Culture of Human Glomerular Cells

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Human glomeruli were routinely cultured in Waymouth's medium supplemented with insulin and conditioned medium. Three cell types were seen in the culture of both adult and infant kidneys, but the morphology of the glomerular cellular outgrowths depended on the age of the patient from which the kidney was obtained. Cultures of glomeruli from older individuals resulted in more "differentiated" cells, but both adult and infant glomerular cells rapidly became "dedifferentiated" as the length of time in culture increased. Outgrowths of cultured glomeruli did not contain fibroblasts or tubular cells. Finally, synthesis of basement membrane material by these cultured glomerular cells was demonstrated. (Am J Pathol 96:101-120, 1979)

TO DEVELOP *in vitro* cellular and molecular models for assessment of renal glomerular cell responses in disease, several investigators have established cultures of glomeruli or glomerular cells.[1-9] For such culture systems to be meaningful, they must be reproducible, artifacts must be well defined, and agreement must be reached regarding optimal culture conditions and the identity of cultured glomerular cells. Unfortunately, there is considerable controversy concerning the feasibility of culturing adult glomeruli [1] and the precise identity of cells (epithelial, mesangial, endothelial, or Bowman's capsule cells) in cultures of young or adult glomeruli.[1,3,6] This situation is complicated by the possibility that tubular cells or fibroblasts may contaminate or dominate cellular outgrowths. Scheinman et al [10] and Norgaard [7] demonstrated that biochemical and morphologic characteristics of glomerular epithelial cells are dramatically altered as a consequence of culture conditions, additionally complicating identification of cell types in culture. The present study presents a reliable way to culture human glomeruli of all ages and presents morphologic and cytochemical characterization of the cultured cells. A preliminary report of this work has been presented previously.[11]

Materials and Methods

Tissue Procurement

Fresh kidneys were obtained from autopsies or as the result of resection for surgical treatment of various conditions, including congenital renal malformation or hypernephroma.

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Isolation and Culture of Glomeruli

Glomeruli were isolated according to a modification of a technique previously described.[9] Briefly, the renal cortex was dissected from renal medulla and minced into a fine paste which was then pestled successively through nylon sieves whose pore size depended on the age of the patient (adult: 450, 277, and 130 μm; infant: 450, 130, and 73 μm). Intact glomeruli were retained on the final screen and comprised both encapsulated (with Bowman's capsule) and nonencapsulated glomeruli in proportions which varied with each individual isolation. In a typical adult glomerular preparation, 70–75% of the glomeruli were nonencapsulated. However, scanning electron microscopy often revealed that glomeruli that appeared nonencapsulated by light microscopy often contained small fragments of Bowman's capsule. After washing, isolated glomeruli were pipetted directly into Tissue-Tek slide culture chambers (Miles Laboratories, Elkhart, Ind). Media consisted of Waymouth's medium (MB 752/1 with L-glutamine) supplemented with sodium pyruvate and nonessential amino acids (Gibco Diagnostics, Chagrin Falls, Ohio), 20% fetal bovine serum, 10^{-6} M insulin, penicillin (100 units/ml) and streptomycin (100 μg/ml), and one-half volume of conditioned medium. Conditioned medium was obtained by filtration of media harvested from cultures of confluent guinea pig glomerular cells. Culture medium was replaced daily. After confluency of cell growth was reached, the cells were detached by exposure to 0.01% EDTA (5 seconds) in PBS and 0.025% trypsin for approximately 5 minutes and were subcultured in plastic flasks.

Isolation of Tubular Cells and Fibroblasts

Renal cortical nonglomerular cells were prepared from human cortex by modifications of the methods of Dechenne et al [8] and of Cade-Trayer and Tsuji.[12] Briefly, human cortex was treated with 0.25% trypsin for 60 minutes at 37 C, and the cell suspension was centrifuged at low speed. The supernatant was saved, and the pellet was resuspended and again centrifuged. The two supernatants were combined and passed through a series of nylon screens designed to entrap glomeruli. Single cells which passed through the screens were plated in Tissue-Tek chambers. Both transmission and scanning electron microscopy have shown that the cells prepared in this way are almost entirely tubular cells [13]; the initial cells have prominent mitochrondria, dense bodies, and microvilli along one pole of the cell. After culture, these cells form confluent sheets which are easily distinguishable from glomerular cells. Although this technique yields tubular cells, in this report the cells prepared in this way will be designated "nonglomerular renal cortical cells."

Human WI 38 fibroblasts were purchased from Grand Island Biological Company (Grand Island, NY).

Electron Microscopy

Electron microscopy was performed as previously described.[9] Isolated cells or cultured glomeruli were prepared for transmission electron microscopy (TEM) by initial fixation for 4 hours at 4 C in 4% cacodylate-buffered glutaraldehyde and subsequent centrifugation in a 12-ml disposable polypropylene culture tube (Falcon Plastics, Oxnard, Ca). The pelletted cells and glomeruli were secondarily fixed in 1% osmium tetroxide (OsO_4) in *s*-collidine buffer, dehydrated in a graded ethanol series, and embedded in an Epon 812-Araldite-epoxy resin mixture. Ultrathin sections were doubly stained with lead hydroxide and uranyl acetate and examined at 50 keV with a Hitachi HS-8F electron microscope.

Alternatively, if only small quantities of suspended cells or glomeruli were available, they were preembedded by centrifugation in a 4% glutaraldehyde, 6% bovine serum albumin gel (BSA, Cohn's fraction V) to avoid the possibility of loss during the preparative procedure. Blocks of the gel pellet containing cells and glomeruli were carefully trimmed under a dissection microscope and then processed for microscopy.

In some cases cells and glomeruli were stained with tannic acid according to the

procedure of Cohn et al.[14] Cells or glomeruli were fixed in freshly prepared 1% tannic acid and 1% glutaraldehyde in 0.075 M phosphate buffer at pH 7.2 for 4 hours at room temperature and stored in 0.18 M sucrose at 4 C in the same buffer. The tissues were rinsed three times for 10 minutes in the sucrose solution and postfixed in 1% OsO_4 in 0.075 M phosphate buffer at pH 7.2 containing 0.18 M sucrose for 1.5 hours. The cells or glomeruli were rinsed twice for 10 minutes each in 25% ethanol, dehydrated in a graded series of ethanol, and embedded in Epon. Sections were stained with lead citrate only.

Immunohistochemistry for Light Microscopy

Limulin (Sigma Chemical Co, St Louis) or wheat germ agglutinin (Miles Laboratories) was conjugated with horseradish peroxidase by the periodate procedure of Nakane and Kawaoi.[15] Limulin-HRP or wheat germ agglutinin-HRP conjugates were separated from free HRP by chromatography on Sephadex G-100. The immunoglobulin fraction of serum containing anti-basement membrane antibody (anti-GBM antibody) from a patient with Goodpasture's syndrome was isolated by precipitation from whole serum in 50% saturated ammonium sulfate followed by chromatography on DEAE cellulose. This immunoglobulin fraction was also conjugated to HRP by the periodate procedure, and antibody-HRP conjugates were separated from free HRP by Sephadex G-100 chromatography.

Six-micron sections of human kidney, quick-frozen in isopentane, were cut on a cryostat microtome. The sections were fixed in the periodate-lysine-paraformaldehyde fixative described by McLean and Nakane.[16] The fixed sections were washed and then treated with HRP-Limulin, HRP-wheat germ agglutinin, or HRP-Goodpasture's antibody for 120 minutes at 37 C. The diaminobenzidine (DAB)-H_2O_2 reaction was then performed. The slides were mounted in Permount. Controls included preincubation of Limulin with 0.2 M sialic acid, preincubation of wheat germ agglutinin with 0.2 M *N*-acetyl D-glucosamine, or the substitution of HRP-labeled nonimmune human immunoglobulins for HRP-anti-GBM antibody from the patient with Goodpasture's syndrome.

Cells grown in tissue culture were fixed with PLP, washed, and then stained with HRP-lectin for 120 minutes at 37 C. The DAB-H_2O_2 reaction was then performed, and the slides were mounted with Permount.

D-Amino Acid Oxidase Histochemistry

Wohlrab's method was used to stain for D-amino acid oxidase.[17]

Results

Culture of Human Glomeruli

Culture of adult human kidney was first attempted using autopsy kidney in the absence of conditioned media, and under these circumstances only 2 of 25 preparations resulted in cellular outgrowths. However, when fresh tissue and conditioned medium were used, 10 of 10 adult preparations and 5 of 5 infant preparations resulted in cellular outgrowths, all of which were subcultured. Thus, the following conditions were found necessary to culture adult human glomeruli: 1) The tissue must be fresh; glomerular cells from tissue removed at autopsies several hours after death did not grow well or at all, whereas glomerular cells from fresh surgical specimens of any age grew well. 2) The use of nylon screens for glomerular isolation avoided the damage to epithelial cells that

is frequently caused by sieving techniques.[18] 3) The culture medium used was critical. Most satisfactory was Waymouth's medium (MB 752/1 with L-glutamine) supplemented with nonessential amino acids and sodium pyruvate, 20% fetal bovine serum, 10^{-6} M insulin, and conditioned medium. If large quantities of glomeruli were available for establishing a culture, conditioned medium was not necessary. Infant glomeruli grew much more readily than adult glomeruli, and it was possible to grow infant cells without insulin or conditioned medium.

Morphology of Human Glomerular Cells

Culture of adult human glomeruli usually resulted in growth of three cell types. Predominant in early cultures of whole glomeruli was a cell with long cytoplasmic extensions (Figure 1A). As the culture aged, the numbers of extensions decreased dramatically and the cell flattened (Figure 1B). Also present in primary cultures were a large circular cell and a more rectangular cell (Figure 1B). The proportion of each cell type varied with each isolation, but as a rule the rectangular cell was much more prominent in kidneys from young patients.

Identification of the cell with cytoplasmic extensions was much more difficult in kidneys from young patients, since the number of cytoplasmic extensions were always greatly reduced as the age of the kidney decreased (Figure 2A). As cells from young patients were maintained for prolonged periods in culture, the number of cytoplasmic extensions became even fewer than in the subcultured adult kidney (Figure 2B). TEM of these cells showed that they appeared to grow from the epithelial side of the basement membrane (Figure 3A), and although cytoplasmic extensions were not seen, microvillus-like protrusions were often seen abutting the basement membrane (Figure 3B).

When mixed cultures of adult glomerular cells were allowed to reach confluency, it became more difficult to identify cell types. At confluency, one could easily discern that a large circular cell was present, but the identity of other smaller cells was more difficult to ascertain (Figure 4A). However, even at confluency, cytoplasmic extensions were visible (Figure 4B). After confluency was reached, cells in some areas grew over each other (Figure 4C), and what appeared to be an extracellular matrix often was seen. At this time, subculturing was performed. After subculturing, some cells were readily identifiable as the rectangular cell (Figure 4D), but unless the cell with cytoplasmic extensions still had extensions, it was often difficult to distinguish the circular cell from a cell which no longer had cytoplasmic extensions.

While adult glomerular outgrowths were predominantly the cell with

cytoplasmic extensions, infant glomeruli grew three cell types which could usually be distinguished from each other on the basis of size rather than morphology (Figure 5). These were designated as a large circular, a small circular, and a rhomboid cell. The rhomboid cell was predominant in cultures of infant glomeruli.

Transmission Electron Microscopy of Subcultured Cells

The predominant cell (cell with cytoplasmic extensions) which evolved in subculture of glomerular cells from intact adult glomeruli had prominent rough endoplasmic reticulum (Figure 6A) and intracellular microfilaments. The predominant cell evolving from subcultured infant glomerular cells (rhomboid cell) showed prominent branched rough endoplasmic reticulum (Figure 6B); these cells also had prominent microfilaments, usually located just beneath the cell membrane.

Cytochemical Characterization of Human Glomerular Cells

Light microscopic examination of sections of normal adult kidney treated with HRP-Limulin revealed prominent staining of glomeruli with almost no tubular staining (Figure 7A). Control sections in which HRP-Limulin was preincubated with 0.2 M sialic acid before reaction with tissue showed no staining. Both adult and fetal glomerular cells in culture showed prominent staining with HRP-Limulin (Figures 7B and C), while renal cortical, nonglomerular cells did not stain at all. WI 38 fibroblasts stained lightly with HRP-Limulin.

Staining with HRP-wheat germ agglutinin was studied only on secondary, subcultured cells. The predominant cell from subcultured adult glomerular cells (cell with cytoplasmic extensions) showed only very light diffuse cytoplasmic staining. In contrast, subcultured infant glomerular cells showed moderate to strong staining (Figure 5), with the rhomboid cell and the small circular cell staining more strongly then the large circular cell. Human WI 38 fibroblasts showed strong diffuse cytoplasmic staining with HRP-wheat germ agglutinin.

To eliminate the possibility that the cells studied in glomerular culture were contaminating tubular cells or fibroblasts, D-amino acid oxidase histochemical studies were performed.[19] Enzyme histochemical staining for D-amino acid oxidase showed *in vitro* grown renal cortical, nonglomerular (tubular) cells to be strongly positive (4+), while cultured WI 38 fibroblasts did not stain at all; these results are in agreement with those of Gilbert and Midgeon.[19] Adult glomerular cells (cell with cytoplasmic extensions) stained lightly (1+), while fetal cells (rhomboid cell) stained more strongly (2+), but in both cases the staining intensity was clearly

intermediate between the high level of the tubular cell and the complete absence of staining of the fibroblast.

A summary of the morphologic and biochemical characteristics of cultured adult and infant glomerular cells is provided in Table 1.

Cytochemical Characterization of Extracellular Matrix Produced by Human Glomerular Cells in Culture

When mixed cell cultures of glomeruli reached confluency, the cells appeared to be embedded in an extracellular matrix. Reactivity of the extracellular matrix with HRP-labeled Goodpasture's serum (anti-glomerular basement membrane antibody) was demonstrated by staining glomerular cells at confluency (Figure 8). Controls treated with HRP-nonimmune serum did not show staining.

Discussion

This report presents methods for reproducible culture of adult and infant human glomerular cells. A previous study had suggested that culture of adult glomeruli was not possible,[1] but results in other laboratories have disproved this.[4,5] Dechenne et al [4] stressed the importance of using fresh tissue to obtain cellular outgrowths from adult kidney. The use of insulin and conditioned medium in preparation of culture media for this study definitely increased the rate of growth of human adult glomerular cells, and we feel these are crucial for culturing of cells from small numbers of glomeruli.

Cytochemical studies suggested that the glomerular outgrowths were not composed of contaminating tubular cells or fibroblasts. First, the present study demonstrated that Limulin reacts with glomerular cells but not tubular cells, both *in vivo* and *in vitro*. Second, enzyme histochemical examinations have revealed the D-amino acid oxidase content of glomerular cells to be intermediate between the high levels of tubular cells and the undetectable levels of the fibroblast. Therefore, we feel confident that the glomerular cell outgrowths are not actually tubular cells or fibroblasts.

It is evident from our work that adult human glomerular cells are morphologically different from infant glomerular cells in culture. These results certainly make it easier to understand why separate laboratories have reported different morphologies for cultured glomerular cells. Scheinman et al [2] stated that the predominant cell in culture of infant kidneys is the mesangial cell, while Dechenne et al [3] stated that the predominant cell in culture of adult kidney is the epithelial cell. Holdsworth et al [6] believe that both cell types immediately grow from adult

Table 1—Summary of Characteristics of Adult and Infant Glomerular Cells in Culture

	Morphologic characteristics		% Cells in secondary culture	D-amino acid oxidase (secondary culture)	HRP-wheat germ agglutinin reactivity	HRP-Limulin reactivity
	Phase microscopy	Electron microscopy				
Adult Glomerular cells						
Cell with cytoplasmic extensions	Long cytoplasmic extensions	Prominent rough endoplasmic reticulum, numerous microfilaments	~95	1+	1+	3+
Large circular cell	Large squamous-appearing cell	Numerous mitochondria	~2	ND*	1+	ND
Rectangular shaped cell	Cell with peripheral areas of thinned cytoplasm	Golgi more prominent than either of above	~2	ND	2+	ND
Infant Glomerular cells						
Rhomboid cell	Rectangular	Branched rough endoplasmic reticulum, numerous microfilaments	~90	2+	3+	4+
Small circular cell	No distinguishing feature other than circular shape and small size	Prominent rough endoplasmic reticulum but without branching	~5	ND	4+	ND
Large circular cell	No distinguishing feature other than circular shape and large size	Numerous mitochondria	~5	ND	1+	ND

* ND = not determined.

glomeruli. Although the present work did not resolve this controversy, it did show that the morphology of the glomerular cells obtained depended on several factors, including age of the cultured kidney and length of time that the cells have been in culture; glomeruli from younger individuals or cells that had been in culture for prolonged periods of time were less differentiated than primary cells from adult glomeruli. The "dedifferentiation" of cultured glomerular epithelial cells was recently described and discussed by Norgaard.[7] The proportion of each cell type in primary culture of the adult kidney depended both on the number of encapsulated glomeruli and the number of "damaged" glomeruli; encapsulated glomeruli produced more large circular cells, whereas "damaged" glomeruli produced more rectangular cells. However, the cell with cytoplasmic extensions always predominated. In the case of the infant kidney, the rhomboid cell predominated both in primary and secondary cultures.

The present study demonstrated that the predominant cell in cultures of infant glomeruli was different morphologically from the cells in cultures of adult glomeruli. While we cannot be sure of the identity of the predominant cell growing from infant glomeruli, we feel certain that the predominant cell growing from adult glomeruli is the capillary epithelial cell, since we feel the long cytoplasmic extensions seen are the *in vitro* equivalents of the glomerular capillary epithelial cell arbor. Future biochemical work will be directed at resolving the controversy of the origin of glomerular cells observed in culture.

Scheinman and Fish [20] used antibodies to basement membrane, fibroblast surface antigen, actomyosin, and antihemophilic factor to tentatively identify cell types from human infant glomeruli. On the basis of a comparison of the *in vivo* distributions of these antigens with their distributions *in vitro*, the large circular cell has been tentatively identified as the glomerular epithelial cell and the rhomboid cell as the mesangial cell. We feel less certain of the identity of these cell types, since we have recently shown that the immunofluorescent localization of antigens can often be misleading. By light microscopy, it appears that fibronectin is localized to the glomerular mesangium,[21] but we recently demonstrated with the use of immunoelectron microscopy that fibronectin is actually present in all glomerular cell types.[22] We feel that the use of immunoelectron microscopy will give more valid insights into the origin of glomerular cells than will fluorescence microscopy.

The extracellular matrix produced by glomerular cells contains two immunochemically detectable macromolecules. We recently demonstrated that the matrix contains fibronectin.[22] The present study demon-

strated that the matrix also probably contains a form of basement membrane, since it binds anti-GBM antibody from a patient with Goodpasture's syndrome. These results suggest that *in vitro* culture of glomeruli may provide a useful system for study of the regulation of basement membrane synthesis by glomerular cells.

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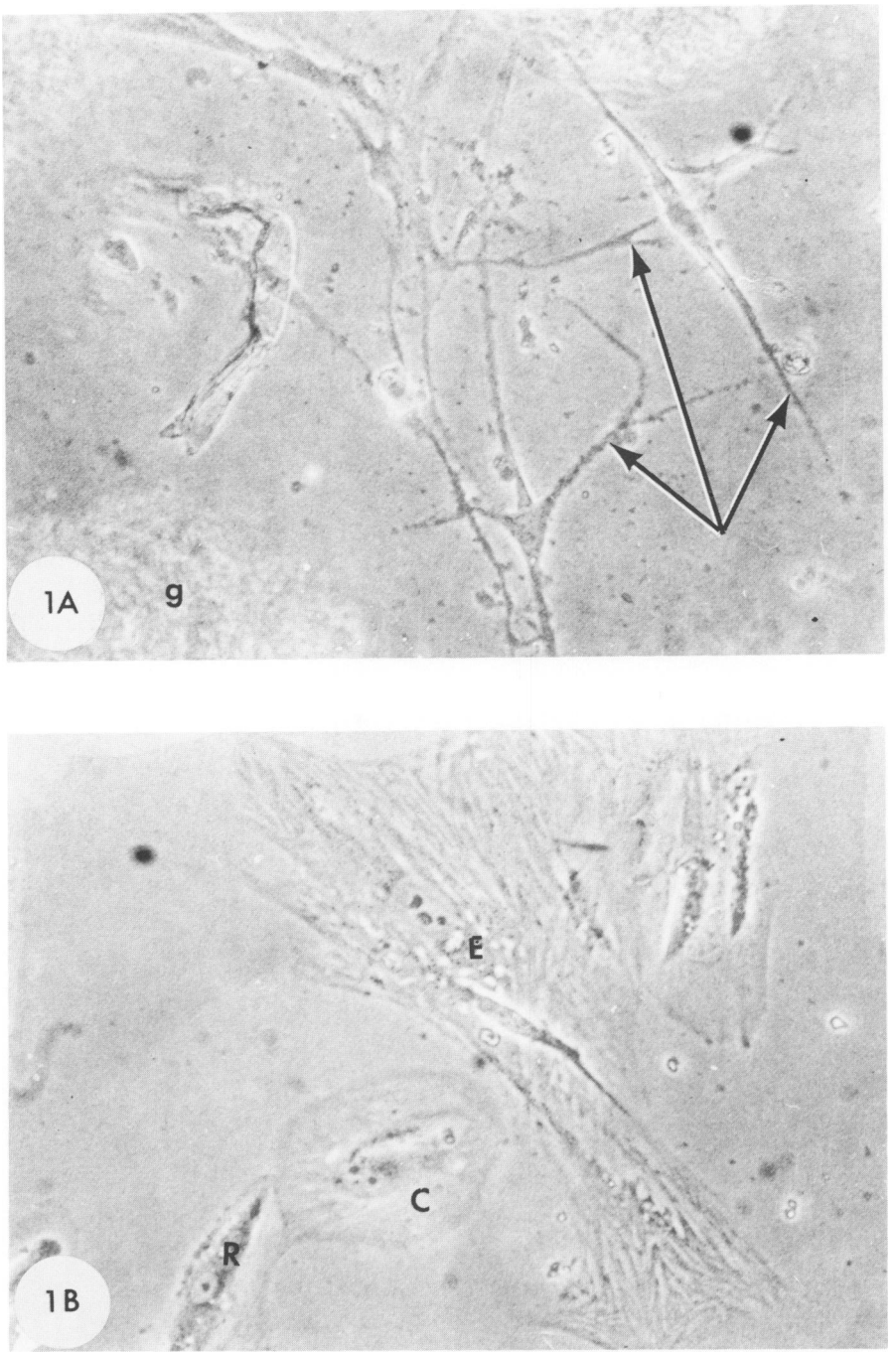
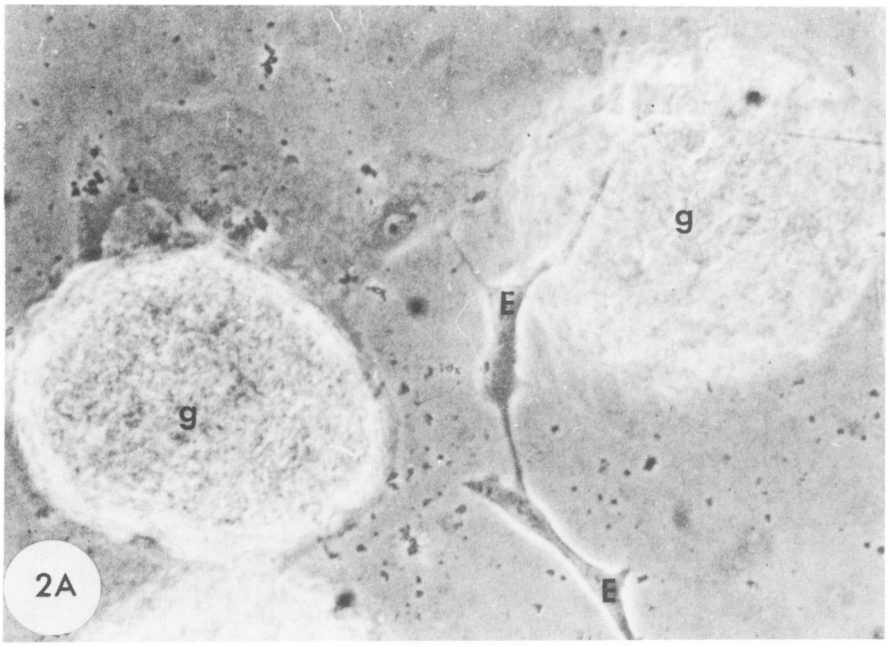


Figure 1—Phase microscopy of cellular outgrowths from a kidney resected from a 50-year-old male with hypernephroma. **A**—The cells initially growing from glomeruli (*g*) in culture have long cytoplasmic extentions (*arrows*). (×266) **B**—After the first subculture, the cell with cytoplasmic extensions (*E*) has flattened and has numerous microfilaments. Also present are a circular cell (*C*) and a rectangular cell (*R*). The rectangular cell always has an area of thinned cytoplasm surrounding the perinuclear area. (×266)



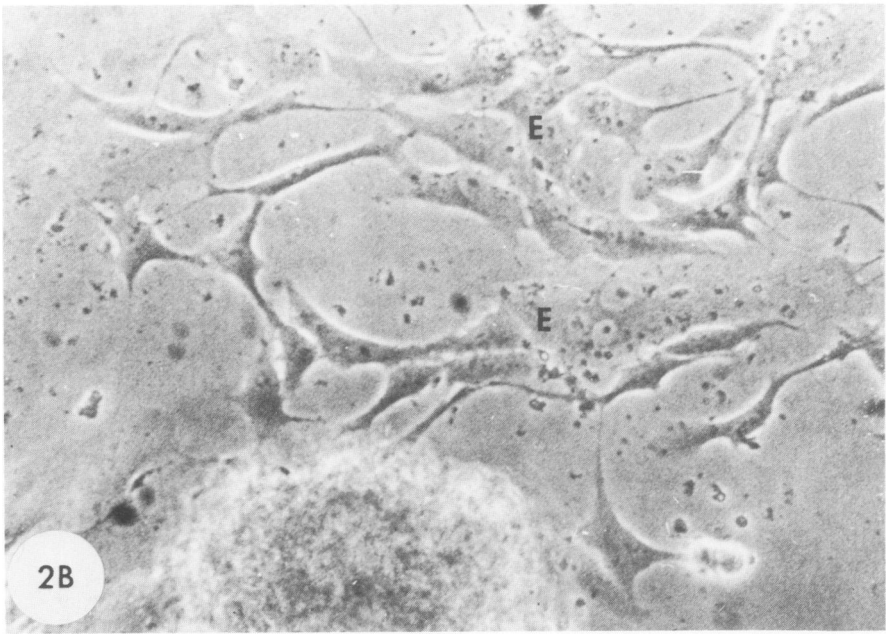


Figure 2—Phase microscopy of cellular outgrowths from an autopsy kidney of an 11-year-old with myoclonus epilepsy. **A**—Cells with cytoplasmic extensions (*E*) are seen around the glomeruli (*g*), but these cells have fewer extensions than their adult counterparts in primary culture. (×266) **B**—Since the cell with cytoplasmic extensions (*E*) was present in culture for longer periods, some of the cells no longer had extensions. (×266)

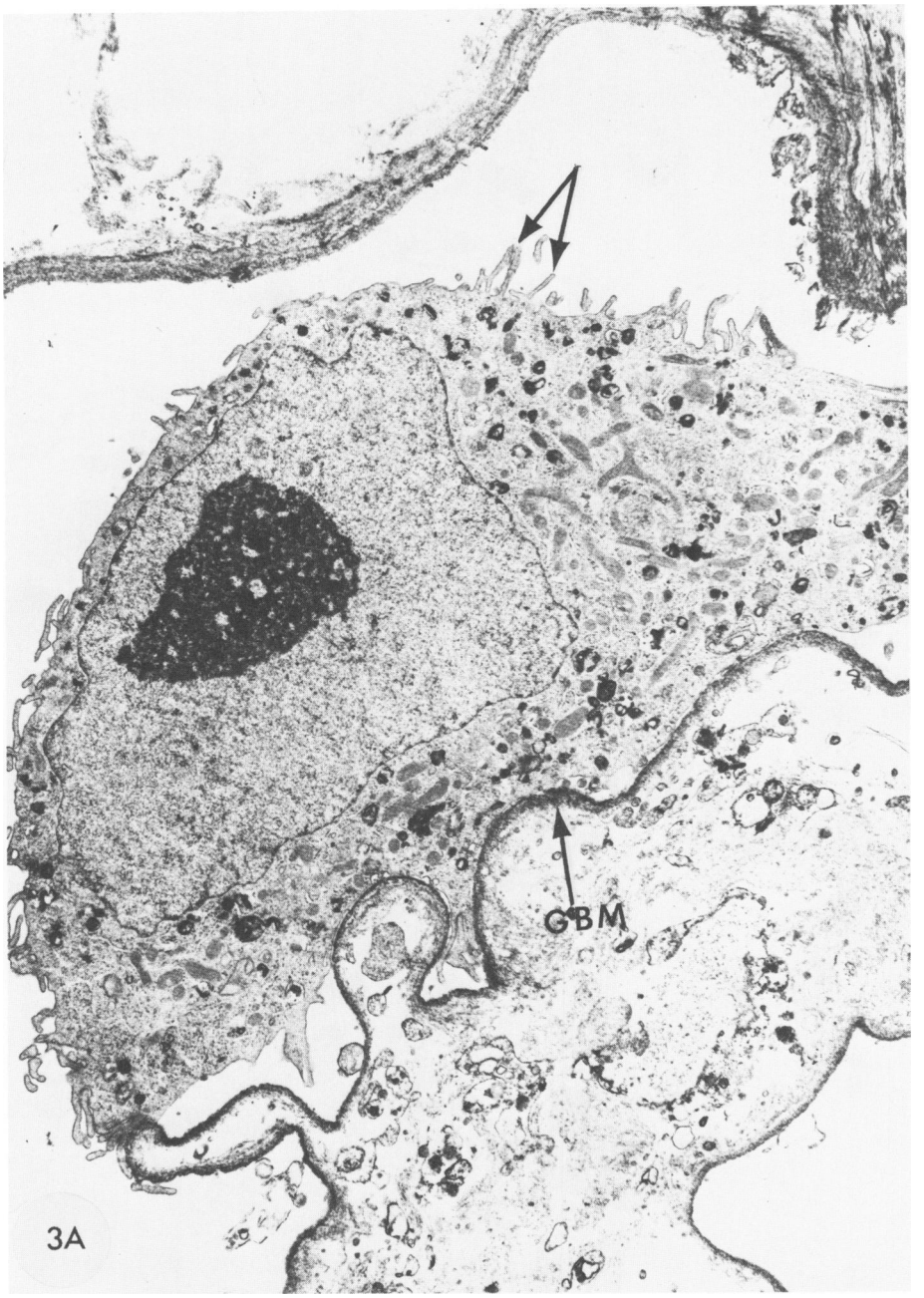
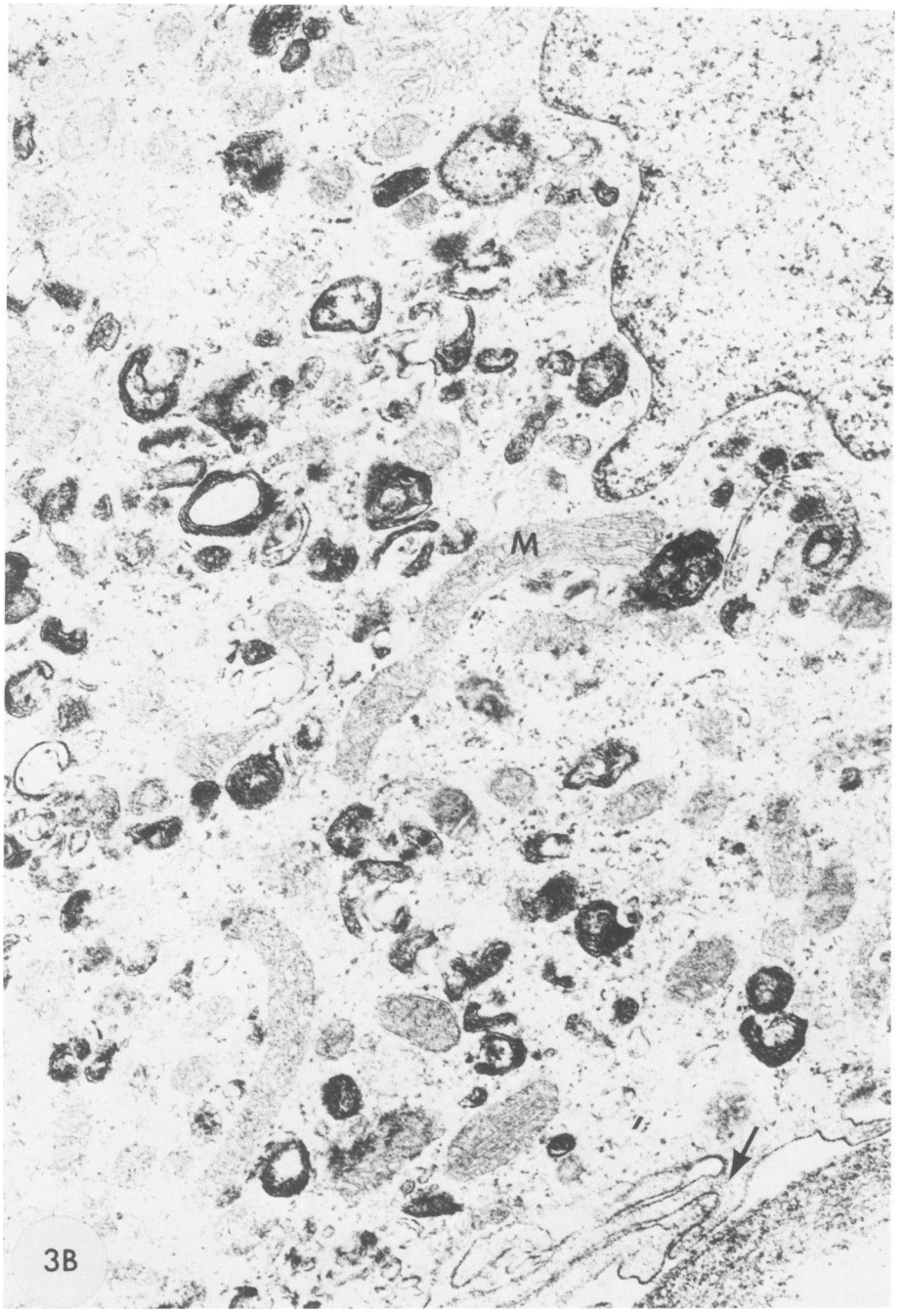


Figure 3—Transmission electron microscopy of tannic acid-fixed glomeruli from the kidney of an 11-year-old with myoclonus epilepsy, which had been cultured for 2 weeks. Staining is with lead citrate only. **A**—It appears that the cell is growing from the epithelial side of the glomerular basement membrane (*GBM*). The cell has numerous microvillus-like protrusions (*arrows*). Large nucleolus indicates cell has adapted to conditions of culture, since these nucleoli are only seen in cultured cells. The lipid inclusions might be related to the enzymatic defect postulated to be present in this disease. (×8179) **B**—High magnification shows the microvillus-like protrusions are often branched (*arrows*). Numerous mitochondria are present (*M*). (×22,610)



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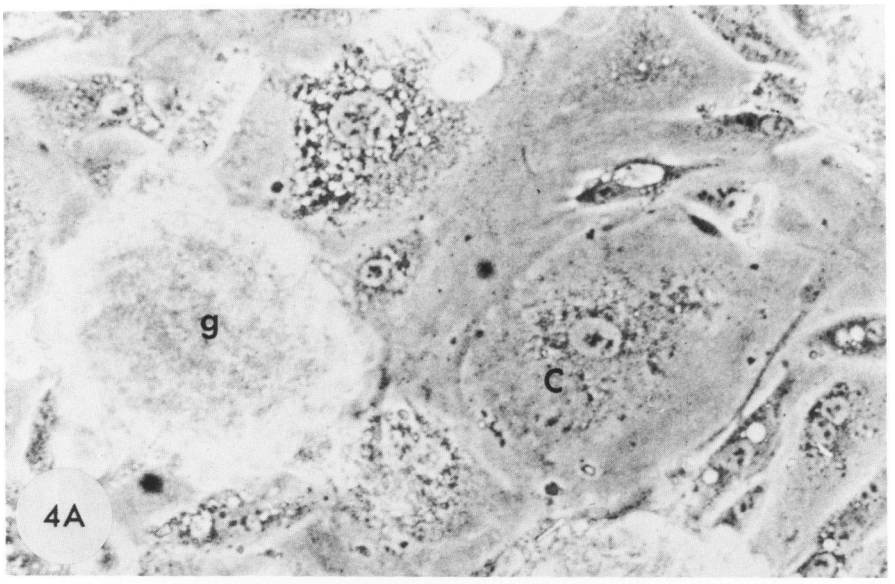


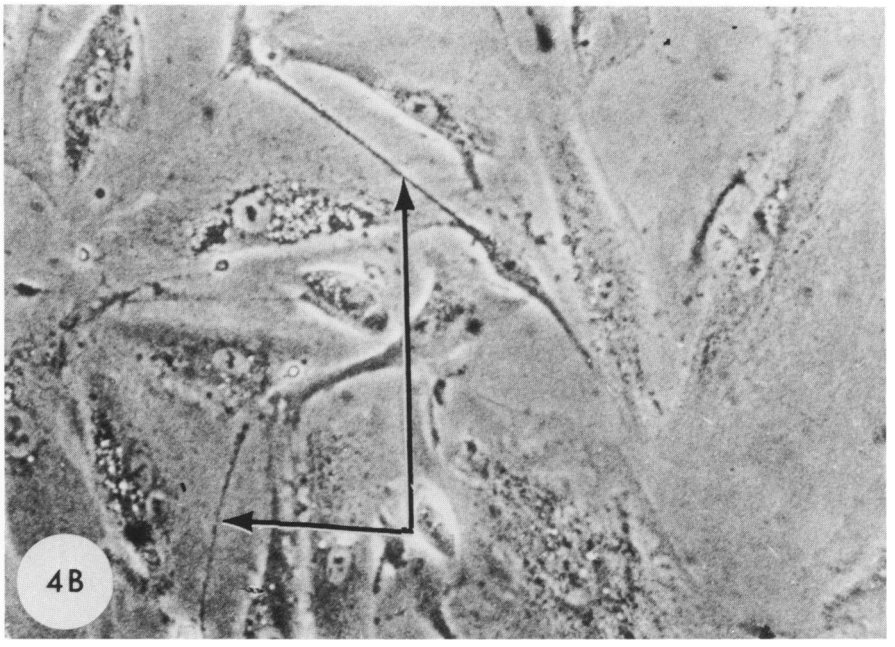
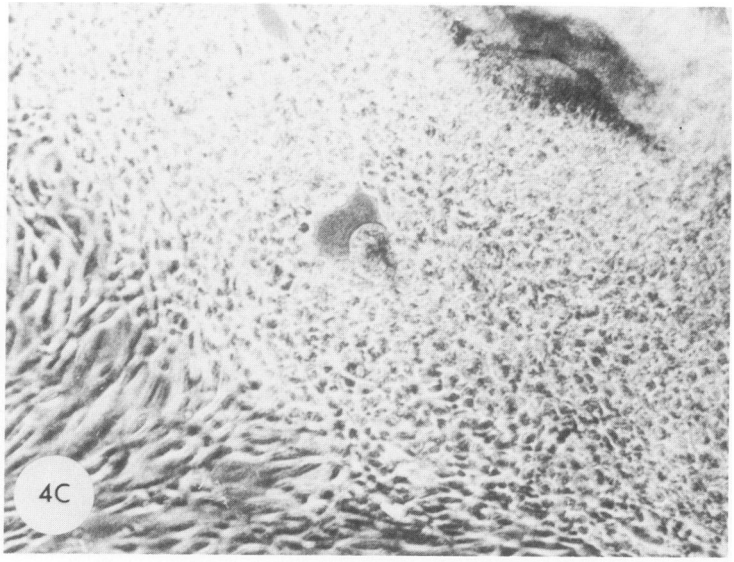
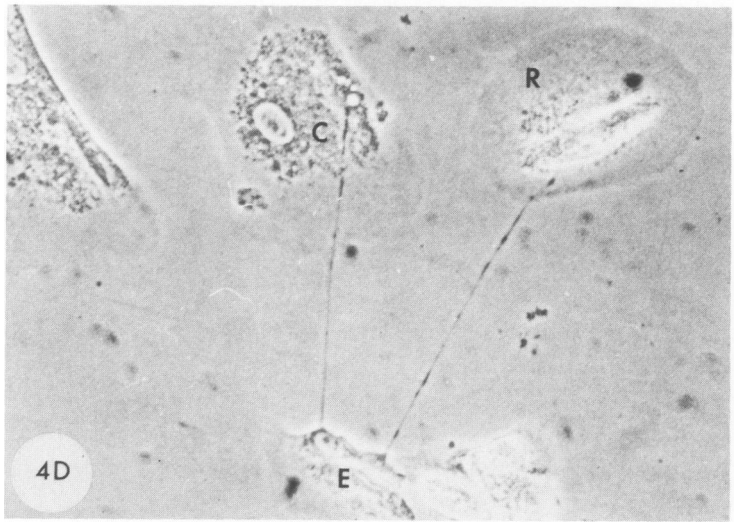


Figure 4—Phase microscopy of cellular outgrowths from a congenitally hypoplastic kidney which was resected from a 65-year-old because of hypertension. **A**—At confluency it was more difficult to distinguish cell types. Especially prominent around the glomerulus (*g*) is a large circular cell (*C*). The nature of the smaller cells is much more difficult to ascertain. (×266) **B**—However, at confluency some of the cells retain their original properties, with some cells with cytoplasmic extensions being seen (*arrows*). (×266) **C**—After confluency was reached, the cells would pile up on top of each other in certain areas, and what appeared to be an extracellular matrix could sometimes be seen. (×266) (With a photographic reduction of 17%) **D**—After subculturing, it was still easy to identify the rectangular cell (*R*), since it always had the peripheral areas of thinned cytoplasm. However, it was often difficult to differentiate between a cell with cytoplasmic extensions (*E*) which had flattened and a circular cell (*C*). One clue that was often of help was the presence of cytoplasmic extensions in the former (*arrows*). (×266) (With a photographic reduction of 17%)





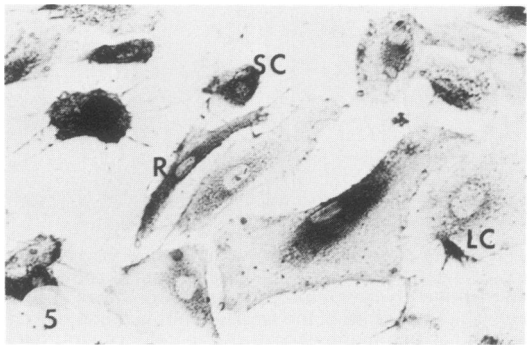


Figure 5—Light microscopy of infant glomerular cells stained with peroxidase-labeled wheat germ agglutinin. Cultures from infant glomeruli showed a large circular cell (*LC*), a small circular cell (*SC*), and a rhomboid cell (*R*). (×650) (With a photographic reduction of 17%)

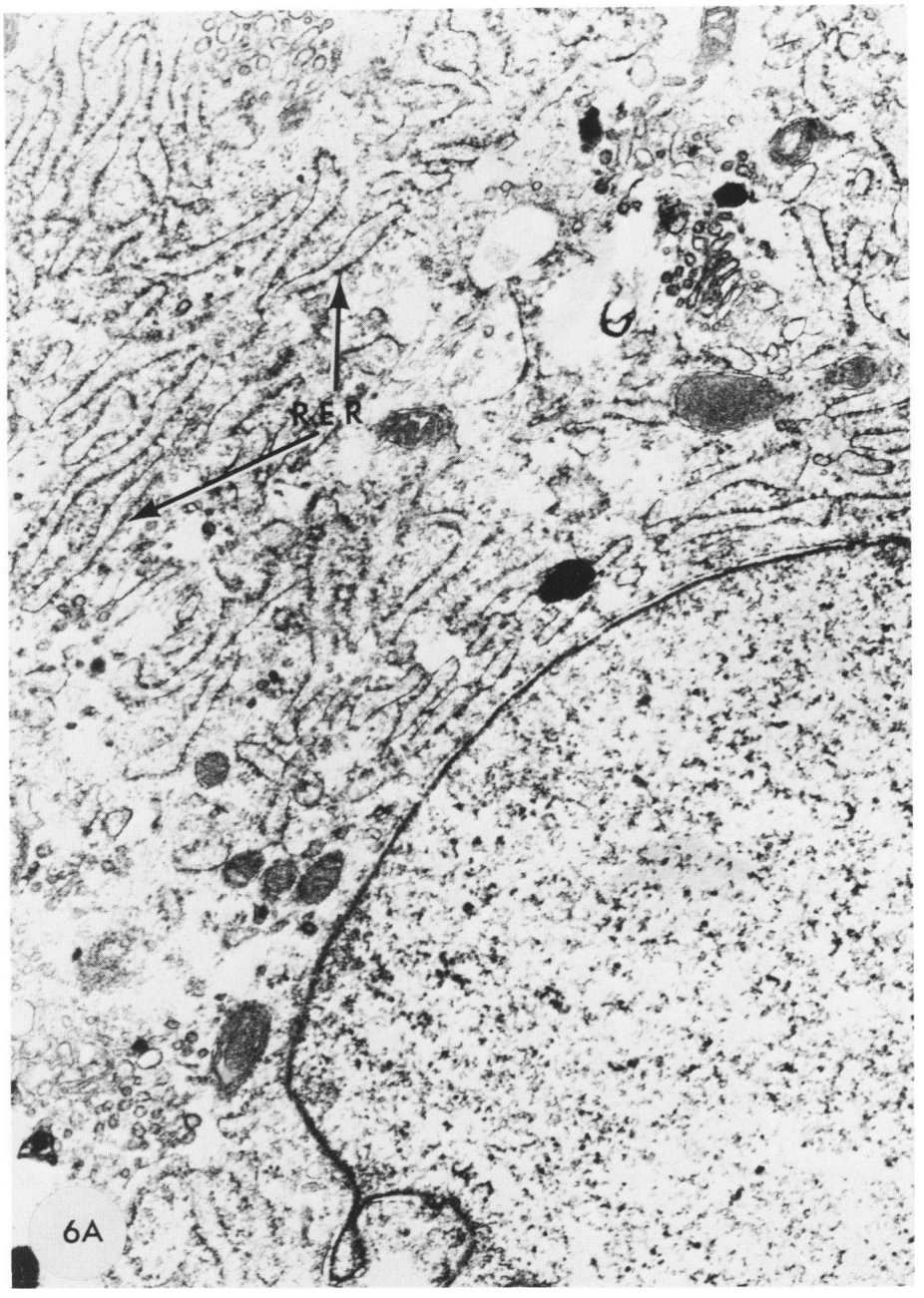
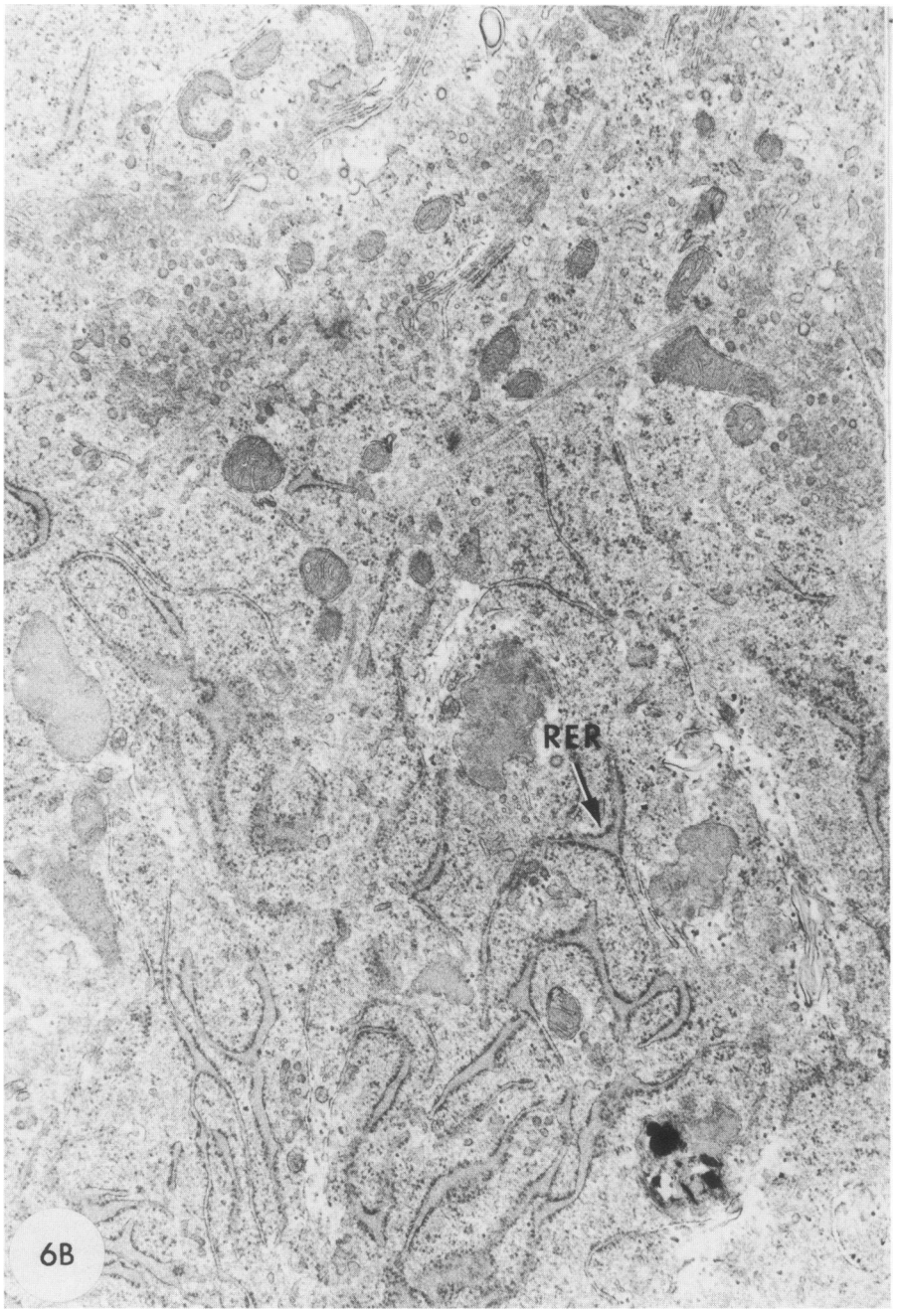


Figure 6—Transmission electron microscopy of subcultured human glomerular cells. **A**—Transmission electron microscopy of subcultured adult glomerular cell. Prominent rough endoplasmic reticulum is seen (*RER*). (×9538) **B**—Transmission electron microscopy of subcultured infant glomerular cell. Note prominent branching rough endoplasmic reticulum (*RER*). (×27,060)



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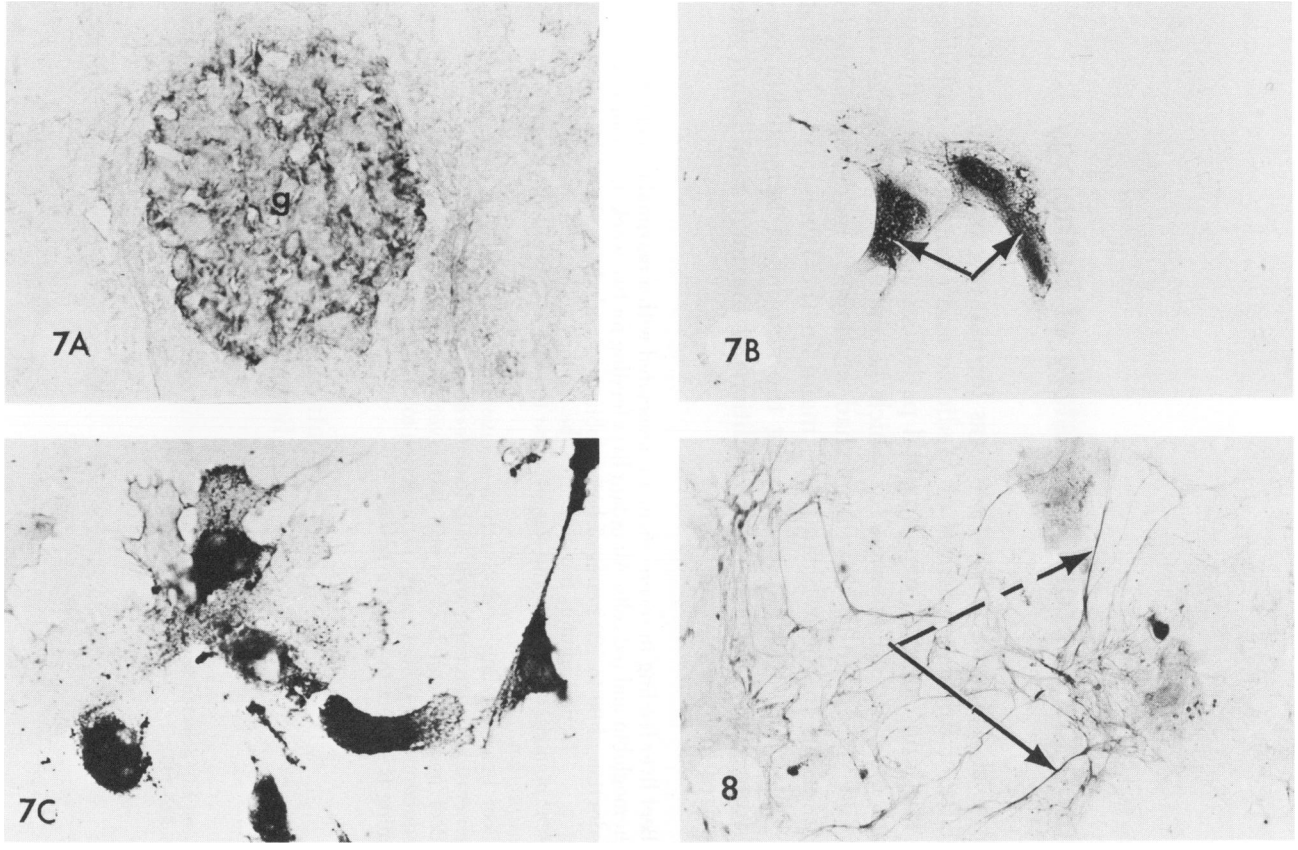

Figure 7—Light microscopy of staining of tissue and cells with peroxidase-labeled Limulin. **A**—Autopsy kidney stained with HRP-Limulin. Only the glomerulus (*g*) showed staining. (×900) **B**—Adult glomerular cells show cytoplasmic staining (*arrows*). Primary cultures stained darker than secondary cultures. Tubular cells did not stain at all. (×570) **C**—Infant glomerular cells show cytoplasmic staining. (×900)

Figure 8—Reactivity with peroxidase-labeled antibody from patient with Goodpasture's syndrome of extracellular matrix produced by infant glomerular cells. Note extracellular matrix staining (*arrows*). (×400)