and larger immunofluorescent peripheral nodular deposits were present.

Electron Microscopy. The endothelial cells were still unremarkable and were separated from the lamina densa by the usual thin lamina rara interna. Often, mesangial cell cytoplasmic projections separated the attenuated endothelial cytoplasm from the GBM. The endothelial side of the lamina densa was still smooth, whereas the epithelial side was irregular, owing to multiple basement membrane projections which separated many of the electron-dense deposits from each other and/or from the epithelial processes. The latter were wider and in many areas fused together.

Group IV (Proteinuria, 12–24 Weeks)

Light Microscopy. The GBM revealed many projections from the epithelial side, and in many areas these projections surrounded completely a PAS-negative area (Fig. 5). The mesangial areas were still prominent. The epithelial cells revealed vacuoles containing occasional PAS-positive or PAS-negative material. The endothelial cells revealed no appreciable changes, and the capillary lumens contained many red blood cells.

Immunofluorescence. The immunofluorescence deposits at the periphery were more numerous, larger, and irregularly shaped. Many were bisected, multisected, or surrounded by a zone which did not react with the fluoroscein conjugated serums. When the section was stained with PAS iron hematoxylin, the deposits reacted weakly or not at all with the Schiff reagent, but the surrounding zone, which did not fluoresce, took the PAS stain positively (Fig. 5 and 6). Most of the round homogeneous vacuoles described under the light microscope reacted with the fluorescein conjugated serums.

Electron Microscopy. The mesangial changes persisted. The endothelial cells were unchanged. The frequent electron-dense deposits were still separated from the endothelial cells by a lamina rara interna and lamina densa. The epithelial side of the GBM, however, revealed striking irregularity. This consisted of multiple knob-like projections giving the cross section of the capillary a cogwheel appearance (Fig. 12 and 13). The center of each knob or cog was made up of electron-dense deposits surrounded by GBM material. The fused foot processes covered and filled the spaces between the cogs. Rarely, a few strands of dense ma-

terial with the periodicity of collagen, were seen around these deposits (Fig. 13 and 14).

Discussion

Endothelial Cells

This study demonstrates that the endothelial cells do not participate in the morphogenesis of autoimmune nephrosis—no appreciable endothelial swelling or proliferation was encountered. The capillary lumens were always patent and contained the usual circulating elements.

Mesangial Cells

The prominent mesangial areas observed in this experiment appear to result from an increase in cell size rather than cell proliferation, since only rarely were more than 2 mesangial cells found in a single area. This change antedated clinical proteinuria. Peripheral cytoplasmic extensions persisted through all stages of the disease and had many intraluminal projections. Farquhar and Palade [24] noticed similar changes in rats having proteinuria induced by aminonucleoside and, to a lesser extent, in the noninjected controls. They considered intraluminal cytoplasmic projections as evidence of phagocytic activity of the mesangial cell, which increased with the proteinuria. Also, these investigators mentioned that Zimmerman, in 1929, had observed these projections with the light microscope and called them "intrakapillarhöckerchen." Our findings establish that the mesangial cells do have cytoplasmic intraluminal projections. The marked reaction with antiglobulin serum seen in the A/K and A rats that received injections may reflect an increase in pinocytotic activities. Faith and Trump [25] reported peripheral mesangial cell processes, similar to those we have described here, surrounding partially or completely the capillary lumen, and adding another cell layer to the periphery of the capillary wall. They stated that this was more marked in acute glomerulonephritis and eclampsia and probably in lupus nephritis. Our experience with human biopsy material revealed similar mesangial changes as well as the presence of intraluminal cytoplasmic projections in those diseases, and in the nephrotic syndromes of unknown etiology.[18] It is difficult to relate this prominent mesangial alteration to a single disease. It may reflect the increased phagocytic (or pinocytotic) activity of these cells. If this is so, one may explain the presence of rat globulins as a result of uptake of circulating rat globulins alone, or antigen-antibody complexes caused by a variety of antigenic stimuli. Obviously, proof of the latter depends upon finding the antigen in the same location—a problem which concerns many investigators.

Epithelial Cells

No appreciable epithelial cell proliferation was noticed at any stage of this disease. Therefore, the reduction of the Bowman's space was considered the result of the increase in the epithelial cell size. The epithelial cells themselves revealed more osmiophilic membrane-limited droplets than those seen in the controls. No other cytoplasmic or nuclear changes were observed. The droplets increased with time, and with the immunofluorescence technique, most of them were shown to react with antiglobulin and anticomplement serums. They appeared similar to the droplets seen in the epithelial cells of the proximal convoluted tubules.[26] Fusion of the epithelial cell foot processes developed only after the appearance of the electron-dense deposits, and even in the late stages, intact processes were encountered away from the deposits. No correlation in this model was found between the extent of the fusion and the severity of the proteinuria. However, the extent of the fusion did correspond to both the size of the deposit and the duration of the disease. Fusion was seen in areas of large deposits and was more marked in late stages of the disease.

Basement Membrane

In the early stages the GBM is of the usual thickness. In the late stages, the spaces between the epithelial and endothelial cells became markedly widened and contained many large deposits surrounded by irregular masses of GBM. Two interesting conclusions can be drawn from the changes noted in the GBM: (1) In the early stages the subepithelial localization of the deposits without intervening GBM and the presence of GBM zones between them and the epithelial cells in the late stages suggest that the epithelial cells themselves form at least part of the GBM. (2) The persistence of a uniform layer of GBM with a smooth surface along the lamina rara interna suggests that if there is a turnover in GBM the endothelial cells themselves contribute in part to the formation of the new GBM. Otherwise, if the epithelial cells alone were forming the new GBM, one would expect, in the late stages of the disease, that the deposits would be found along the endothelial side of the GBM.

Therefore, this study offers further evidence for the concept that both the epithelial and the endothelial cells contribute to the formation of the GBM.[27,28]

Lack of Hyalinization

The absence of necrosis, of macrophages, of polymorphonuclear leu-

kocyte infiltrate, and of fibrin deposits was a prominent feature of this disease. This may explain the lack of hyalinized glomeruli even in very late stages. Electron microscopic examination of the late stages, however, sometimes showed collagen fibers adjacent to the deposits. The absence of fibroblasts or macrophages suggests that the adjacent epithelial cells produced this collagen.[29] Chemically, the basement membrane, in part, is collagen,[30] and if the epithelial cell can form basement membrane, then under abnormal circumstances, it may form banded collagen. This is further supported by our observation that in renal glomerular diseases characterized by epithelial cell proliferation, intraglomerular collagen deposition of the usual periodicity is seen, with the electron microscope, between the epithelial cells and the basement membrane.[29] It is thought that the presence of fibrin in Bowman's space plays an important role in inducing epithelial cell proliferation, visceral and parietal, and both may form collagen in the chronic stage of the disease. The absence of necrosis and fibrin deposition in this study may explain the lack of epithelial cell proliferation and, hence, the paucity of collagen formation.

Development of Electron Dense Deposits Containing Globulin and Complement

In this sequential study, the electron-dense deposits appeared at least 2 weeks before development of the clinical proteinuria and rarely extended beyond the lamina rara externa. With time, the deposits became larger and more frequent and irregular. At this time staining with anti-rat globulin and complement revealed in both A/K rats and A rats only mesangial deposition of host gamma globulin with extension toward the periphery of the capillary lumens. Comparing the photographs of these deposits with those published by Glassock and his colleagues,[13] we interpreted this as corresponding to the peripheral deposition of gamma globulin along the capillary walls, which they described as being seen 1–2 weeks prior to the demonstration of the complement and antigen deposits. In addition to the rarity of these deposits at this stage, their size, seen with the electron microscope, was not more than the width of the lamina rara externa and, therefore, they are beyond the resolution of the light microscope. Our comparative immunofluorescence, histochemical, and electron microscopy study demonstrates that the electron-dense deposits are not Schiff-positive, in contrast to a recent report [12] in which it is stated that they are PAS-positive. From the published figures, what they identify as PAS-positive deposits seem to correspond to the spikes of the GBM in our study. Such deposits may assume a very faint PAS-positive reaction but can be easily differentiated in our 0.5-μ thick

sections from the PAS-positive GBM and its spike. The GBM and its spike are always denser and take the PAS stain more deeply than the deposits (Fig. 5 and 6). This is very similar to the histochemical description of human membranous glomerulonephritis.[17,18]

This comparative, thin-section, and ultrastructural study confirms that the deposits observed by the electron microscopists are the same as the immune complexes seen by the immunomorphologist. Both appear at the same time, progress similarly, and are related in an identical way to both epithelial cells and basement membrane.

Summary

Sprague-Dawley and Lewis-Mai rats received repeated injections of kidney homogenate emulsified with complete Freund's adjuvant and were killed either before or after the development of proteinuria. The longest period of observation was 6 months after the onset of proteinuria. The morphologic findings in the two strains of rats were similar. The study showed that subepithelial electron-dense deposits appeared at least 2 weeks before the development of significant proteinuria. With thin sections and immunofluorescence and histochemical techniques, these deposits were seen 2 weeks after the development of proteinuria and contained gamma globulin and complement. The lag was thought to be an artifact due to the size of the deposits, since in the preproteinuria stages the deposits occupied the lamina rara externa and were beyond the resolution of the light microscope. With time, these deposits became larger, more irregular, and were surrounded partially or completely by basement membrane. This study of thin sections, comparing the results of immunofluorescence, histochemical, and ultrastructural techniques presented further evidence that the deposits revealed by electron microscopy are the same as the "lumps" described by the immunomorphologist. The fusion of the epithelial foot processes was not *sine qua non* to the disease, and the extent of the fusion was not predictable from the amount of proteinuria. The study furnished further evidence of the role of the epithelial and endothelial cells in the formation of new basement membrane. No necrosis or fibrosis was noticed in this disease. Very rarely, with the electron microscope, collagen fibers were seen adjacent to the deposits in the GBM next to the epithelial cells. The role of the epithelial cells in the formation of the collagen was thus suggested.

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[*Illustrations follow*]

Legends for Figures

Sections for light microscopy (Fig. 1, 3, and 5) were stained with PAS iron hematoxylin. Electron micrographs (Fig. 7–14) were made of sections stained with uranyl acetate and lead hydroxide.

Fig. 1. Portion of glomerulus from an A rat which received 10 injections, with no proteinuria and normal BUN, cholesterol, and total lipids. Note prominent mesangial area *(M)* with extension of cytoplasm into adjacent periphery of capillary walls. × 3000.

Fig. 2. Same section as Fig. 1, reacted with anti-rat gamma globulin. Note diffuse cytoplasmic reaction with extension to periphery of capillary walls. × 3000.

Fig. 3. Portion of glomerulus from an A/K rat which received 10 injections, developed proteinuria 2 weeks after the last injection, and was killed 1 week later. Proteinuria, 78 mg./24 hr.; cholesterol, 110 (110–120 mg./100 ml. is normal); total lipid, 450 (300–430 mg./100 ml. is normal). Note epithelial swelling *(EP)*, prominent mesangium *(M)*, and PAS-negative focus *(arrow)*, limited on either side and at the base by PAS-positive GBM and its spikes. Capillary lumen, *CAP*. × 3000.

Fig. 4. Same section as Fig. 3 reacted with anti-rat complement. Note peripheral granular reaction *(arrow)* surrounded by nonreactive zones corresponding to GBM and its spikes seen in Fig. 3. Cytoplasmic reaction surrounds mesangial nucleus *(M)*. × 3000.

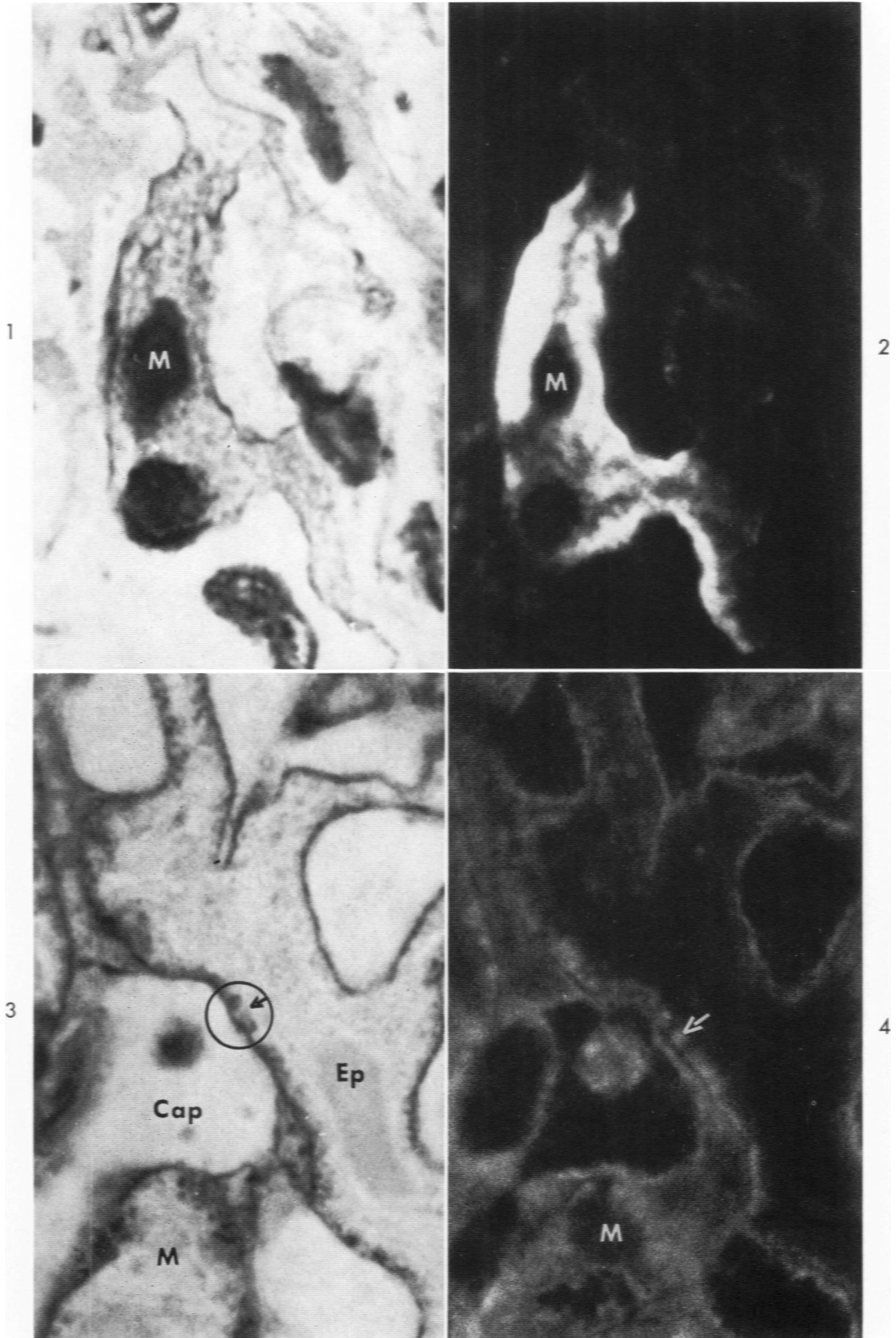
1
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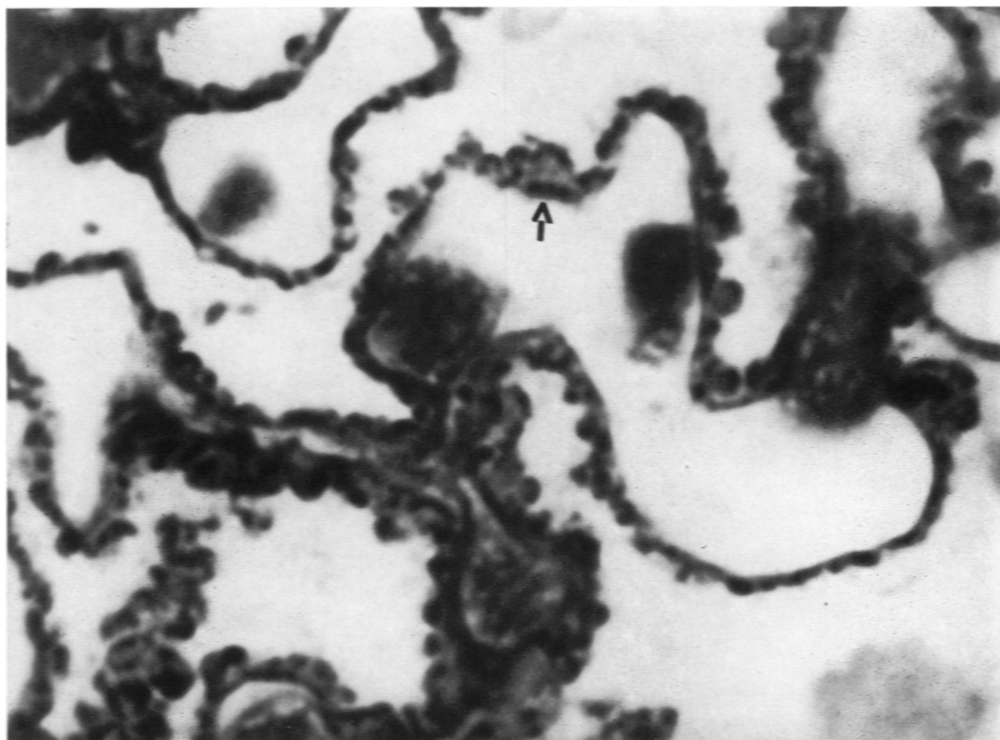
Fig. 5. Portion of glomerulus from an A/K rat which received 5 injections and was killed 4 months later. Proteinuria (mg./24 hr.): 135, 393, and 354 at 1 week, 2 months, and 4 months. BUN, 18 mg./100 ml.; cholesterol, 285 mg./100 ml.; and total lipids, 1470 mg./100 ml. at 4 months. Field shows marked irregularity caused by multiple spikes and curves from GBM and many PAS-negative deposits. Note PAS-negative deposit surrounded by GBM *(arrow)*. × 3000.

Fig. 6. Same section as Fig. 5, reacted with anti-rat gamma globulin, illustrates granular appearance of deposits. Compare area indicated by arrow to same area in Fig. 5. The PAS-negative area in Fig. 5 fluoresced in Fig. 6, where it was surrounded by nonfluoresced PAS-positive GBM. × 3000.



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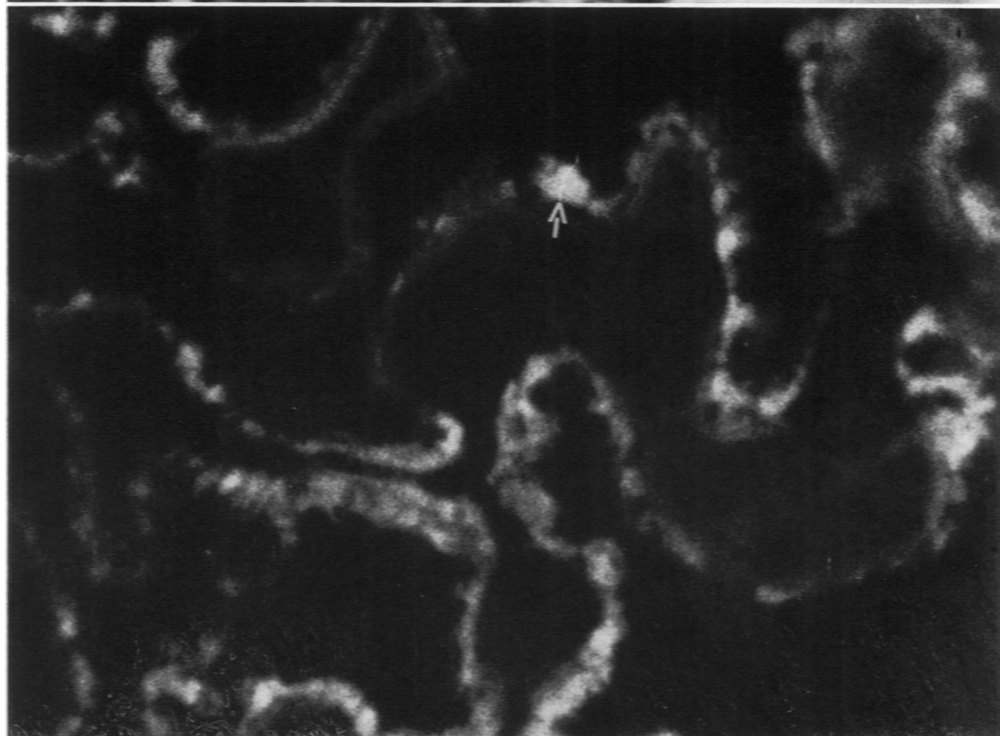

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Fig. 7. Portion of glomerulus from an A/K rat which received 5 injections and was killed 5 days later with no proteinuria. Note prominent mesangial area *(M)* consisting of many cytoplasmic projections into capillary lumen at arrows, and peripheral extensions at open arrows. Many epithelial microvilli are seen at the urinary space *(US)*. BUN, serum protein, cholesterol, and total lipids within normal limits. × 7750.

Fig. 8. Portion of glomerulus of an A/K rat similar to that in Fig. 7 illustrates early electron-dense deposits *(open arrows)* seen at lamina rara externa. Note slit pore membranes at arrows and their continuity with outer layer of epithelial cell membrane. × 64,000.

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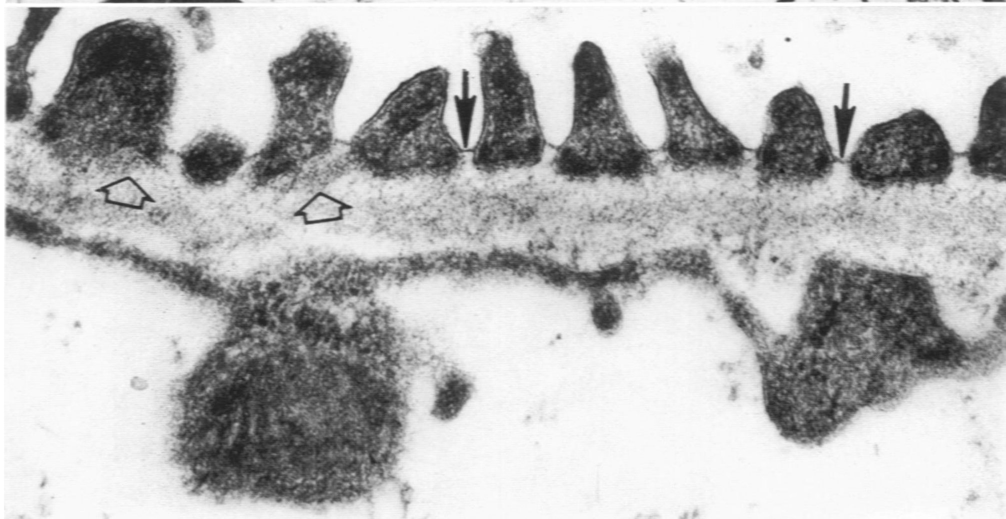

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Fig. 9. Electron micrograph from A/K rat seen in Fig. 3 and 4 to illustrate prominent mesangial area and its extensions to luminal side *(M)* and their interdigitation with endothelial cell cytoplasm *(E)*. Compare with Fig. 10. Red blood cell, *R*. × 22,000.

Fig. 10. Electron micrograph of adjacent section to that seen in Fig. 9 illustrates clearly the mesangial cytoplasmic projection into capillary lumen. Compare circles and squares in Fig. 9 and 10. Note continuity of intraluminal projections to deeper portion of mesangial cytoplasm. × 22,000.

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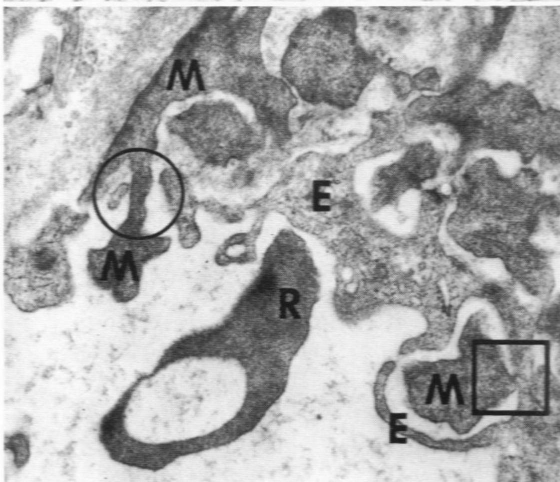
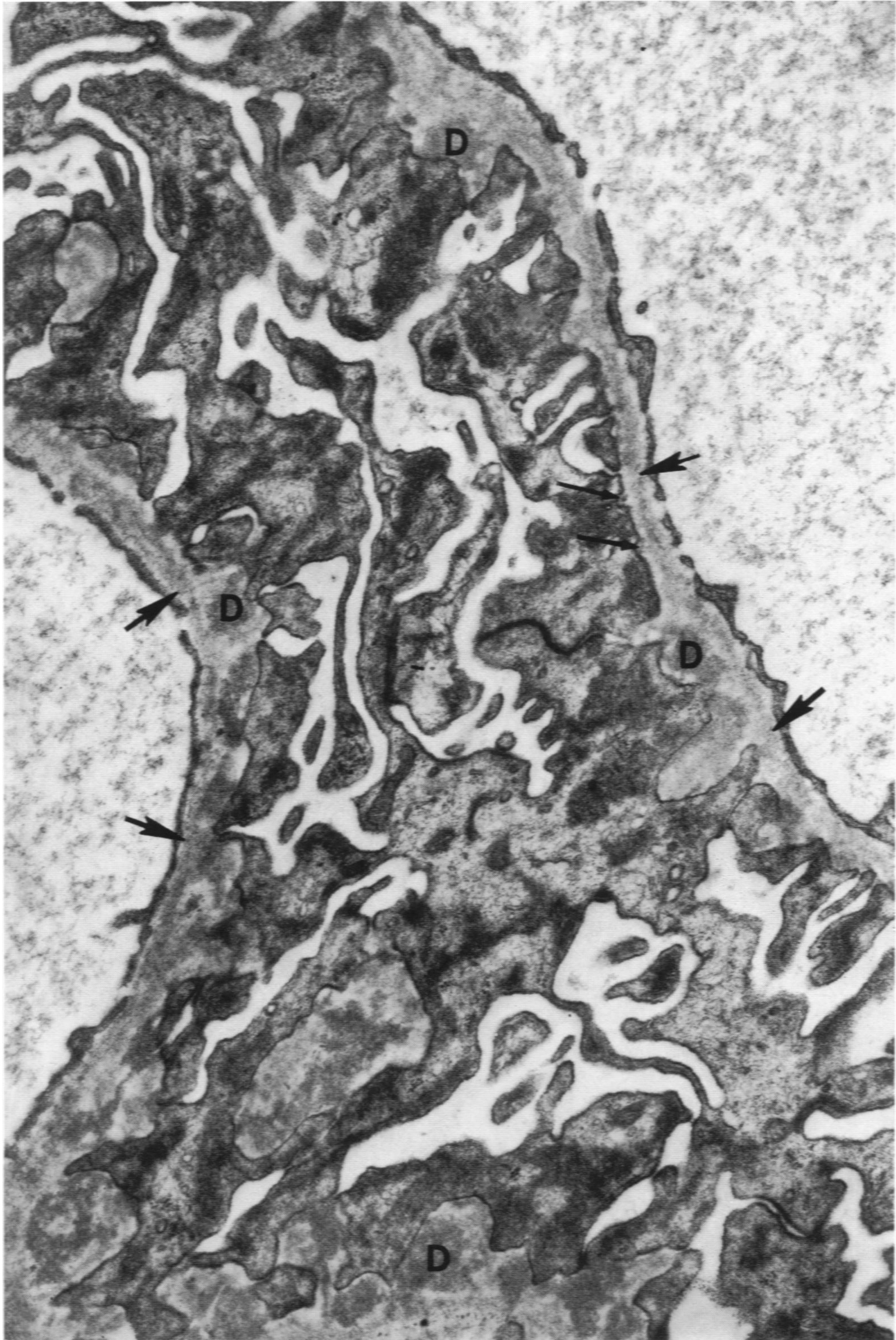


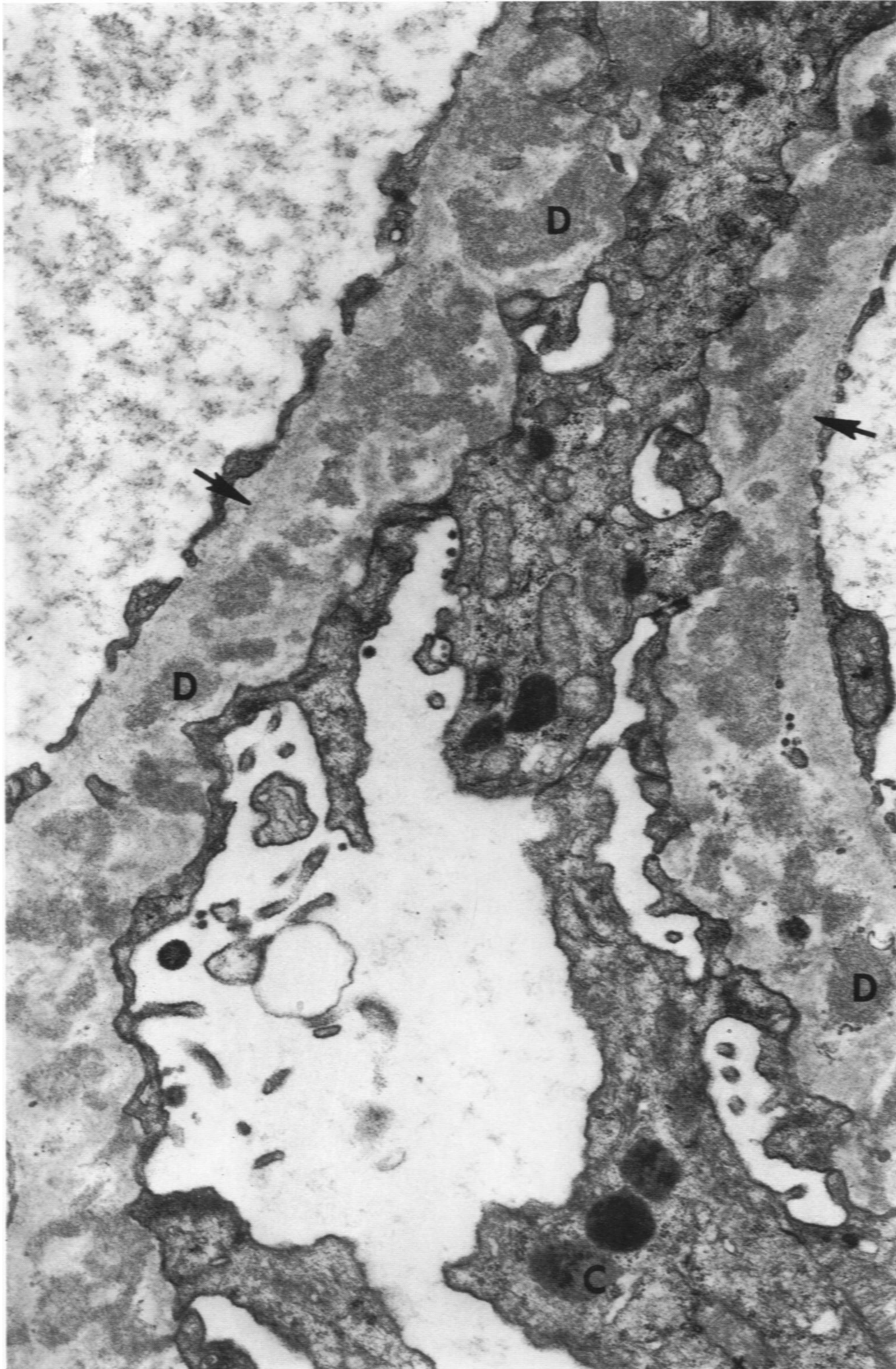
10

Fig. 11. Electron micrograph from same rat in Fig. 9 and 10 illustrates peripheral portion of glomerular capillaries. GBM *(arrows)* is of usual thickness. Note frequent subepithelial electron-dense deposits *(D)* and concavities of epithelial foot processes around them. A very small deposit appears at small arrows. Intracytoplasmic condensation in epithelial cell foot processes adjacent to deposits is marked. × 21,700.



11

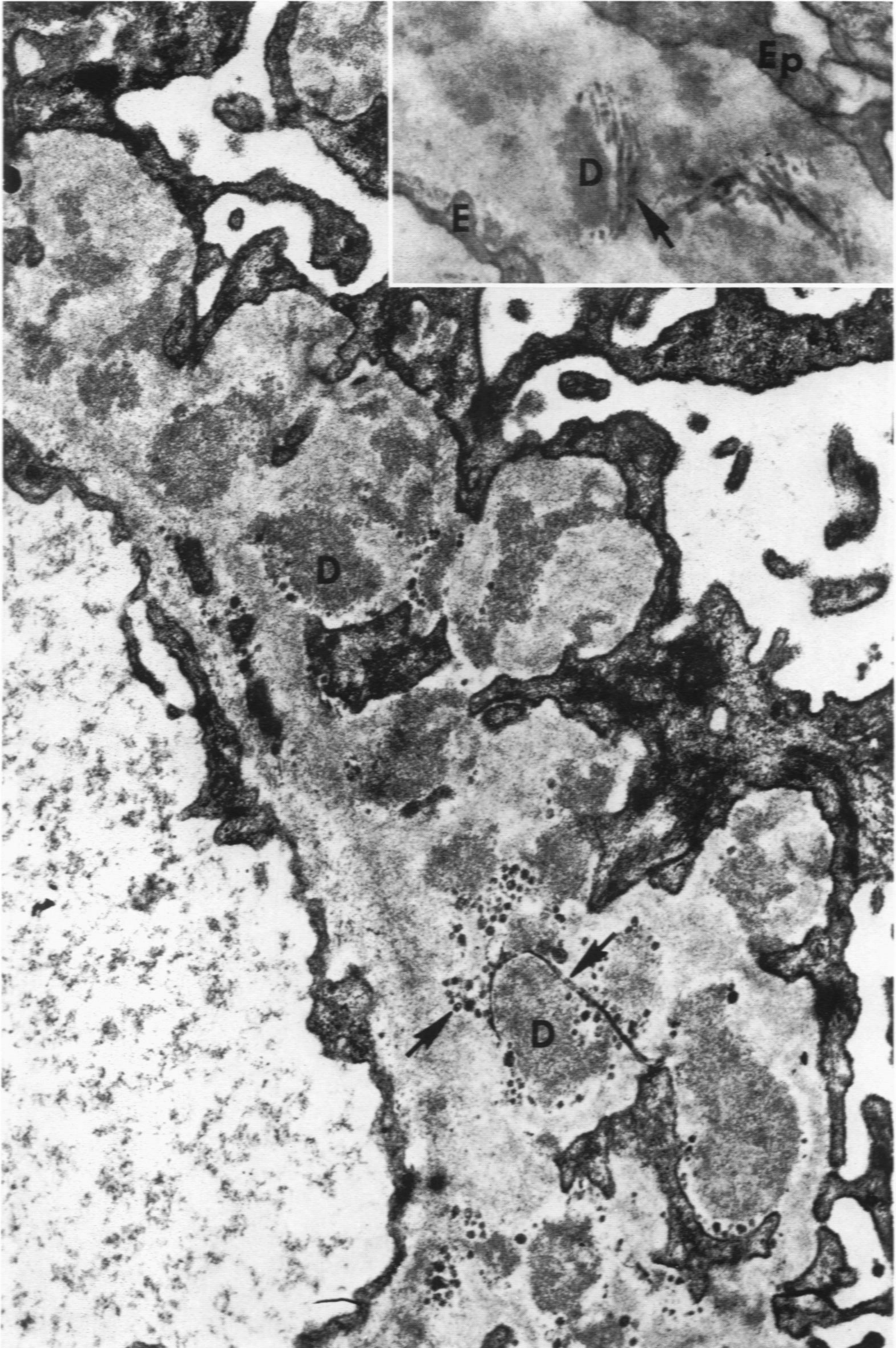
Fig. 12. Electron micrograph of portion of glomerulus from same A/K rat seen in Fig. 5 and 6 (4 months of disease) illustrates persistence of uniform layer of GBM *(arrows)* adjacent to endothelial cells (of the same thickness as seen in Fig. 11). There are frequent electron-dense deposits *(D)*, some completely surrounded by GBM, which separates them from fused epithelial foot processes. Epithelial cell cytoplasm contains frequent lipid droplets and cytolysomes *(C)*. × 22,200.



12

Fig. 13. Electron micrograph of portion of glomerular capillary wall from same A/K rat seen in Fig. 12 illustrates cogwheel appearance of capillary wall where electron-dense deposits *(D)* form core of these cogs surrounded by GBM and fused foot processes. Most of the frequent collagen fibers adjacent to these deposits *(arrows)* in this field appear in cross section. × 30,000.

Fig. 14. Electron micrograph from same A/K rat seen in Fig. 13 illustrates portion of glomerular capillary wall. GBM is limited by epithelial cell *(EP)* and endothelial cell *(E)* and contains deposits *(D)* with longitudinal cuts of adjacent collagen *(arrow)* illustrating the usual collagen periodicity. × 35,000.


Ep
D
E
D
D
14
13