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Cytomegalovirus replicates efficiently in human kidney mesangial cells

(glomerulonephritis/renal transplantation/congenital infection)

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ABSTRACT **Human cytomegalovirus (HCMV) is a major renal pathogen in congenitally infected infants and renal allograft recipients. We postulated that a specific renal cell type was involved in HCMV infection and reactivation. Human fetal kidney cortex cell cultures were assayed for their ability to support HCMV infection. Infectious center assays indicated that the low level of viral replication observed by virus yield assay occurred from a fraction of the cells in the mixed cultures. Virus-specific immunofluorescence and *in situ* hybridization documented the presence of HCMV-specific protein and nucleic acid, respectively, in a morphologically distinct cell type. These cells were purified, were identified as kidney mesangial cells, and were observed to support efficient HCMV replication. Our research identifies mesangial cells as a renal cell type that supports HCMV replication and provides evidence to implicate these cells in the pathogenesis of HCMV-induced renal disease.**

Human cytomegalovirus (HCMV) can produce life-threatening complications in immunocompromised patients and can induce pathologic changes in a number of human organ systems. HCMV inclusions are found in the liver, lungs, and kidneys of HCMV viremic patients examined by biopsy or at autopsy (1–3). High titers of HCMV are shed in urine during congenital or neonatal HCMV infection, and histologic examination of kidney sections from congenitally infected infants has revealed viral inclusions in the renal tubules (3). HCMV infection is a significant covariate for graft failure and mortality in the renal allograft recipient (4). Indeed, the donor kidney itself has been implicated as a major source of HCMV infection for the transplant recipient (3). Generalized CMV infections were observed in 60–100% of mice receiving renal allografts from murine CMV-infected donors. The allografts had been perfused with saline to exclude the possibility of blood-borne viral transmission (5, 6). HCMV nucleic acid sequences have been demonstrated in human kidney glomerular and tubular cells by *in situ* hybridization (7). These studies suggest that the kidney parenchyma is a reservoir for infectious virus. However, the exact sites of viral replication for HCMV in the kidney are not known.

Mixed human kidney cortex cell cultures (MKC) contain mesangial cells, glomerular visceral epithelium (GVEC), and tubular epithelium. All three cell types are morphologically distinct and have been characterized in culture (refs. 8 and 9; F.J.v.d.W., Y. Kim, and A. Michael, personal communication). GVEC and tubular epithelium exhibit pavementing and swirling patterns, respectively, and can be distinguished from one another by using a battery of monoclonal antibodies (ref. 9 and F.J.v.d.W., Y. Kim, and A. Michael, personal communication). Kidney mesangial cells are stellar, multilayered, and produce hillock formation *in vitro* (10). Mesangial cells can be induced to contract in the presence of angiotensin II and most closely resemble vascular smooth muscle cells (11). Collagen type III is deposited by mesangial cells in culture as shown by immunofluorescence (10). This matrix component is absent in glomerular and tubular epithelial cultures and, therefore, can be used as a marker for mesangial cells in MKC.

Prevention of fulminant CMV replication in renal transplant patients requires an understanding of the virus-cell interactions resulting in organ rejection and failure. Since evidence presented here suggests that there should be a productive cell type in the kidney, three major cell types present in MKC cultures were studied for their ability to support replication of CMV.

MATERIALS AND METHODS

Virus. Towne strain of HCMV was obtained as vaccine from Merck Sharp & Dohme. Virus was plaque-purified and propagated in human foreskin fibroblasts (SF). Stocks were prepared as cell-free or cell-associated virus. A clinical HCMV isolate, designated RN, was recovered from an SF culture seeded with peripheral blood leukocytes from a renal transplant patient and used at passages 3–7. Viral supernatant was pooled with infected cells and frozen at −70°C. Thawed stocks were sonicated briefly and centrifuged to remove cell debris. Cultures were infected with dilutions of the sonicated stock in Hanks' balanced saline solution (HBSS). Virus stocks were routinely tested for mycoplasma contamination.

Cells. SF cells were prepared from foreskin tissue. Tissue was minced and digested with trypsin 0.025% (GIBCO) for 20 min at 37°C. Digested tissue was passed through sterile gauze to separate undigested fragments. Cells were collected and grown in Eagle's minimal essential medium (MEM) supplemented with 10% (vol/vol) fetal calf serum and 2 mM glutamine. Primary cultures received penicillin at 100 units/ml, streptomycin at 100 μg/ml, and amphotericin B at 0.25 mg/ml (GIBCO). Cells were used between passages 7 and 12. MKC cultures were prepared from minced human fetal kidney cortex issue after a 15-min digestion with trypsin 0.012% and collagenase at 2 mg/ml, 1:1 (vol/vol). Digested cells were washed and plated in 20% (vol/vol) fetal calf serum-supplemented Dulbecco's modified Eagle's medium (DMEM)/Hanks' F12 (GIBCO), 1:1 (vol/vol), containing 0.1 mM nonessential amino acids, penicillin, streptomycin, and amphotericin B as described above. MKC cells were used on primary passage only.

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Abbreviations: HCMV, human cytomegalovirus; moi, multiplicity of infection; MKC, mixed human kidney cortex culture; GVEC, glomerular visceral epithelium; SF, human foreskin fibroblasts; pfu, plaque-forming unit(s).

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Mesangial cells were prepared from MKC cultures by removing mesangial hillocks, digesting briefly with trypsin, and growing cultures to confluency. Mesangial cells used in these experiments were derived from human fetal kidneys at 18–22 weeks of gestation, two infants with congenital nephrotic syndrome, and one adult whose kidneys were not used for transplantation as approved by the Committee on the Use of Human Subjects in Research (University of Minnesota). Mesangial cells were used at passages 3–6.

Glomerular cultures depleted of mesangial cells were prepared by isolating glomeruli from kidney cortex tissue (11). Glomeruli were digested briefly with 1% collagenase (type 1A, Sigma) in 10% (vol/vol) fetal calf serum-supplemented DMEM/Hanks' F12. The suspension was passed though a 400-mesh screen to separate the dissociated cells (mostly epithelial) from the cores containing mesangial cells. Cells were grown to confluency, trypsinized, washed, and layered into 10–40% Percoll gradients. A cell band containing predominantly GVEC cells was removed, cultured, and monitored for mesangial growth by type III collagen immunofluorescence. GVEC cultures were used during passages 3–6 as well.

Virus Yield and Infectious Center Assays. One-step growth curves using HCMV strains Towne and RN were used to assess viral yield over time. SF, mesangial cells, or MKC cells were grown in Leighton tubes to confluency. Cultures were infected with either Towne or RN strain at a multiplicity of infection (moi) of 3–5 plaque-forming units (pfu) per cell. Inoculum was allowed to adsorb 1 hr at 37°C. Infected monolayers were washed three times with HBSS and incubated with 1 ml of fetal calf serum supplemented MEM or DMEM at 37°C. At various intervals after infection duplicate tubes were frozen at −70°C. Time points were assayed as sonicated pooled intra- and extracellular virus by plaque assay on SF monolayers (12).

Infectious center assays tested the susceptibility of the cell types to support growth of HCMV strains Towne and RN. Cells were grown to light confluency and infected with either Towne or RN as described above. After adsorption, cells were washed three times in HBSS and incubated for an additional 24 hr at 37°C in the appropriate medium as described earlier. We used the infectious center assay method described by Gonczol *et al.* (13) with the following changes. At 24 hr after infection, the cells were trypsinized, washed twice in HBSS, and counted. Cells (5×10^3) were incubated with a 1:8 dilution of pooled patient CMV-immune sera (complement fixing titer >256), non-immune sera (complement fixing titer <4), or HBSS alone and with a 1:24 dilution of guinea pig complement. Samples were incubated for 45 min at room temperature. Cells were washed in HBSS and resuspended in 10% (vol/vol) fetal calf serum-supplemented DMEM and plated onto SF monolayers at the following cell densities: 3×10^2 SF cells per 30-mm^2 dish, 3×10^2 mesangial cells per dish, 10^3 MKC cells per dish, and 10^3 GVEC cells per dish. Cells were allowed to settle overnight and were overlaid with agarose as described (12). Plaques were counted 10 days after infection.

Immunofluorescence. Cultures were tested for CMV antigen and type III collagen reactivity by immunofluorescence at all passage levels. Cells were seeded onto either glass coverslips or multichambered glass slides (Miles Scientific, Naperville, IL). Approximately 5 days after plating, cells were infected at a moi of 1–3 pfu per cell, washed following adsorption, and incubated at 37°C an additional 24 or 120 hr. Slides were rinsed in isotonic phosphate-buffered saline (PBS), air dried, and fixed in either acetone or ethanol at 4°C. HCMV-specific fluorescence staining used a combination of monoclonal antibodies to early and late HCMV antigens at a 1:100 dilution (New England Nuclear) followed by fluorescein-conjugated sheep $F(ab')_2$ anti-mouse IgG (1:50 dilution). Preparation and staining for collagen type III were described (10). Phenylenediamine in PBS and 90% (vol/vol) glycerol was applied to the stained slides to retard fluorescent decay (14).

HCMV Immediate-Early Protein Expression. Monolayers of MKC, SF, mesangial cells, and MKC with mesangial overgrowth were infected with Towne strain HCMV at a moi of 5–10 pfu per cell. Following adsorption, cycloheximide at 30 μg/ml (Sigma) was added to inhibit translation. Prior to labeling, medium containing cycloheximide was removed, and monolayers were washed three times in HBSS. Cells were incubated for 2 hr in methionine-free MEM (GIBCO) containing 5% (vol/vol) fetal calf serum, 2 mM glutamine, actinomycin D at 10 μg/ml, with a total NaCl concentration of 210 mM, and [^{35}S]methionine at 20 μCi/ml (1 Ci = 37 GBq) as described by Stinski (15). Cells were rinsed, harvested by scraping, washed, and lysed in 20 mM Tris·HCl/1% Nonidet P-40/2 mM phenylmethylsulfonyl fluoride. Cells were separated into nuclear and cytoplasmic fractions by centrifugation (16). Levels of [^{35}S]methionine incorporated into protein were determined by trichloroacetic acid precipitation of cytoplasmic lysates. Aliquots containing equivalent cpms were loaded onto 10–15% gradient $NaDodSO_4$/polyacrylamide gels as described by Laemmli (17). Following electrophoresis, gels were dried and exposed to Kodak X-Omat AR film at −70°C.

Purification and Labeling of HCMV Probes. Plasmid PACYC 184 containing a 32-kilobase *Xba* I Towne DNA fragment in *Escherichia coli* was obtained from Mark Stinski (Dept. of Microbiology, University of Iowa, Iowa City) and represented 13% of the HCMV genome. *E. coli* cultures were grown in LB medium containing chloramphenicol at 25 μg/ml. Plasmids were amplified using spectinomycin as described by Thomsen and Stinski (18). Methods for plasmid DNA isolation and insert purification are described elsewhere (18, 19). Purified inserts were nick-translated with [^{32}P]dCTP by using a commercially available kit (Bethesda Research Laboratories). The specific activity of the labeled fragment used was at least 10^8 cpm/μg of DNA. Biotinylated CMV probes used for *in situ* hybridization were purchased from Enzo Biochemicals (New York) and consisted of two clones representing 18% of the Towne genome.

***In Situ* Hybridization.** Cells were seeded onto glass coverslips and infected with Towne or RN strain at a moi of 3–5 pfu per cell. Five days after infection, coverslips were rinsed with PBS, air dried, fixed in ethanol for 15 min at 4°C, and air dried again. Cells were digested with proteinase K (0.001 mg/ml in 50 mM Tris·HCl, pH 7.4/0.5 mM EDTA at 37°C for 10 min) (20). Coverslips were washed in PBS containing glycine at 2 mg/ml and dehydrated through graded concentrations of ethanol. Biotinylated HCMV probes were hybridized to prewetted slides at 94°C for 3 min and 37°C for 2 hr. Methods for biotin/streptavidin colorimetric detection followed those described by Unger *et al.* (21).

Southern Blot Hybridization. SF, MKC, mesangial cells, and mesangial cell-depleted glomerular cell cultures were grown to confluency. Cell cultures were infected with Towne or RN strain at a moi of 0.5–1 pfu per cell. After a 1-hr adsorption period, cultures were washed and refed. Four days after infection, infected and mock-infected controls were trypsinized, washed, and counted. Total DNA was isolated from 10^6 cells from each culture by phenol/chloroform extraction, ethanol precipitation, and RNase digestion (19). Aliquots of 5 μg, 1 μg, 0.5 μg, and 0.1 μg from each of the DNA extractions were blotted onto nylon membrane filters and baked at 80°C for 2 hr. Nylon filters were treated with prehybridization solution and hybridized by methods described elsewhere (22, 23).

RESULTS

HCMV Replication in MKC Cultures. It has been suggested that MKC cultures are inefficient at supporting HCMV replication, and initial work with human kidney cells in cultures indicated that these cells did not support viral replication (24, 25). We reassessed the ability of MKC cultures to support HCMV replication by quantitating the amount of virus produced by HCMV strains Towne and RN infected cultures over time. Because human fibroblasts replicate HCMV efficiently, we infected human SF cells at similar mois to serve as productive controls. Clinical strain RN produced consistently lower levels of infectious virus than laboratory strain Towne. Low-level progeny production was observed in MKC cultures 72 hr after infection as compared with efficient viral replication in SF cultures (Fig. 1*A*). To determine if the virus yield observed in MKC cultures was due to low-level replication occurring from a large proportion of the MKC population or was the result of high-titer replication in a few cells, we inoculated MKC cells with both strains of HCMV and quantitated the number of productive cells on SF monolayers by infectious center assays (13). The number of infectious centers in MKC cultures was less than infected SF cultures indicating that only a fraction of the cells supported productive viral replication (Table 1).

Distribution of HCMV Replication in MKC Cultures. In general, human epithelial cell lines are nonproductive for HCMV replication. However, epithelial cells undergoing differentiation in culture may develop the ability to replicate virus (13, 24–26). Differentiation in cultures of glomerular visceral epithelial cells could be ruled out on the basis of (*i*) the absence of podocyte formation; (*ii*) positive staining by monoclonal antibody OKB2, known to be a marker for primitive GVEC; and (*iii*) the absence of complement component 3b receptor antigen (CR1), known to be a marker for mature GVEC (F.J.v.d.W., A. Michael, and Y. Kim, personal communication). Thus differentiation of GVEC cells in culture was unlikely. We looked at the distribution of HCMV-specific antigen expression in infected MKC cultures by using monoclonal antibodies to both early and late HCMV proteins 24 hr and 120 hr after infection. Localization of antibody binding to morphologically distinct cells would suggest that a distinct cell type within the MKC cultures supported productive replication and was responsible for the virus yield observed in Fig. 1*A*. The majority of fluorescence observed in infected MKC cultures was clustered in cells bearing a distinct cell morphology (Fig. 2). Occasional single cells were positive for HCMV antigen as well. GVEC cells could be ruled out since OKB2-positive cells lacked HCMV-specific immunofluorescence. Endothelium was not present in the cultures as evidenced by a lack of immunofluorescent staining for factor VIII and I-A (OK1A, Ortho Diagnostics) antigen expression in primary culture and in cultures of subsequent passages (F.J.v.d.W., A. Michael, and Y. Kim, personal communication). To positively identify the productive cell type, we compared the distribution of viral antigen-specific immunofluorescence with fluorescent labeling for collagen type III. Cells that stained positively for viral-specific antigen deposited collagen type III. Whereas most of the type III collagen-positive cells were clustered together, individual type III positive cells were occasionally observed, which suggested that

Table 1. HCMV infectious center assays of renal cells in culture

Cell type	Viral strain	Percentage of total cells infected: Immune sera	Nonimmune sera	HBSS control
SF	Towne	65.2	64.1	65.7
	RN	74.7	73	75.1
MKC	Towne	1.2	5.0	3.0
	RN	0.08	0.08	0.4
Mesangial	Towne	82	80.4	81.1
	RN	55.3	54.6	53
GVEC	Towne	<1	<1	<1
	RN	ND	ND	ND

The ability of SF, mesangial cells, MKC, and glomerular visceral epithelial cells to replicate HCMV strains Towne and RN was quantitated by modified plaque assay (15). Infected cells were incubated in the presence of pooled patient CMV immune sera (complement fixing titer >256), nonimmune sera (complement fixing titer <4), or HBSS with a 1:24 dilution of guinea pig complement. Cells were plated onto confluent monolayers at 3×10^2 SF cells per dish, 3×10^2 mesangial cells per dish, 10^3 MKC cells per dish, and 10^3 GVEC cells per dish. Plaques were counted 10 days after infection. Susceptibility was expressed as the percentage of infectious cells per total cells plated. ND, no plaques detected.

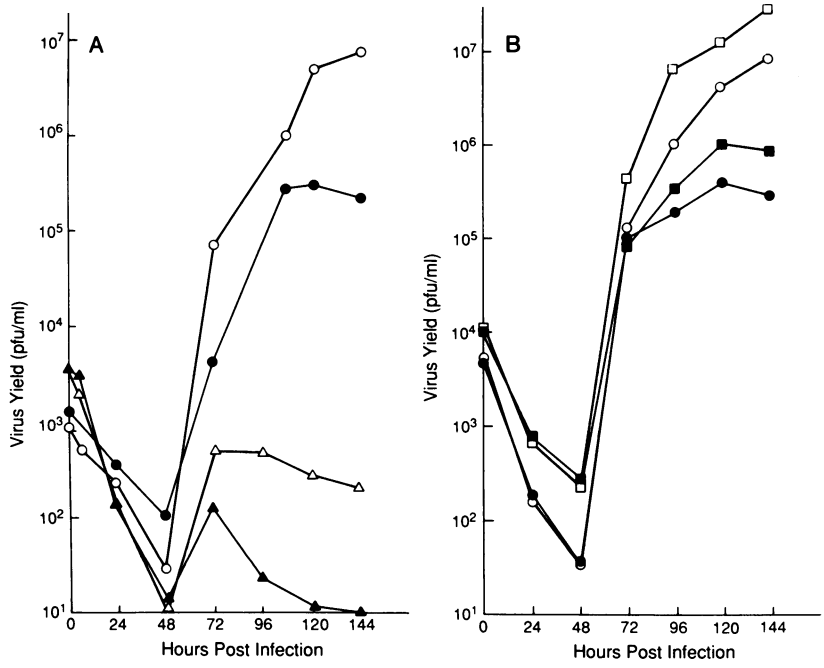

FIG. 1. (*A*) Virus yield assays of HCMV strains Towne and RN over time. Virus yield assay of Towne (○) and RN (●) in SF was compared with Towne (△) and RN (▲) in infected MKC cells. (*B*) Viral titers were quantitated by plaque assay (14) by using sonicated pooled intra- and extracellular virus. Virus yield assay of Towne (□) and RN (■) in mesangial cells was compared with Towne (○) and RN (●) yields in SF cells.

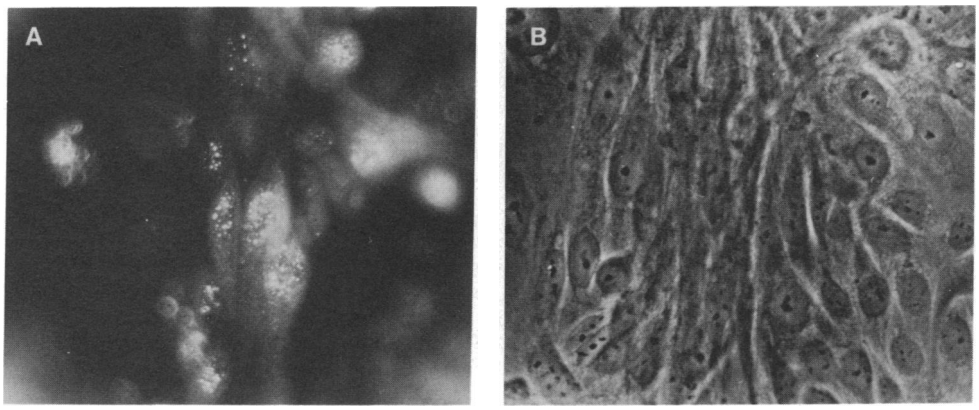

FIG. 2. HCMV-specific monoclonal antibody fluorescence in MKC cultures. (*A*) Immunofluorescent staining of MKC cells with a combination of HCMV monoclonal antibodies to early and late HCMV proteins 24 hr after infection. (×430). (*B*) Phase-contrast photograph of *A* showing a cluster of morphologically similar cells forming a mesangial hillock. Micrographs were taken with a Zeiss Universal microscope equipped with epifluorescence (Zeiss). (×430.)

HCMV-specific antigen expression was restricted to mesangial cells. To extend these findings, we further assessed the correlation between HCMV expression and cell phenotype by using biotin/streptavidin *in situ* hybridization of an HCMV probe to MKC monolayers and observed the distribution of HCMV DNA replication (data not shown) . The distribution of probe hybridization was again limited to a morphologically distinct cell type indistinguishable from the fluorescent-positive cells described above.

Identification of Productive Cells in MKC. To confirm that mesangial cells supported HCMV-specific protein and nucleic acid production, mesangial hillocks were removed from primary cultures and expanded for further investigation (ref. 27; E. Muller, Y. Kim, and A. Michael, personal communication). The cells maintained their original morphology, formed hillocks, and remained positive for type III collagen on subsequent passages. Human arterial smooth muscle cells are reported to have similar growth characteristics to mesangial cells in culture (28). Collagenase treatment of purified glomeruli necessary for mesangial cell isolation did not leave observable hilar arterioles by microscope examination. Therefore, while smooth muscle cell contamination was unlikely, there are no markers available to unequivocally distinguish smooth muscle cells from mesangial cells in culture. Growth curves and infectious center assays of mesangial cell cultures infected with HCMV indicated that viral replication was concentrated in this cell type as compared with MKC cultures infected at similar mois (Fig. 1*B* and Table 1). Mixed cultures contained fewer infectious cells than mesangial cultures but contained more productive cells than cultures of GVEC. To further compare HCMV replication in SF, MKC, and mesangial cells, we looked for the expression of HCMV immediate-early proteins in infected cells by one-dimensional PAGE (Fig. 3). Virus-induced immediate-early polypeptides include a major 72-kDa protein produced by productive cultures during both immediate-early and early phases of viral replication (15, 29). Infected [^{35}S]methionine-labeled SF and mesangial cytoplasmic fractions showed evidence of the major 72-kDa HCMV protein. MKC fractions from cells in culture for 7 days prior to viral inoculation did not exhibit visible immediate-early bands at time intervals from between 4 and 6 hr to between 36 and 38 hr after infection. Infected MKC cells cultured for >10 days that morphologically showed mesangial cell overgrowth did express the 72-kDa protein band.

To assess the ability of these cultures to replicate HCMV DNA, a 32-kilobase Towne strain probe was hybridized to Southern dot blots containing DNA from infected and uninfected cells harvested 96 hr after infection (Fig. 4). The autoradiogram revealed obvious viral DNA in SF and mesangial cells. Minimal hybridization was observed in MKC cultures. The probe did not hybridize to mesangial-depleted cultures containing predominantly GVEC from either 2-hr or 2-week exposures. In addition, there was a direct correlation between moi, immunofluorescent staining, and probe hybridization in the mesangial cultures. Mesangial cells isolated from two infants and an adult also supported virus growth. We concluded that mesangial cells were productive for HCMV replication in culture and that the viral protein synthesis, HCMV DNA replication, and viral progeny production observed in infected MKC cultures were due to HCMV replication in mesangial cells. This is in contrast to reports (24) indicating that human kidney cultures do not support HCMV replication.

DISCUSSION

In addition to their probable role as a support system in the renal corpuscle, mesangial cells have phagocytic properties (30). Experimental studies indicate that macromolecules including aggregate protein and immune complexes accumulate in the mesangium in amounts proportional to their blood concentration (31). Immunoglobulin and CMV antigen are present in the glomeruli of patients with CMV pneumonitis suggesting immune complex deposition and possible CMV antigen accumulation in the mesangium (32). HCMV has been implicated as a cause of glomerulonephritis in infants

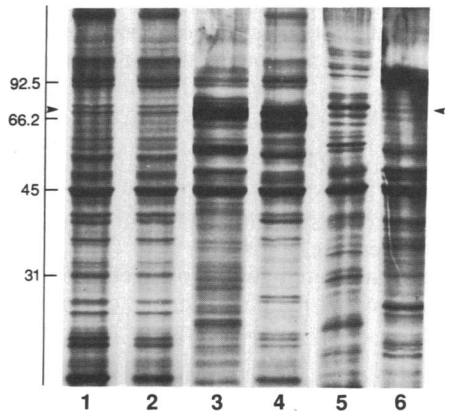

FIG. 3. Towne strain HCMV immediate-early protein expression in cytoplasmic lysates obtained from infected SF, MKC, and mesangial cultures. [^{35}S]Methionine-labeled infected and uninfected cytoplasmic fractions were loaded onto 10–15% $NaDodSO_4$/polyacrylamide gels as microliter aliquots containing equivalent trichloroacetic acid-precipitable cpm and electrophoresed according to Laemmli (19). Arrows indicate the relative position of the major immediate-early 72-kDa protein. Immediate-early protein expression at 4–6 hr after infection in SF cultures, 12–14 hr in mesangium, and 16–18 hr in MKC cultures containing mesangial cell overgrowth is shown. Lanes: 1, MKC uninfected control; 2, infected MKC; 3, infected SF; 4, infected MKC with mesangial overgrowth; 5, infected mesangial cells; 6, SF uninfected control.

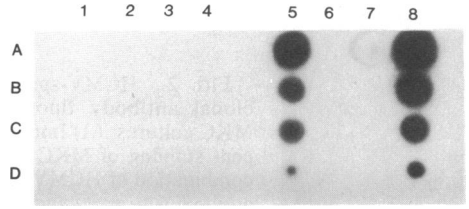

FIG. 4. Southern dot-blot hybridization of infected and uninfected DNA from SF, MKC, mesangial, and GVEC (mesangial depleted) 96 hr after infection. Total DNA was extracted from 10^6 cells from each culture. Aliquots of 5 μg, 1 μg, 0.5 μg, and 0.1 μg (rows A–D, respectively) were blotted onto nylon membrane filters for lanes 1–8. [^{35}P]dCTP nick-translated Towne fragment probe was hybridized to the filters. Lanes 1–4 are uninfected cell fractions. Lanes 5–8 are infected cell fractions. Lanes: 1 and 5, SF; 2 and 6, mesangial-depleted GVEC; 3 and 7, MKC; 4 and 8, mesangial-depleted culture.

and adults (33). Patients with renal allograft dysfunction accompanied by HCMV viremia show mesangial hypercellularity and increased matrix deposition (34). Immune complex deposits in the mesangium of these patients contain IgG, CMV antigen, and complement component 3. Macrophage migration into the mesangium has also been documented (35). It is possible that macrophage migration and immune complex deposition introduce virus into the mesangium.

An experimental mouse model for CMV glomerulonephritis has documented intranuclear inclusions and cytomegaly in the mesangium 1–3 days after infection (36, 37). Electron microscopic analysis showed accumulation of virus in the cells 5 days after the mice were inoculated with murine CMV. This was followed by mesangial hypercellularity accompanied by an increase in mesangial matrix deposition. Mesangial pathology resolved gradually as viral inclusions progressed to the vascular pole and juxtaglomerular region followed by prolonged distal tubule involvement. In this model only 10% of the glomeruli contained observable cytopathology. Histologic analysis of kidneys from HCMV viremic patients indicated that viral inclusions were frequently found in kidney tubules, and *in situ* hybridization studies revealed viral nucleic acid sequences in glomeruli of kidney sections taken from HCMV-infected patients at autopsy (7). If HCMV infection of the human kidney results in pathologic changes similar to those observed in the murine CMV glomerulonephritis model, then mesangial involvement would be transient. Cytomegaly would not be expected to be observed in the mesangium at the time of renal biopsy or autopsy, since histologic examination would take place too late in the course of disease.

Our results indicated that prolonged influx of virus to mesangium by either immune complex deposition or macrophage migration could initiate a progression of viral replication through the kidney similar to that observed in the murine model described above. This could result in trafficking of virus from the mesangium to the tubules leading to tubular infection and chronic viruria in patients with undetectable HCMV viremia. In addition, the cell culture system used in these experiments may provide a powerful *in vitro* tool to identify humoral or cellular factors that are crucial in the initiation, reactivation, or potentiation of CMV disease following renal transplantation.

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