# TISSUE CULTURE OF ISOLATED GLOMERULI IN EXPERIMENTAL CRESCENTIC GLOMERULONEPHRITIS*

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A number of cellular and humoral factors have been investigated as possible mediators of glomerular damage in experimental crescentic glomerulonephritis. Fibrin (1, 2, 3) and polymorphonuclear leukocytes (4) have been firmly established as important in the development of tissue injury. The participation of other inflammatory cells such as the macrophage has not as yet been investigated. By culturing isolated glomeruli we have been able to study the cell types present in the outgrowth from normal animal (5) and human (6) glomeruli. Glomeruli from normal patients and from patients with a wide variety of types of noncrescentic glomerulonephritis, when cultured, regularly show two types of cells in their outgrowth. These cells, which we have designated type I and type II, have the morphological characteristics of epithelial and mesangial cells, respectively. However in patients with rapidly progressive crescentic glomerulonephritis we have noted not only the intrinsic glomerular cells in the glomerular outgrowth but also large numbers of macrophages (7).

To assess further the participation of macrophages in the formation of crescentic glomeruli, isolated glomeruli from two well-established animal models of crescentic nephritis, autoimmune glomerulonephritis in sheep (8), and nephrotoxic nephritis in rabbits (9) have been studied in tissue culture. The culture characteristics of these glomeruli have been compared with glomeruli from normal sheep and rabbits.

## Materials and Methods

*Animals.* Male New Zealand White rabbits weighing between 2 and 2.5 kg were used for studies of nephrotoxic nephritis. Merino-Border Leicester cross sheep were used for studies of experimental autoimmune anti-glomerular basement membrane glomerulonephritis.

*Preparation of Rabbit Particulate Glomerular Basement Membrane (GBM).*[1] Glomeruli were isolated by Spiro's modification (10) of the method of Krakower and Greenspon (11). Isolated glomeruli were sonicated with an MSE Ultrasonicator Disintegrator until all glomeruli were

[1] *Abbreviations used in this paper:* FCA, Freund's complete adjuvant; FITC, fluorescein isothiocyanate; GBM, glomerular basement membrane; H & E, hematoxylin and eosin; NTN, nephrotoxic nephritis; NTS, nephrotoxic serum; PAS, periodic acid-Schiff reagent.

disrupted. The particulate GBM, after centrifugation and washing was stored in portions at −70°C until used.

*Production of Sheep Anti-Rabbit GBM Antiserum (Nephrotoxic Serum [NTS]).* A sheep was immunized with rabbit GBM by the method of Steblay (8). 25 mg of particulate GBM was incorporated into Freund's complete adjuvant (FCA-Difco Laboratories, Detroit, Mich.) and injected intramuscularly into the sheep every 2 wk. Blood samples were taken from the sheep at weekly intervals for estimation of anti-rabbit GBM antibody titers. When a high titer ($1/5 \times 10^6$) of antibody was obtained the sheep was bled out and killed. The crude NTS was decomplemented by heating at 56°C for 30 min and absorbed out against normal rabbit erythrocytes and plasma.

Preliminary experiments, with a salt-fractionated gamma-globulin preparation of this serum labeled with $^{125}I$ established that a dose of 2 ml/kg body weight of NTS, when given intravenously to five rabbits, resulted in the deposition of a mean of 410 μg (range 280–450 μg) of nephrotoxic antibody per kidney. This same dose of NTS given to other rabbits induced a severe proliferative glomerulonephritis with most glomeruli showing some degree of crescent formation by 12–14 days.

*Renal Function.* Serum concentrations of urea and creatinine were determined by the autoanalyzer method.

*Histological and Immunofluorescent Preparations.* Portions of kidney from rabbits and sheep were fixed in Bouin's renal fixative, sectioned at 2 μm and stained with hematoxylin and eosin (H & E) and periodic acid Schiff reagent (PAS). Specimens were also snap frozen in liquid nitrogen, sectioned at 2 μm on a cryostat, and stained with fluorescein isothiocyanate (FITC) conjugated antisera to sheep IgG (for sheep kidneys) and sheep IgG, rabbit IgG, rabbit C3, and rabbit fibrin (for rabbit kidneys). Commercial FITC-antisera were used (Behring-Werke AG, Marburg/Lahn, W. Germany) except for anti-rabbit fibrin which was prepared in our laboratory and conjugated to FITC as described by Wood et al. (12).

*Assessment of the Histological Preparation.* Sections were coded and analysis based on independent assessment. As described previously (2) crescent formation was quantitated by a scoring system in which each glomerulus was graded zero to three according to the surface area of the glomerulus occupied by the crescent. A mean crescent score for each animal was thus calculated.

*Isolation of Glomeruli for Culture.* Animals were killed with an intravenous dose of 60 mg/kg of pentobarbitone (Nembutal). The kidneys were immediately removed and placed in tissue culture medium 199. Glomeruli were isolated and purified by a mechanical sieving and pipetting technique previously described (6). Each glomerulus was individually washed and assessed under phase contrast microscopy to ensure final purity.

*Morphology of Isolated Glomeruli.* Freshly isolated glomeruli from normal and diseased animals were fixed in Bouin's fixative for light microscopy. After ethanol extraction and paraffin embedding, 2–3-μm sections were cut and stained with H & E as well as PAS. Electron microscopic preparation consisted of postfixation with osmium tetroxide, epon embedding, and staining with uranyl acetate. Sections were viewed on a Siemans 1A electron microscope (Siemens Industries, Munich, Germany).

*Culture of Isolated Glomeruli.* Individual glomeruli from normal and diseased animals were placed in drops of medium 199 containing 20% fetal calf serum (Commonwealth Serum Laboratories, Melbourne) 20 mM HEPES Buffer and 1% sodium bicarbonate, in Falcon tissue culture flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). pH was maintained at 7.4 and culture was performed at 37°C.

### Assessment of Tissue Culture Growth

PHASE CONTRAST MICROSCOPY. Each glomerulus was observed on days 3 and 7 for quantitative assessment of cell outgrowth.

TIME LAPSE CINEMICROSCOPY. Randomly selected glomeruli were filmed (1 frame/minute) at × 150 magnification. Glomeruli were filmed for 24–48 h from the time at which attachment to the flask was first observed. This usually occurred between 2 and 6 days in culture. 4 of the glomeruli from kidneys of nephritic sheep, 6 from normal sheep, 8 from normal rabbits, and 12 from nephritic rabbits were studied.

PHAGOCYTIC CAPACITY. To assess the phagocytic capacity of cells in the glomerular outgrowth, heat killed opsonized yeast was added to culture flasks and the culture filmed for phagocytosis at

1 frame/10 s over a period of 4 h. Glomeruli in culture from all nephritic and normal sheep, three nephritic rabbits, and two normal rabbits were studied in this way. After 4 h the cells were fixed in 5% glutaraldehyde with 0.1 M cacodylic acid and processed for ultrastructural examination.

ASSESSMENT OF CELLS FOR Fc RECEPTORS. Isolated glomeruli from normal and nephritic rabbits were cultured for 3 days on glass slides. Rabbit peritoneal macrophages (obtained 6 days after the intraperitoneal instillation of sterile liquid paraffin) were also cultured on slides and repeatedly washed over a period of 3 days to remove nonadherent cells. The cell preparations were washed with phosphate-buffered saline for 15 min and incubated for 30 min with FITC-labeled aggregated human IgG. After final washing and mounting, the cells were examined by fluorescence microscopy.

ELECTRON MICROSCOPY OF THE GLOMERULAR OUTGROWTH. After postfixation in 2% osmium tetroxide the cells still attached to their culture flasks were stained with uranyl acetate. The cells were then rapidly dehydrated in cold alcohols. Epon embedding of individual glomeruli was performed by placing a capsule filled with fresh plastic over each glomerulus and outgrowth. After polymerization the capsule was snapped off, removing the embedded outgrowth and glomerulus from the flask. Sections were then cut on a Reichert OMU3 ultramicrotome (Reichert, Vienna, Austria) and viewed in Siemens 1A electron microscope.

### Experimental Protocols

NEPHROTOXIC NEPHRITIS IN RABBITS (NTN). 19 rabbits were injected intravenously with 2 ml/kg of NTS (day 0). Five control animals were injected with a similar volume of saline. On day 14 all animals were killed and the kidneys were immediately removed for histology, electronmicroscopy, immunofluorescence, and glomerular culture. Blood was drawn on day 0, 7, 10, and 14 for serum creatinine estimation.

EXPERIMENTAL AUTOIMMUNE GLOMERULONEPHRITIS IN SHEEP. Four sheep were injected intramuscularly every 2 wk, with 25 mg of particulate rabbit GBM incorporated into FCA. The animals were bled at weekly intervals for estimation of serum creatinine and sheep anti-rabbit GBM antibody titers. The animals were bled out and killed when they became ill or developed renal failure. The kidneys were immediately removed and processed as above. Kidneys from two normal sheep were also removed and similarly examined.

## Results

### Induction of Glomerulonephritis.  Nephrotoxic Nephritis in Rabbits

RENAL FUNCTION. All animals given NTS developed progressive renal failure from day 5 of the disease. The mean serum creatinine concentration on day 14 was 0.67 mmol/liter (range 0.15–1.32 mmol/liter). The mean serum creatinine of control animals was 0.11 mmol/liter (range 0.09–0.13 mmol/liter).

HISTOLOGY. A crescentic glomerulonephritis of varying severity developed in all rabbits given NTS (Fig. 1 a). The mean crescent score for all rabbits on day 14 was 1.58 (range 0.16–2.96). The mean percentage of glomeruli showing crescents was 69% (range 16–100%).

IMMUNOFLUORESCENCE. Sheep IgG, rabbit IgG, and rabbit C3 were deposited in a linear fashion along the GBM in all animals with NTN. Extensive glomerular fibrin deposition was also present in these animals. IgG, C3, and fibrin were not detected in the glomeruli of normal animals.

### Autoimmune (Steblay) Nephritis in the Sheep

RENAL FUNCTION. Two sheep (sheep A1 and A2) developed severe acute renal failure 20 and 14 wk, respectively, after commencing the immunization with rabbit GBM. At the time the animals were sacrificed the serum creatinine concentrations were 1.6 mmol/liter and 0.66 mmol/liter, respectively. The two other sheep (B1 and B2) became ill after 18 and 26 wk, respectively, but only
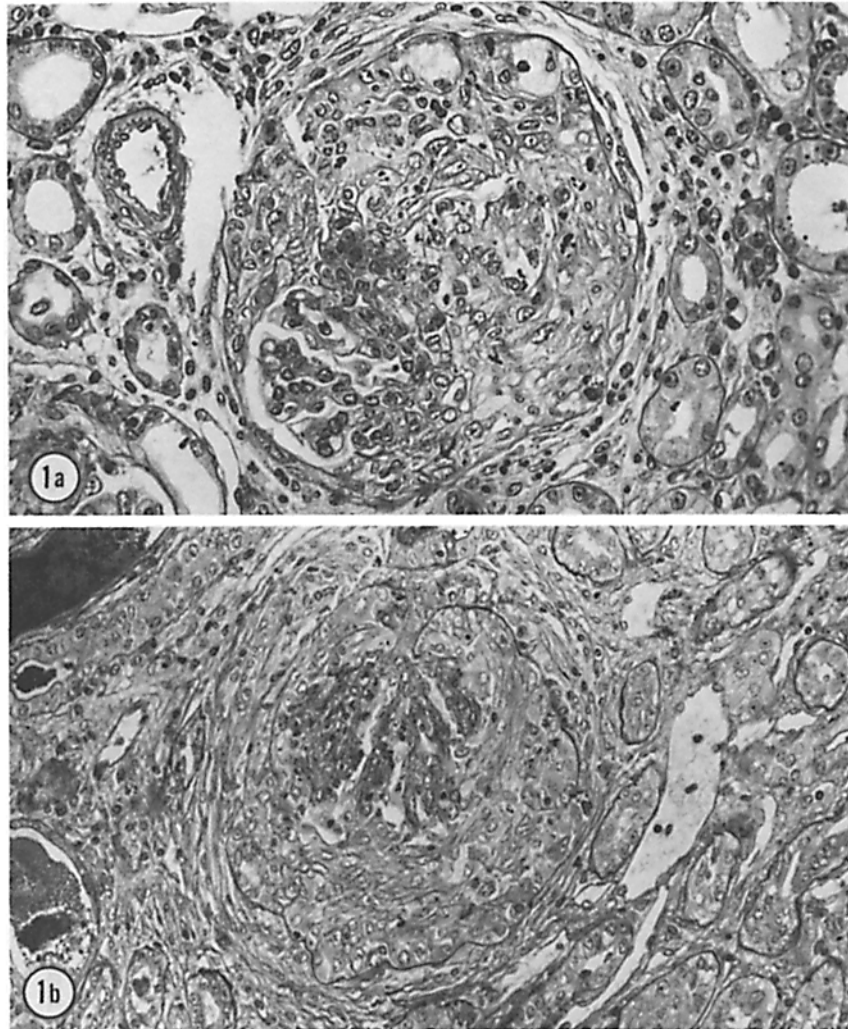
FIG. 1a.  Section of rabbit kidney showing a typical glomerulus at day 14 of NTN. Extensive extracapillary crescent formation is present. PAS × 320. Fig. 1b. Section of sheep kidney. All glomeruli in this animal were similarly affected by extensive crescent formation. PAS × 320.

moderate renal impairment had developed (peak creatinine 0.26 mmol/liter and 0.27 mmol/liter, respectively). The latter animals were severely nephrotic with pulmonary and generalized tissue edema.

HISTOLOGY.   Sheep A1 and A2 both developed a severe crescentic nephritis (Fig. 1 b) with 100 and 88% of glomeruli showing extensive crescent formation. Sheep B1 and B2 developed considerable glomerular damage with exudation of polymorphonuclear leukocytes and mesangial proliferation. However, crescent formation was not seen in these latter animals.

IMMUNOFLUORESCENCE.   Glomeruli from all animals showed heavy linear deposition of sheep IgG along the GBM.
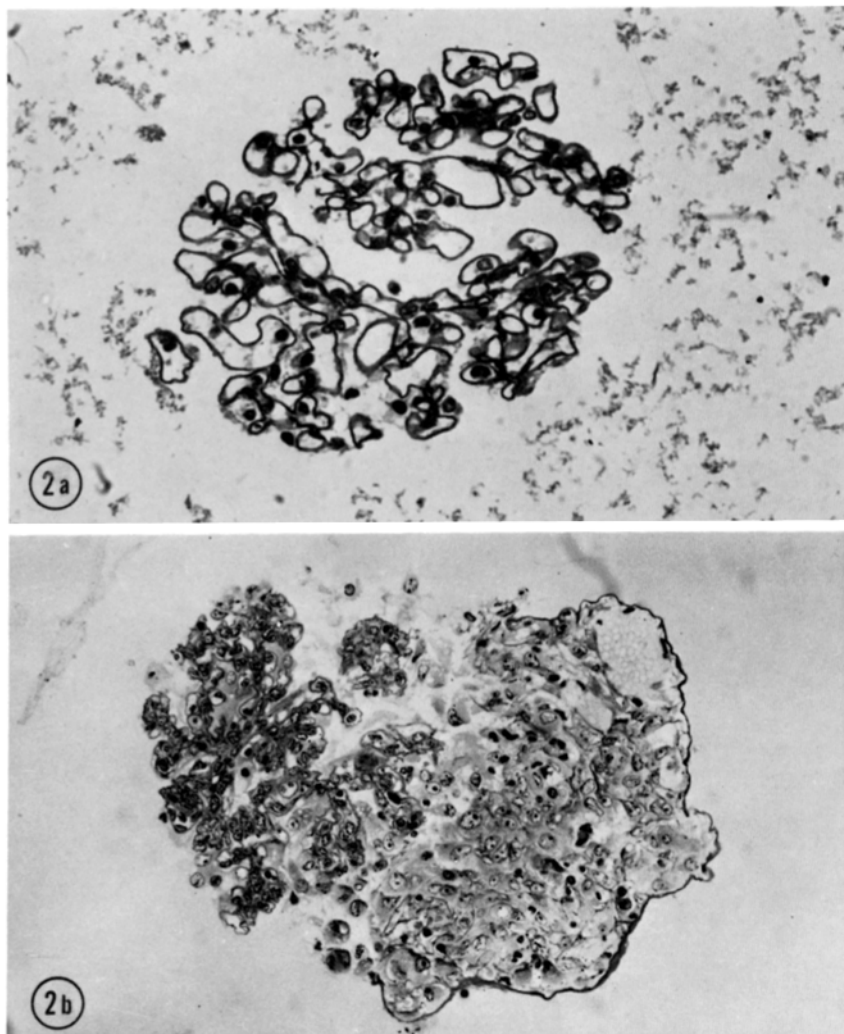
FIG. 2a.  A glomerulus from normal sheep demonstrating the morphology of glomeruli after the isolation process. Capillary loops, endothelial, epithelial, and mesangial cells remained intact. PAS × 410. Fig. 2b. A section of freshly isolated glomerulus and its associated crescent. PAS × 200.

*Tissue Culture of Isolated Glomeruli*

ISOLATION OF GLOMERULI.  It was observed that isolated glomeruli from diseased animals were significantly larger than normal in both species.

MORPHOLOGY OF ISOLATED GLOMERULI.  Isolated normal glomeruli (Fig. 2 a) were found to consist of capillary loops with endothelial and epithelial cells, supported by mesangium. In the majority, Bowman's capsule had been removed. An occasional marginated leukocyte was observed. Ultrastructural examination demonstrated that no significant cell damage or loss of cell relationships was brought about by the isolation procedure. Glomeruli from animals with crescentic nephritis (Fig. 2 b) showed the presence of the same three intrinsic glomerular cells, epithelial, endothelial and mesangial, although the capillary loops
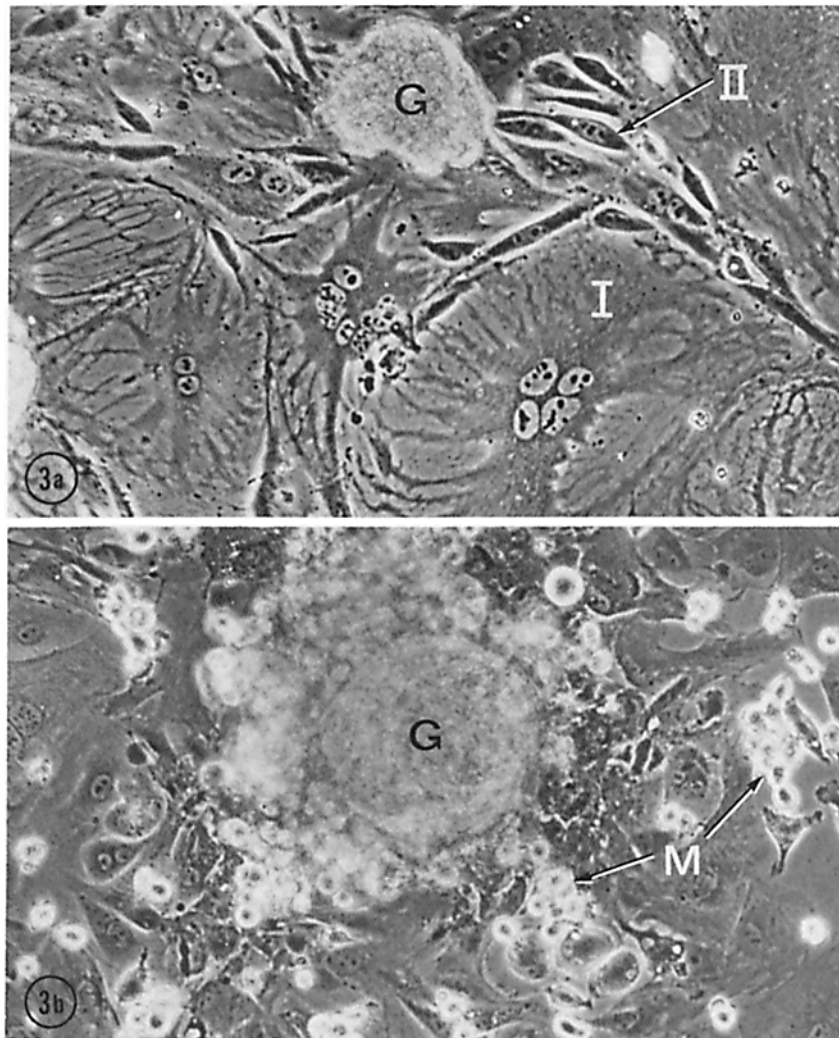
Fig. 3a.  Phase contrast photomicrograph of normal rabbit glomerulus (G) in tissue culture. Two cell populations, large type I and smaller type II cells are present. × 100. Fig. 3b. Phase contrast photomicrograph of isolated crescentic glomerulus of sheep in tissue culture. Macrophages (M) make up the predominant cells in the outgrowth. × 100.

were often compressed and damaged. In addition cellular crescents were seen, affecting the same proportion of glomeruli as on the routine histological sections. The crescent cells were attached to capillary loops and invaginated between them.

## Assessment of Tissue Culture

PHASE CONTRAST MICROSCOPY.  Normal glomeruli from both species attached to the culture flask and developed cellular outgrowths which were composed of two cell types, designated type I and type II (Figs. 3 a and 4 a). As previously reported (5) type I cells were very large (50–150 μm) arborized cells with the ultrastructural features of epithelial cells. Type II cells were fusiform, 30–50
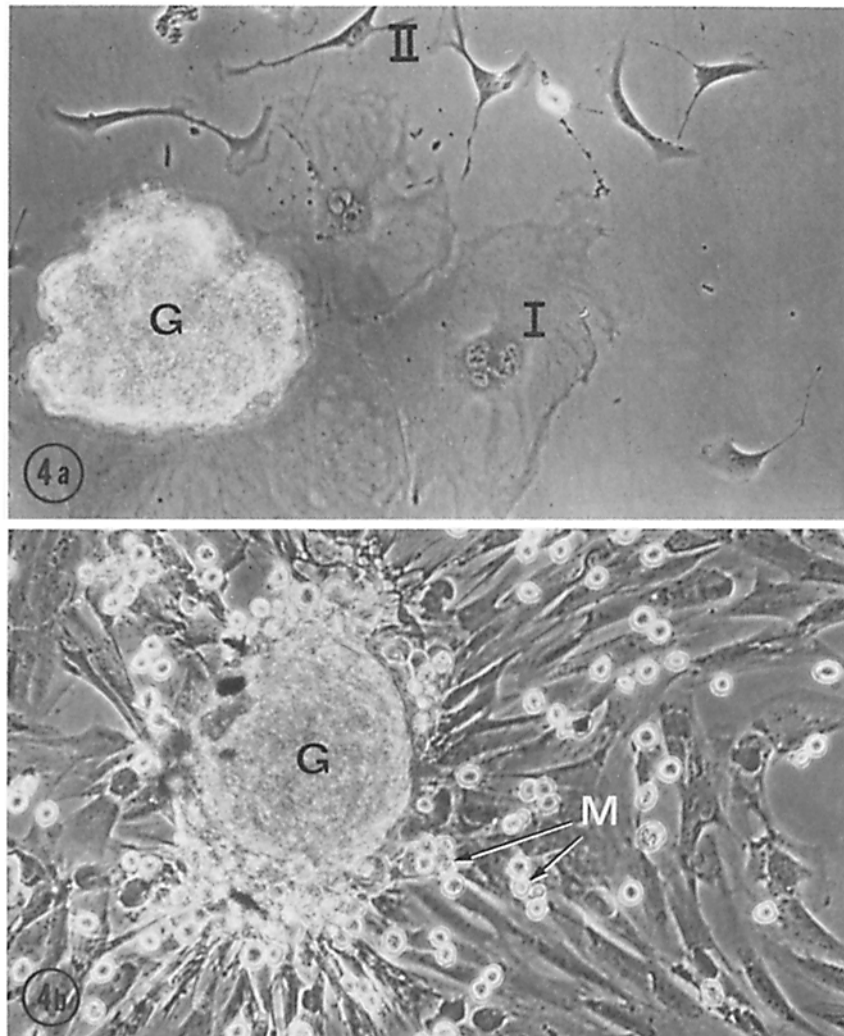
FIG. 4a.   Phase contrast photomicrograph of isolated normal sheep glomerulus in culture with type I cells and a few fusiform type II cells. × 100. Fig. 4b. Phase contrast photomicrograph of isolated crescentic sheep glomerulus in tissue culture showing numerous macrophages (M) in the outgrowth. × 100.

μg in diameter and had electronmicroscopic characteristics of mesangial cells.

In the outgrowth of glomeruli with crescents, type I and type II cells were also seen but in addition a third cell type, called type III, was observed. This cell became numerically the predominant cell in the culture. Type III cells (Figs. 3 b and 4 b) were small, 3-6 μm in diameter, refractile, and round. This cell type was not observed under phase contrast microscopy in normal glomeruli.

Type III cells were seen in the outgrowths of glomeruli from all rabbits with crescentic glomerulonephritis (Fig. 3 b): a mean of 60 ± 23 (SEM) type III cells per glomerulus was observed. All glomeruli from sheep A1 and A2 (with
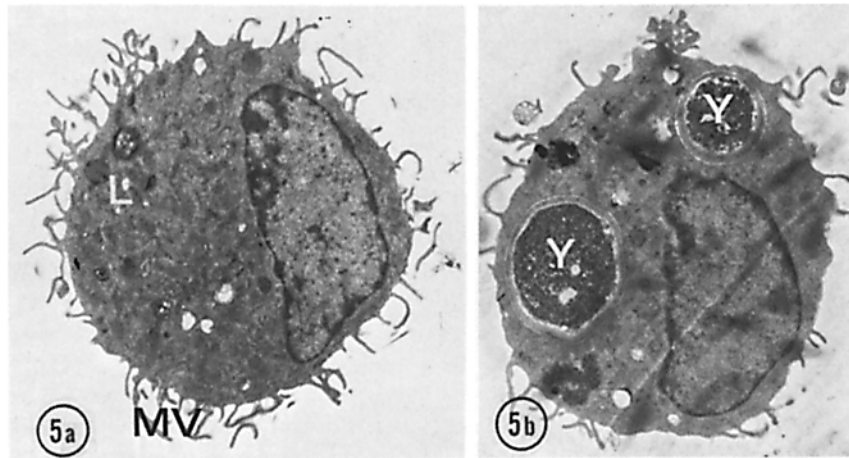
Fig. 5a.  Transmission electron micrograph of type III cell from tissue culture outgrowth of an isolated crescentic glomerulus from a rabbit. The cell had lysosomes (L) and microvilli (MV) characteristic of a macrophage. × 4,000. Fig. 5b. Transmission electron-micrograph of a macrophage from tissue culture of an isolated crescentic glomerulus after culture with yeast. Phagocytosed yeast (Y) is present. × 4,000.

severe crescentic nephritis) showed large numbers of type III cells in culture (Fig. 4b) with a mean of 170 ± 30 cells per glomerulus being recorded. Glomerular outgrowths from sheep B1 and B2 which developed noncrescentic nephritis, contained no type III cells.

CINEMICROSCOPY.  Cinemicroscopic examination of cultured glomeruli revealed that the three cell types had differing characteristics. The outstanding feature was the mobility of type III cells seen in the outgrowths of glomeruli with crescents. These cells demonstrated active movement by pseudopodia. Macrophages cultured from rabbit peritoneal fluid were identical in size, shape, rate, and style of movement with the type III cells observed in culture from glomeruli with crescents. By cinemicroscopy this macrophage-like cell was very occasionally observed in the outgrowths of normal glomeruli. Only three type III cells were seen emerging from the culture of a total of 12 glomeruli from normal rabbits and 2 such cells from cultures of 6 glomeruli from normal sheep.

PHAGOCYTIC CAPACITY OF GLOMERULAR CELLS.  Only type III cells were observed to phagocytose opsonized yeast. Phagocytosis was avid and each of the type III cells actively engulfed many yeast particles.

ELECTRON MICROSCOPY OF GLOMERULAR CELLS.  Electron microscopy of type I cells confirmed their likely origin as epithelial cells while type II cells had an ultrastructural appearance consistent with mesangial cells. Type III cells (Fig. 5a) had the characteristic features of macrophages. Prominent lysosomes and microvilli were present in all type III cells. Electron microscopic review of cultured cells after addition of yeast to the culture confirmed that yeast particles had been phagocytosed by the type III cells (Fig. 5b). Over 90% of type III cells contained yeast, most having ingested many particles. Yeast was not observed in the other cell populations.

*Examination for Fc receptors.*  Fc receptors were detected by fluorescence on the surface of type III cells and rabbit peritoneal macrophages. Thus the characteristics of the type III cell, namely its mobility, ultrastructure, phagocytic capacity, and presence of Fc receptors establish the cell to be a macrophage.

## Discussion

Experimental crescentic glomerulonephritis in animals, induced either by the administration of anti-GBM antibody (NTN) or the repeated injections of GBM (Steblay nephritis) closely resembles human rapidly progressive crescentic glomerulonephritis in morphology, the degree of crescent formation, and the severity of renal failure.

Our studies reported in this paper have demonstrated that when glomeruli from both sheep and rabbits with experimental crescentic glomerulonephritis were cultured, large numbers of macrophages appeared in the cellular outgrowth and rapidly became the predominant cell type of the outgrowth. Macrophages were rarely seen in the outgrowth of normal glomeruli or of glomeruli from the sheep with noncrescentic glomerulonephritis (sheep B). We have recently shown that the cellular outgrowth of human glomeruli from patients with rapidly progressive crescentic glomerulonephritis also contained numerous macrophages (7). Thus the demonstration of macrophage accumulation in crescentic glomerulonephritis in three different species, all of differing immunological etiology, strongly suggests that the macrophage is an important cell type in the pathogenesis of both experimental and human crescentic glomerulonephritis.

The accumulation of macrophages in isolated crescentic glomeruli suggests that macrophages may play a role, either damaging or protective, in the genesis of glomerular injury. Although the role of fibrin (1–3), polymorphonuclear leucocytes (4), and complement (13) have been intensively investigated in the pathogenesis of crescentic glomerulonephritis, the role of the macrophage has not been studied, though its presence in crescents has been previously noted (14). Macrophages have the potential to induce tissue damage either directly or by stimulating other cellular or humoral inflammatory mediator systems. In response to a variety of immunological and nonimmunological stimuli, macrophages release enzymes capable of inducing tissue damage (15). Pyrogen (16) and prostaglandins (17) are also produced thus potentially altering vascular flow and permeability. Macrophages are able to trigger other inflammatory mediator systems including coagulation (18), complement, and kinin systems. As well as in the induction of tissue injury, macrophages may play a role in tissue repair by stimulating fibroblast proliferation (19), thus influencing the fibrosis occurring in response to chronic inflammation. They may have a healing effect by removing fibrin and phagocytosing debris, and thus limiting tissue damage.

The accumulation of macrophages within Bowman's capsule may occur in response to a number of chemotactic substances including polymorphonuclear leucocyte products and activated components of complement (20). These substances have all been identified in the glomeruli of experimental and human

crescentic glomerulonephritis (21) and thus may account for the macrophage accumulation in crescentic glomerulonephritis.

It is most likely that the macrophages found in crescentic glomerulonephritis are derived from circulating cells as it has been shown that the inflammatory tissue macrophage is derived from the circulating monocyte and ultimately from the bone marrow cells (22). Chronic inflammatory tissue damage is associated with the transformation of macrophages into epithelioid cells in animals (23) and man (24). The accumulation and transformation of macrophages forms a palisade around the area of damage. These transformed macrophages were originally termed epithelioid cells because of this epithelial appearance (25). Chronic glomerular inflammation, like inflammatory processes elsewhere in the body, may well be associated with the accumulation of macrophages. The accumulation and epithelial transformation of these macrophages in the presence of persistent tissue damage may produce or contribute to the formation of crescents.

The cells of the crescent have been traditionally viewed as being derived from epithelial cell proliferation (26). The evidence for this has been largely based on the similar appearance of crescent cells and epithelial cells. However, the morphological appearance of crescent cells would also be consistent with a macrophage origin.

In conclusion, the macrophages which we have demonstrated to be present in large numbers in culture of glomeruli with crescents, have the potential to influence profoundly the tissue injury occurring in crescentic glomerulonephritis. Such influence may affect the degree of primary tissue damage, the chronicity of inflammation, and the occurrence eventually of glomerular fibrosis. Circulating macrophages may move into Bowman's space through injured capillary loops where they then accumulate and undergo epitheliod transformation producing the crescentic appearance of those glomeruli in glomerulonephritis.

## Summary

As a means of studying mechanisms of response to injury in glomerulonephritis, glomeruli from normal sheep and rabbits and from sheep and rabbits with experimental crescentic glomerulonephritis have been isolated and grown in tissue culture. The cellular outgrowths from the normal and diseased glomeruli have been compared.

The outgrowth of glomeruli from normal animals contained only two cell populations whose microscopic and ultrastructural appearances were of epithelial and mesangial cells. The same cells were also observed in the outgrowths of glomeruli from animals with crescentic nephritis but in addition a third population of cells was present in large numbers. These cells were identified as macrophages by their mobility, ultrastructure, phagocytic capacity, and presence of Fc receptors. Glomerular outgrowth from sheep with crescentic glomerulonephritis contained 170 ± 20 (SEM) macrophages and outgrowths from rabbits with crescentic nephritis contained 64 ± 6 (SEM) macrophages per glomerulus.

We have previously observed large numbers of macrophages in the outgrowth

of isolated glomeruli from humans with rapidly progressive crescentic glomerulonephritis. The predominance of the macrophage in cultures of glomeruli from both human and animal crescentic glomerulonephritis suggests that this is an important cell in the inflammatory reaction occurring in crescentic glomerulonephritis and may comprise a substantial proportion of the cells forming the crescent.

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