HIV-associated immune-mediated renal disease

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HIV-associated immune-mediated renal disease. Although focal glomerulosclerosis is the most common renal disease, other proliferative glomerulonephritides are encountered in HIV-infected patients. We studied four HIV-infected patients with renal insufficiency, proteinuria, and proliferative glomerulonephritis, consistent with immune-mediated disease, to investigate the role of the virus and immune complexes in the pathogenesis of the nephropathy. Circulating immune complexes (CICs) and HIV-reactive antibodies were measured and characterized in each patient. Renal biopsy tissue was acid eluted, and the eluate analyzed. DNA extracted from biopsies was subjected to the polymerase chain reaction (PCR) to detect HIV genome. CICs were detected in each patient: an IgA-p24 HIV antigen complex and an IgG antibody-gp120 HIV antigen complex in two patients; two patients had an IgG-p24 HIV antigen complex. Identical complexes were eluted from renal tissue in the first three patients; p24 HIV antigen, and complement from the fourth. The eluted antibodies reacted with the HIV antigens from the isolated CICs. Direct immunofluorescence for viral antigen in the eluted glomerular tissue revealed HIV antigens; PCR confirmed the presence of gag genome in all four biopsies. We conclude both circulating and *in-situ* HIV antigen-specific immune complexes may be associated with glomerulonephritis in HIV infected patients. Viral incorporation into renal tissue may be important in the pathogenesis of HIV-associated renal disease.

Several renal syndromes have been described in patients with HIV infection [1–7]. Patients with chronic renal insufficiency and nephrotic syndrome associated with HIV infection often have focal and segmental glomerulosclerosis [1–5, 8–10]. Some investigators have suggested this finding constitutes a distinct, clinico-pathological entity, termed either AIDS-associated or HIV-associated nephropathy [1, 2, 4, 8, 9]. In contrast, however, other histological findings such as membranoproliferative and diffuse proliferative glomerulonephritis [6, 11, 12], membranous glomerulonephritis [13], and IgA nephropathy [14–17], noted in patients with HIV infection and chronic renal dysfunction or nephrotic syndrome [1–3] all possess pathological characteristics strongly suggestive of an immune-mediated etiology. Recent biopsy series in patients with HIV infection and renal disease have suggested that approximately one-quarter to one-half of patients may have glomerulonephritis [18, 19]. Since HIV-related antigen-antibody complexes have been demonstrated in the circulation of HIV infected patients [20–22], it is unclear whether these pathologic findings relate to immune complex deposition, the formation of *in-situ* complexes, infectious complications of HIV infection, manifested as post-infectious glomerulonephritis [12], or to other, as yet unknown, mechanisms.

We studied four patients with HIV infection, proteinuria and renal insufficiency in whom the renal pathologic findings were consistent with immune-mediated glomerulonephritis. HIV-associated antigen-antibody complexes were recovered from the circulation of all four patients, and identical complexes were recovered and identified from eluates of renal tissue in three of the four. In all four of the patients' biopsy material HIV antigens could be immunologically detected within glomeruli, and polymerase chain reaction (PCR) examination confirmed the presence of HIV genome in the renal tissue.

Methods

Case reports

Patient 1. A 31-year-old Black homosexual male had HIV infection for two years prior to an increase in serum creatinine concentration from 88.4 to 283 μmol/liter over a year, four months before admission. He had *Pneumocystis carinii* pneumonia two years prior to admission, treated with sulfamethoxazole-trimethoprim. Zidovudine, started at the time of the diagnosis of HIV infection, was discontinued six months prior to admission. *Mycobacterium avium intracellulare* lymphadenitis was diagnosed one year prior to admission. Medications at the time of evaluation consisted of pentamidine, nadolol, ranitidine, triamterene-hydrochlorthiazide, hydroxyzine hydrochloride, terfenadine and triazolam. There was no history of transfusions, intravenous drug use, or family history of kidney disease.

Physical examination revealed BP of 130/100 mm Hg, a clear chest examination and no edema. There was bilateral axillary adenopathy and a diffuse hypopigmented rash. Urinalysis revealed 4+ proteinuria, trace hematuria, pH 6.0. Two to three tubular epithelial cells, and rare granular and cellular casts per high power field were seen on microscopic exam. Serum urea and creatinine concentrations were 38.6 mmol/liter and 928 μmol/liter, respectively. Creatinine clearance was 0.12 ml/sec; there was 10.2 g non-selective urinary protein excretion/day. A complete blood count revealed a hematocrit of 32.4%, platelet count 205 × 10^9/liter, and 5.4 × 10^9/liter leukocytes. There was lymphopenia with an absolute CD4 count of 6/μl, (normal >

400/μl), low percentage of CD4 positive cells (1%), and an extremely diminished CD4/CD8 ratio of 0.02 (normal 1.0 to 2.2).

Serum electrolyte concentrations were within normal limits. Serum aspartate and alanine aminotransferase (AST and ALT) levels were slightly elevated, but bilirubin and alkaline phosphatase levels were normal. Serum protein electrophoresis revealed diminished total protein and albumin levels but increased alpha globulins. There was no monoclonal spike. CH50, C'3 and C'4 levels were either normal or slightly increased. Serologic tests for syphilis were strongly positive. Hepatitis B virus surface antigen was negative, but hepatitis B surface and core antibody and hepatitis C virus antibody screens were positive.

Patient 2. A 55-year-old Black male presented with a one month history of pedal edema, fatigue, and nocturia, and a two week history of nausea, vomiting and a 10 pound weight loss. Two years prior to evaluation the patient had normal renal function. He had taken ibuprofen over the previous three weeks. Evaluation revealed serum creatinine concentration of 433 μmol/liter, creatinine clearance 0.42 ml/sec, and 29.4 g urinary protein excretion/day. Serum protein electrophoresis revealed diminished total protein concentration, with severe hypoalbuminemia, normal alpha 1, 2, and beta globulin levels, and elevated gamma globulin concentration. Antinuclear antibody, antistreptolysin O serology, latex fixation, hemoglobin A1C percentage, C'3 and C'4 were either negative or within normal limits. The patient had had an oligoclonal gammopathy for two years. There was a history of mitral regurgitation and paroxysmal supraventricular tachycardia, treated with diltiazem, longstanding well-controlled hypertension, most recently treated with verapamil, and gout. There was no history of opportunistic infection. The patient was bisexual, and denied use of intravenous drugs. There was a family history of diabetes and kidney disease.

Physical examination showed BP of 140/100 mm Hg, fundi with rare hemorrhages and arteriovenous nicking, an apical holosystolic murmur, and trace pedal edema. Urinalysis revealed specific gravity 1.020, pH 6.0, glucosuria, proteinuria, rare amorphous crystalluria and glitter cells. Serum urea and creatinine concentrations were 11.4 mmol/liter and 451 μmol/ liter respectively. Complete blood count revealed 7.5 × 10⁹/liter leukocytes, hematocrit of 30%, platelet count 251 × 10⁹/liter. Absolute lymphocyte count was within normal limits and the absolute CD4 count was 488/μl, but the percentage of CD4 positive cells was diminished (helper/suppressor ratio of 0.4).

Serum electrolyte concentrations were within normal limits. AST and ALT levels were slightly elevated, but bilirubin and alkaline phosphatase levels were normal. ELISA screen for HIV was positive, confirmed by Western blot analysis. Serological tests for syphilis were negative. Hepatitis B virus surface and A virus antibody screens were negative, but hepatitis B core antibody was positive. Cytomegalovirus titers were markedly elevated. Anti-double stranded DNA antibody titer was slightly elevated but anti-single stranded DNA antibody, Sm and RNP antigens were within normal limits or negative.

Patient 3. A 44-year-old Black male had proteinuria and hematuria discovered two months after the diagnosis of HIV infection was made. He had known of HIV infection for two years before the renal evaluation. He denied homosexual encounters, but had had sexual relations with prostitutes. He

had been asymptomatic, but a routine urinalysis revealed hematuria. Creatinine clearance was 1.07 ml/sec and urinary protein excretion was 0.3 g/day. He had a fifteen year history of hypertension, treated with various medication regimens, and had syphilis diagnosed and treated fourteen years prior to evaluation. Ulcerative colitis was diagnosed seven years prior to evaluation, but had been well-controlled after treatment. Medications at the time of evaluation were zidovudine, azulfidine, nadolol, triamterene/hydrochlorthiazide and sulfamethoxazole/trimethoprim.

Physical examination revealed BP of 150/100 mm Hg, hypertensive retinopathy, and bilateral axillary adenopathy. There was no edema. Urinalysis revealed pH 6.0, 3+ proteinuria, and 10 to 20 red blood cells per high power field, without red blood cell casts. Serum urea and creatinine concentrations were 7.5 mmol/liter and 115 μmol/liter, respectively. Complete blood count revealed 5.1 × 10⁹/liter leukocytes, hematocrit of 37.4%, platelet count 236 × 10⁹/liter. Absolute lymphocyte count was within normal limits, but there was a relative lymphocytosis. The absolute CD4 count was 183/μl. The percentage of CD4 positive cells was diminished (6%) and the helper/suppressor ratio was 0.08.

Serum electrolyte, ALT and AST, bilirubin and alkaline phosphatase concentrations were within normal limits. Creatinine clearance was 1.22 ml/sec. Urinary protein excretion was 1.4 g/day. Serological tests for syphilis were positive. Hepatitis C virus and hepatitis B virus surface antigen serologies were negative, but hepatitis B core antibody screen was positive. C'3 and C'4 concentration were at the lower level of normal. Serum protein electrophoresis showed mild hypoalbuminemia and increased total gamma globulin concentration, beta-gamma bridging and oligoclonal banding. IgA, IgG and IgM levels were all increased. There was no evidence of cryoprecipitation. Urinary protein electrophoresis was consistent with selective proteinuria. There were no monoclonal bands present.

Patient 4. A 35-year-old Black homosexual man had HIV infection for three years. He was treated for two years with zidovudine, which was discontinued nine months before evaluation because of hematologic complications. Two months before evaluation he complained of intermittent bilateral pedal edema. His serum creatinine concentration increased from 124 μmol/liter nine months prior to admission to 256 μmol/liter two months prior to admission. Past medical history was remarkable for candida esophagitis, *E. coli* urinary tract and *Giardia lamblia* gastrointestinal infections and recurrent sinusitis. He had been treated in the past with steroids for allergic dermatitis. There was a history of epididymitis. There was a family history of renal failure. He was taking furosemide and acetaminophen/ oxycodone at the time of evaluation.

Physical examination revealed BP of 110/70 mm Hg. He was afebrile, and had scant rales at the right base. There was a systolic murmur at the lower left sternal border, and marked bilateral lower extremity edema. Urinalysis revealed specific gravity 1.013, pH 5.0, protein concentration greater than 3 g/liter. There was no glucosuria, ketonuria or bilirubinuria. Microscopic examination revealed a few red and white blood cells per high power field and scattered granular casts. Twenty-four-hour urinary protein excretion was greater than 3 g/day. Serum urea and creatinine concentrations were 30 mmol/liter and 602 μmol/liter, respectively. Creatinine clearance was 0.11

ml/sec. Complete blood count revealed 5.0×10^9/liter leukocytes, hematocrit of 21%, platelet count 1012×10^9/liter. There was absolute lymphopenia. CD4 count was $18/\mu l$, with a reduction in both the percentage of CD4 positive cells (4%) and the helper/suppressor ratio (0.1).

Serum sodium concentration was 135 mmol/liter, potassium 5.1 mmol/liter, chloride 113 mmol/liter, bicarbonate 12.7 mmol/liter. Serum albumin concentration was 14 g/liter. AST level was slightly elevated, but bilirubin was normal. Alkaline phosphatase level was markedly elevated. Hepatitis B core antibody and CMV titers were positive. C'3 was 0.7 g/liter (normal 0.8 to 2.0 g/liter) but C'4 concentration was slightly above normal limits. Serum protein and immunoelectrophoresis showed decreased alpha 1, beta, and total gamma globulin concentration and an IgG kappa monoclonal band. IgG and IgA levels were normal, but IgM concentration was slightly decreased. Antinuclear antibody screens and rheumatoid factor evaluation were negative.

All patients underwent renal biopsy after informed consent was obtained. These studies were approved by the George Washington University Medical Center Committee on Human Research.

Histopathology

Paraffin embedded sections were stained for routine light microscopy with hematoxylin-eosin, periodic acid Schiff, periodic acid methenamine silver, Masson's trichrome and Congo red [16]. Immunopathologic studies were performed on frozen sections using fluorescein-conjugated monoclonal antibodies (Dako Corp, Santa Barbara, California; Chemicon International Inc., Temecula, California, USA), directed against albumin, fibrinogen, C'1q, C'3, properdin, IgA, IgE, IgG, IgM, and kappa and lambda light chains. Osmium-treated epon-embedded sections of glutaraldehyde-fixed tissue were examined with electron microscopy [16].

Detection of HIV antibodies

Patients' plasma samples were screened for the presence of anti-HIV antibodies by ELISA (Organon Teknika Corp., Durham, North Carolina, USA) and positive reactions confirmed by Western blot analysis (BioRad Laboratories, Richmond, California, USA). Reactive anti-HIV antibodies were classified using monospecific, peroxidase-labelled rabbit antibodies directed against human IgA, IgG, and IgM in the secondary phase of the blot (Chemicon International, Inc.) [16].

Circulating immune complex detection and isolation

Circulating immune complexes (CICs) were measured by a solid-phase C'1q Enzyme Immunoassay kit (Cytotech, San Diego, California, USA), according to the manufacturer's instructions and isolated by a previously described polyethylene glycol (PEG) precipitation- sedimentation technique [23]. Following isolation, each CIC was recovered, washed in cold PEG, and dissolved in 0.01 M phosphate buffered saline, pH 7.2 (PBS), prior to analysis of the immunoglobulin content by single radial immunodiffusion using commercially available plates (Kallstad, Austin, Texas, USA).

Western blot analysis of CIC antigens

Each PEG-isolated CIC was denatured in SDS and separated on a 10% T, 2.7% C PAGE gel at 40 mA for four hours, using 0.025 M Tris/0.192 M glycine, pH 8.4, as the running buffer. The separated gel maps were blotted to nitrocellulose and incubated overnight at 4°C with 1:1000 dilutions of murine antibodies directed against HIV p18, p24, gp41, p55, p51/56, gp120 and gp160 antigens (Epitope, Inc., Beaverton, Oregon, USA). Antibody localization was visualized by incubation with a 1:500 dilution of alkaline phosphatase-labeled rabbit anti-mouse IgG followed by a 30 minute development in 0.2 mg/ml nitroblue tetrazolium-0.04 mg/ml 5-bromo-4-chloro-3-indolyl phosphate [24]. Non-immune murine IgG, from the same fusion partners, and specific absorption of the anti-HIV antibodies with the appropriate antigen were used as specificity controls.

Dot-blot analysis of urine

Urine samples from each patient were concentrated fifty times by negative pressure dialysis prior to placing 20 μl aliquots into each of seven wells in a BioDot dot-blot apparatus (Bio-Rad Laboratories) [25]. The samples were allowed to diffuse onto a nitrocellulose membrane for 18 hours at 4°C before incubating them for 18 hours with 100 μl of a 1:1000 dilution of mouse monoclonal anti-HIV p18, p24, gp41, p55, p51/56, gp120 and gp160 (Epitope, Inc.). Bound antibodies were detected as described above in the Western blot procedure and identical specificity controls were incorporated.

Elution of kidney biopsies

Six μm thick frozen sections of the patients' renal biopsy material were acid eluted in 50 μl 0.33 M citric acid, pH 2.0 per section, for 17 hours at 4°C [26]. Eluates were sampled at 0, 1, 4, 8, and 17 hours and separated by block electrophoresis, using 1% SeaKem HEEO agarose (FMC BioProducts, Rockland, Maine, USA). Anodal and cathodal migrating materials were recovered, dialyzed against PBS and concentrated fivefold prior to PAGE separation and Western blot analysis. Antibodies and antigens recovered from the 17-hour elution samples were reconstituted in 50 μl of normal human serum, which had previously been shown to be CIC negative by the C'1q assay and retested to assess the degree of enrichment caused by the renal deposition. C'3 levels were measured in the plasma and in separated renal eluates as an additional enrichment marker.

Immunochemical analysis of eluted and complexed materials

Isolated antigens from both biopsy eluates and CICs were separated by PAGE, blotted to nitrocellulose and immunochemically stained with anti-HIV monoclonal antibodies. Isolated antibodies from the same material were reacted against blotted PAGE maps of whole HIV using 1:500 dilutions of enzyme-labeled rabbit anti-human antisera as reporter reagents. In addition, the immunoreactivity of the antibodies was tested by immunodiffusion in 1% SeaKem LE agarose (FMC BioProducts) plates. Each gel pattern consisted of a central well (5 mm diameter) surrounded by six equidistant peripheral wells (2 mm diameter). Twenty-five μl of antibody isolated from either the renal eluate or the CIC were placed into the central well and 10 μl of the eluate antigen, the CIC antigen, the urine antigen, normal renal tissue extract, and two PBS controls were placed

in the peripheral wells. The plate was placed in a moist chamber and examined at 24, 48, and 72 hours.

Immunocytochemical localization of intracellular HIV antigens in eluted biopsy sections

The eluted sections from each patient's renal biopsy were stained with rhodamine-labeled anti-human IgA, IgG, IgM, C'3 and C'4 (Chemicon International, Inc.), to check the efficiency of the elution. The sections from each biopsy were then stained with fluorescein-labeled anti-HIV antibodies (Epitope, Inc.) and the sections examined for the presence of intracellular HIV antigens using laser-enhanced fluorescence microscopy (Meridian Instruments, Inc., Okemos, Michigan, USA). The sections were scanned at 250 diameters magnification and the images of the glomerulus captured by a charged coupled device camera and viewed as a computer-enhanced 256 gray-scale map. This map was analyzed by the instrument's analytical software. Following background subtraction, morphological areas demonstrating high intensity staining were re-examined at 400× power. The fluorescent image was photographed directly using Polaroid 665 film.

Polymerase chain reaction

Five μm thick histologic sections of renal biopsy tissue from the patients and from a control HIV-negative patient with glomerulonephritis were scraped from glass slides. The DNA was extracted using a modification [27] of the method of Saiki et al [28] and subjected to a nested PCR, briefly described below, in order to amplify target sequences within the gag and env regions of the HIV-1 proviral genome. The tissue from the patients and the various controls were processed in a parallel manner. To assure the presence of tissue and that the reaction was not inhibited by the sample, a region from exon 1 of the human cardiac myosin heavy chain (MyHC) gene was co-amplified with the gag and env genes in both the inner and outer nests. The primers used for the amplification of the gag gene were SK100 and SK105 for the outer nest, and SK18 and SK 39 for the inner nest. The primers used for the amplification of the env gene were SK 68 and SK 69 for the outer nest, and SK 682 and SK 692 for the inner nest. For the MyHC gene, MHC1 and MHC2 were used in the outer nest, and MyHC20 and MyHC40 were used for the inner nest. The amplification reaction for the outer and inner nests was repeated forty times. Each cycle consisted of denaturation for one minute at 92°C, annealing for two minutes at 52°C, and extension for three minutes at 72°C, resulting in amplification of a fragment of 137 bps for the gag gene, 79 bps for the env gene, and 185 bps for the MyHC gene. After liquid hybridizing the inner nest reaction product with an end labeled probe (SK19 for the gag gene, SK 689.1 for the env gene and MyHC30 for the MyHC gene), the samples were run on a 10% polyacrylamide gel, using PhX as a molecular weight marker. The gels were exposed to X-ray film for the visualization of bands. Several control samples were amplified simultaneously with the DNA from the patient's renal biopsies, including a reagent control that contained no DNA template, a negative control sample containing DNA known to be negative for HIV-1 proviral DNA, and dilutions of a plasmid containing a 9 kb fragment of the HIV-1 target genome, as a positive control.

All primers and probes were synthesized in an oligonucleotide synthesizer (Milligen-Biosearch Cyclone, Bedford, Massachusetts, USA) using phosphoramitide based chemistry [28].

Results

Histopathology

Patient 1. Of the 25 glomeruli found in the biopsy, one was normal, and two showed mild to marked mesangial expansion, prominent visceral epithelial cells, and cellular crescents (Fig. 1A). The rest were obsolescent. There was microcystic tubular dilation and atrophy, interstitial fibrosis and interstitial infiltration of lymphocytes and plasma cells. Immunofluorescent microscopy showed faint intramembranous deposits of IgA, C'3 and albumin, but when the sections were etched by trypsin digestion for 10 minutes at 37°C, staining for IgA, C'3, C'4 and albumin was seen. In addition, the etching revealed faint IgG staining at the periphery of the tubules. Electron microscopy in two obsolescent glomeruli showed the presence of finely granular, subendothelial and intramembranous electron dense deposits (Fig. 2A). Tubular reticular structures were not identified in obsolescent glomeruli.

Patient 2. Six of twelve glomeruli showed increased cellularity with lobular transformation, segmental consolidation of the tuft, and synechiae. A few obsolete glomeruli with fibrocellular crescents were also present. Peripheral capillary loops in uninvolved segments appeared normal. In some glomeruli, segmental increase of mesangial cells and matrix was prominent (Fig. 1B), while in others sclerosis of tuft, including total obsolescence were noted. Masson's trichrome stain revealed mesangial deposits. There were scattered dense interstitial collections of mononuclear cells, mostly lymphocytes and plasma cells, accompanied by polymorphonuclear leukocytes and eosinophils. The tubular epithelium was permeated by inflammatory cells, resulting in tubular destruction. Microcystic tubular dilation was present, as were foci of tubular atrophy, periglomerular and interstitial fibrosis. Immunofluorescence of the single available glomerulus revealed IgM and C'3 present in abundance as coarse granular mesangial deposits. Properdin and C'1q were present in small amounts in mesangial granular deposits and albumin and IgG were present within the proximal tubular epithelium. There was insufficient glomeruli-containing tissue for trypsin etching and immunoperoxidase studies. Electron microscopy of one glomerulus, indicated as slightly abnormal on light microscopy, showed glomerular basement membrane of normal thickness and composition. There were no peripheral intramembranous or subepithelial deposits noted. There was extensive fusion of foot processes. The expanded mesangium contained increased matrix, cells, and finely granular electron dense deposits (Fig. 2B). Tubuloreticular structures were abundant within the cytoplasm of endothelial cells.

Patient 3. All of the six glomeruli exhibited diffuse mesangial expansion (Fig. 1C). There was a dense mononuclear interstitial infiltrate consisting mostly of plasma cells. The infiltrating cells in some instances permeated tubular epithelium, resulting in destruction of the tubular basement membrane. A few tubules showed dilation with proteinaceous material within lumina. Immunofluorescent microscopy showed glomeruli contained C'3, IgG, IgM, IgA, kappa and lambda light chains and C'1q,
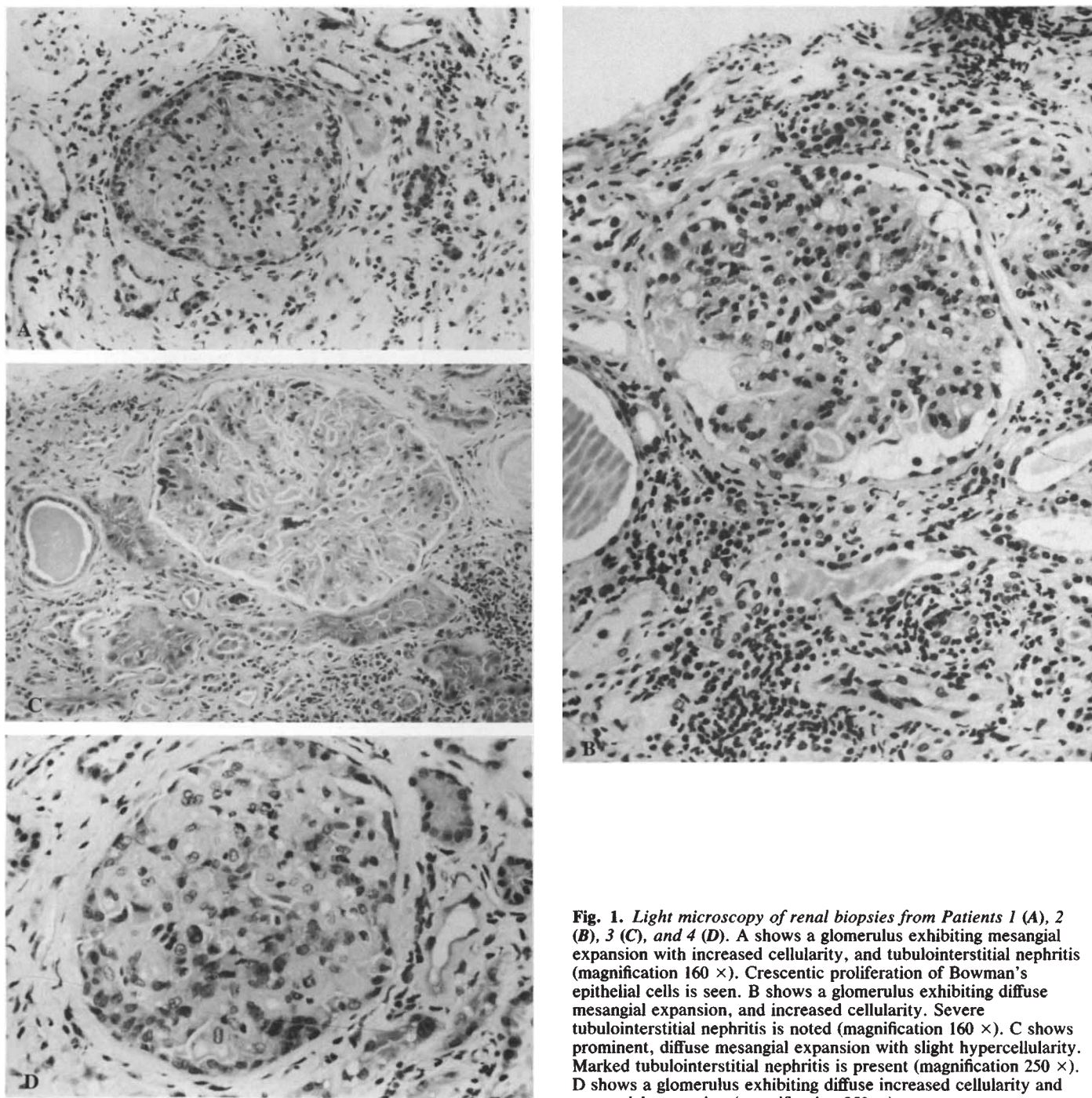
Fig. 1. *Light microscopy of renal biopsies from Patients 1 (A), 2 (B), 3 (C), and 4 (D).* A shows a glomerulus exhibiting mesangial expansion with increased cellularity, and tubulointerstitial nephritis (magnification 160 ×). Crescentic proliferation of Bowman's epithelial cells is seen. B shows a glomerulus exhibiting diffuse mesangial expansion, and increased cellularity. Severe tubulointerstitial nephritis is noted (magnification 160 ×). C shows prominent, diffuse mesangial expansion with slight hypercellularity. Marked tubulointerstitial nephritis is present (magnification 250 ×). D shows a glomerulus exhibiting diffuse increased cellularity and mesangial expansion (magnification 250 ×).

primarily in a mesangial distribution (Fig. 3). Electron microscopy revealed one obsolescent glomerulus with scanty electron-dense deposits, while a second glomerulus showed extensive segmental approximation of foot processes of visceral epithelial cells, finely granular electron-dense deposits within mesangial cells, basement membranes of normal thickness, and variably sized peripheral subepithelial electron-dense deposits (Fig. 2C). Tubular reticular structures were present.

Patient 4. Light microscopy revealed two of seven glomeruli with proliferative changes (Fig. 1D). The remaining glomeruli showed global or segmental proliferative and sclerosing changes, with simplification of the glomerular tufts and synechiae. There were many atrophic tubules, with dilated lumina and flat, vacuolated epithelium, and interstitial edema. Foci of mononuclear cell collections were seen mostly around venules. Immunofluorescence showed albumin and complement within
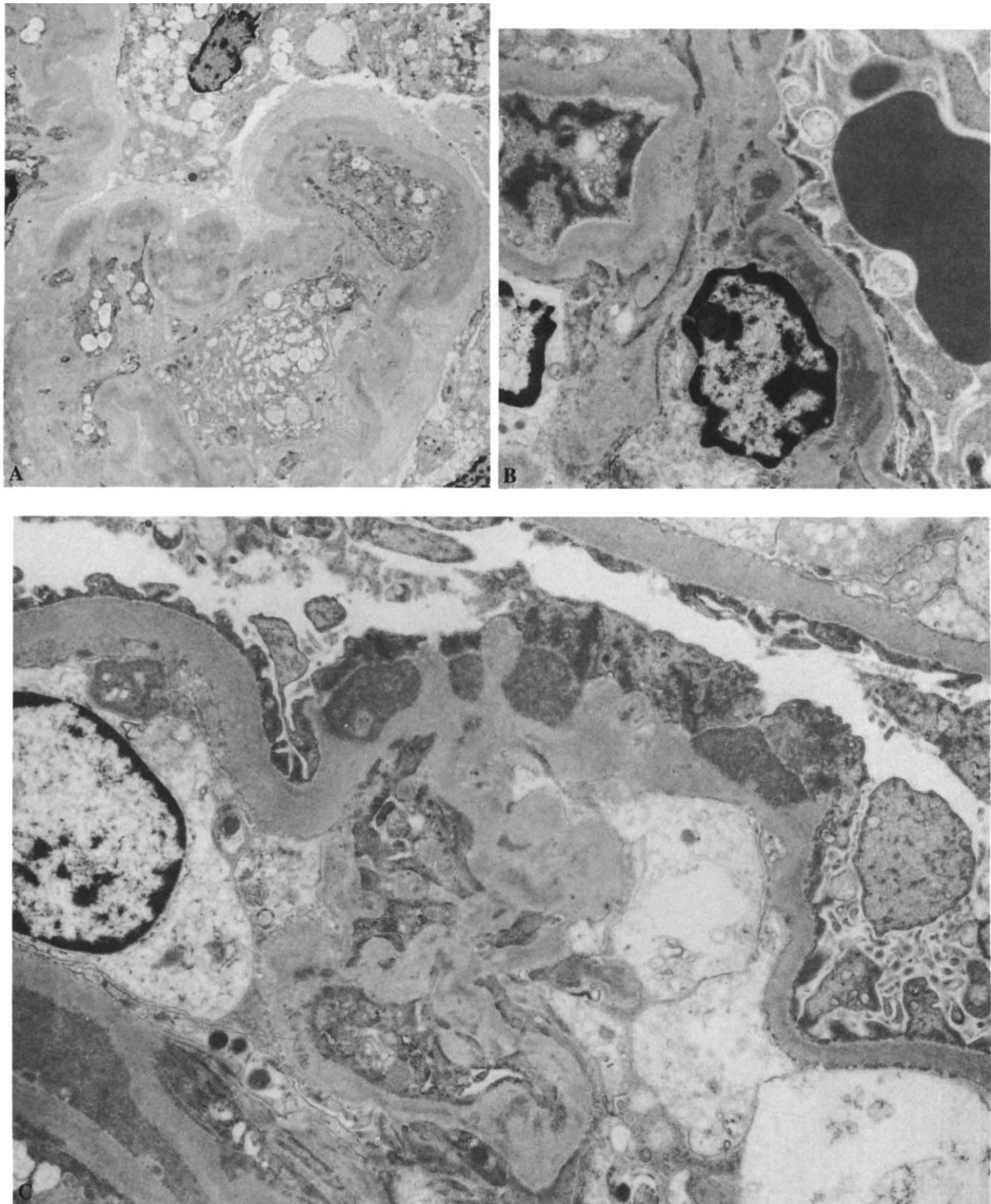
Fig. 2. *Electron micrographs of glomerular tissue from biopsies of Patients 1 (A), 2 (B), 3 (C), and 4 (D).* A shows a consolidated segment of tuft with denuded visceral epithelial cell foot processes and subendothelial deposits (magnification 9,000 ×). B shows fused foot processes and electron-dense deposits within the mesangium (magnification 15,000 ×). C shows prominent, variably-sized subepithelial deposits, and scanty mesangial electron dense deposits. Tubular reticular structures are present (magnification 18,000 ×). D shows fusion of foot processes and intramesangial and subendothelial electron-dense deposits (magnification 17,000 ×).
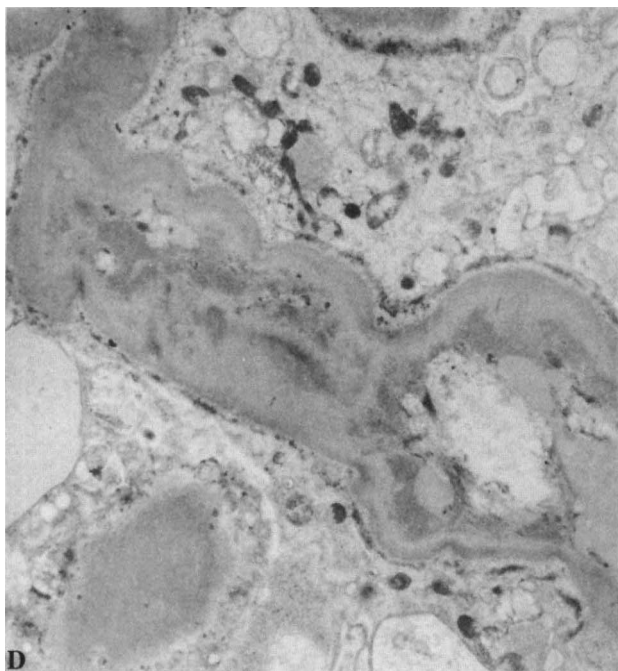
Fig. 2. *Continued.*

dilated tubules and resorption droplets. C'3, C'1q, and properdin were present in all glomeruli studied in a segmental distribution, as were traces of IgA. No fibrin, IgG, IgM or IgE was detected in specific glomerular distribution. Electron microscopy (Fig. 2D) showed a glomerulus with collapsed, consolidated loops, with many small and some large subendothelial deposits within capillaries obliterated by mononuclear cells. There were extensive, large mesangial electron-dense deposits. Foot processes were fused. Tubular reticular structures were appreciated in endothelial cells.

Immunologic studies

Patient 1. The patient's plasma demonstrated the presence of IgA antibodies reactive with HIV p24 and gp41 antigens in addition to IgG antibodies which reacted with p24, gp41, gp120 and gp160 HIV antigens. A single CIC was detected at a level of 73 μg IgG equivalent binding/ml and shown by immunochemical analysis to be composed of an IgA antibody complexed to HIV p24 antigen. In addition, dot blot analysis demonstrated the presence of HIV p24 antigen in the patient's concentrated urine (Fig. 4). Elution studies on the renal biopsy tissue demonstrated the presence of IgA associated with a protein antigen plus trace amounts of IgG. Immunochemical analysis of this material revealed that the protein was HIV p24 antigen, and that the IgA, but not the IgG, was reactive with it, forming a soluble immune complex *in vitro*. Studies performed on the *in vitro* complex showed that it possessed a 3.8-fold enrichment when compared to circulating levels. This level was confirmed by the demonstration of a 3.4-fold higher concentration of C'3 in the eluate compared with plasma concentrations. Direct immunofluorescence of the eluted biopsy material demonstrated the presence of HIV p18 and p24 antigens within both

glomerular and tubular epithelial cells and sparse staining in the mesangium (Fig. 5).

Patient 2. Evaluation of Patient 2's plasma demonstrated the presence of IgM antibodies reactive with HIV p18 and gp41 antigens in addition to IgG antibodies which bound to p24, gp41, p55, gp120 and gp160 HIV antigens. A single CIC was detected (231 μg IgG equivalent binding/ml). Immunochemical analysis of this complex showed it was composed of an IgG antibody complexed to a 120 kDa HIV antigen. Examination of the patient's concentrated urine by dot blot demonstrated the presence of both HIV p24 and gp120 antigens (Fig. 4), together with a trace of free kappa light chain. Elution studies on the renal biopsy demonstrated that IgG antibodies against HIV gp120, and HIV gp120 antigen could be differentially recovered over time (Fig. 6). Immunochemical analysis revealed the eluted antibody was reactive with the HIV gp120 antigen recovered from the urine and the isolated CIC. A classical line of identity could be produced when either the eluted antibody or the antibody isolated from the CIC was reacted with the antigen from the biopsy elution, the antigen recovered from the CIC and the antigen recovered from the patient's urine by immunodiffusion (Fig. 7). Western blot analysis confirmed that all three antigens were of HIV origin. Reconstitution studies showed that the 17-hour elution sample contained the equivalent of 1,059 μg IgG equivalent binding/ml, constituting an approximately 4.5-fold enrichment over circulating levels. Analysis of C'3 levels in the eluate confirmed a fourfold increase compared to plasma levels. Direct immunofluorescence for the presence of HIV antigens revealed HIV p18 in the mesangium.

Patient 3. Analysis of the plasma demonstrated the presence of two circulating immune complexes: one complement-fixing complex composed of IgG and a 24 kDa protein antigen and a non-complement-fixing complex composed of IgM and IgG. Further analysis of the complement-fixing complex revealed the protein was HIV p24 antigen and that the IgG was reactive with HIV p24. Analysis of the non-complement-fixing complex demonstrated that it was composed of an IgM anti-antibody reactive against IgG anti-HIV p24 antibody and an anti-HIV p24 IgG antibody. Examination of the patient's concentrated urine by dot blot demonstrated the presence of HIV p24 and gp160 antigens. Elution studies of the patient's renal biopsy revealed the presence of HIV p24 antigen and an IgG antibody reactive against the p24 antigen. Further analysis demonstrated that the eluted complex was identical to the complement-fixing complex isolated from the patient's plasma. Enrichment studies showed a 5.3-fold increase over plasma levels for the immune complex, confirmed by a 4.8-fold increase in C'3 levels in the eluate compared to plasma levels. Direct immunofluorescence of the eluted renal biopsy material demonstrated the presence of HIV p24 antigen in the mesangium (Fig. 5).

Patient 4. The patient's plasma demonstrated the presence of a positive anti-HIV ELISA composed of IgA antibodies reactive to p18 and IgG antibodies reactive against p24, gp41, p55, gp120 and gp160 HIV antigens by Western blot. A single CIC, at a level of 81 μg IgG equivalent binding/ml, was detected by C'1q assay and isolated by PEG precipitation/sedimentation. Immunochemical analysis showed this complex to be composed of an IgG antibody complexed to a 24 kDa HIV antigen. Dot blot analysis of the urine demonstrated the presence of HIV p24

Fig. 3. *Immunofluorescence photomicrograph of IgG staining of a glomerulus from Patient 3.* A finely granular mesangial and scanty peripheral pattern is present (magnification approximately 300 ×).

antigen (Fig. 4). Elution studies on the renal biopsy demonstrated only albumin, HIV p24 antigen, and complement products. Examination of the eluted biopsy tissue by direct immunofluorescence showed the presence of HIV p18 and p24 antigens within the mesangium and glomerular loops.

Western blot analysis of the eluted antibodies from Patients 1, 2, and 3 failed to demonstrate immune reactivity against CMV, hepatitis B, or syphilis antigens.

Polymerase chain reaction

The reagent control (without DNA template) showed no amplification of MyHC or HIV-1 gag gene (Fig. 8, lane 1). The negative control sample containing DNA known to be negative for HIV target sequences showed amplification of MyHC, but no amplification of the HIV-1 gag gene target sequences (lane 2). The positive control sample showed amplification for MyHC
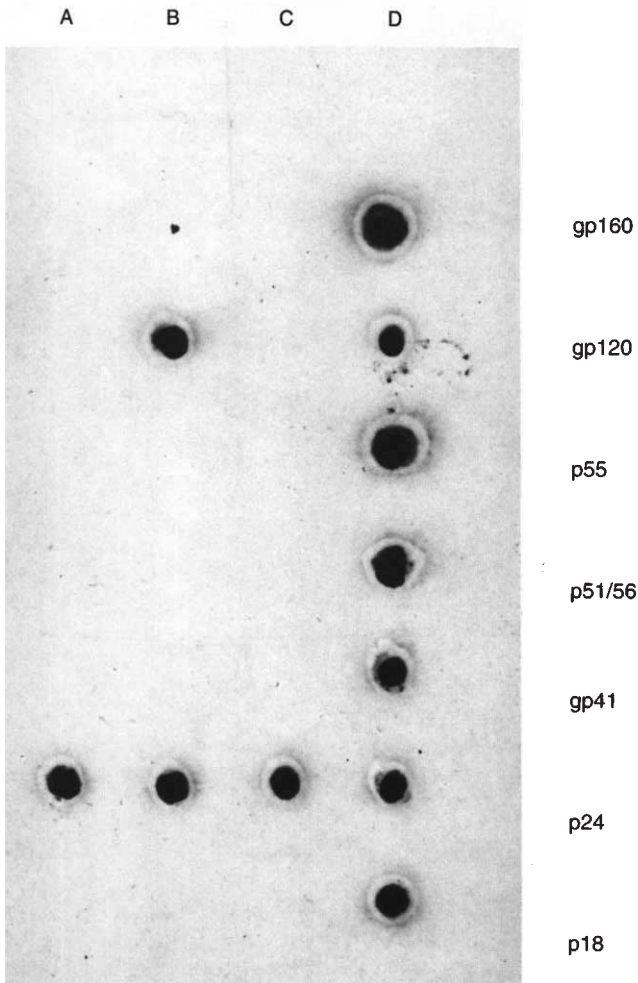
Fig. 4. *Dot blot of concentrated urine samples from all three patients.* Row A represents Patient 1, Row B Patient 2 and Row C Patient 4. Row D is composed of HIV antigen standards (p18, p24, gp41, p51/56, p55, gp120, and gp160). Rows A, B and C all demonstrate the presence of HIV p24 antigen in the urine, while Row B also shows the presence of HIV gp120. Data from Patient 3 are not shown.

Fig. 5. *Laser-enhanced fluorescence photomicrographs of HIV p24 localization in acid-eluted renal biopsy sections.* A shows localization of antigen in a glomerulus from the biopsy of Patient 1, while B shows localization of HIV p24 in a glomerulus from Patient 3's biopsy.

as well as amplification of the HIV-1 gag gene target sequences (lanes 3 to 5) but no amplification of MyHC or HIV-1 gene sequences below 1 copy (lanes 6 and 7). Renal biopsy tissue from an HIV-negative patient with glomerulonephritis showed amplification of the MyHC gene, but not the HIV-1 gag target sequence (lane 8). The renal biopsy specimens of all four patients with glomerulonephritis and HIV infection showed amplification of both MyHc and HIV-1 gag genes (lanes 9 to 12). Amplification of the env gene (not shown) was performed in Patients 1 and 2, and was positive in both cases.

Discussion

Although histopathological findings suggestive of immune complex disease have been reported in patients with renal disease and HIV infection [1–3, 6, 11–19, 29], the significance of these disparate pathologic entities has been unclear. The pathological findings de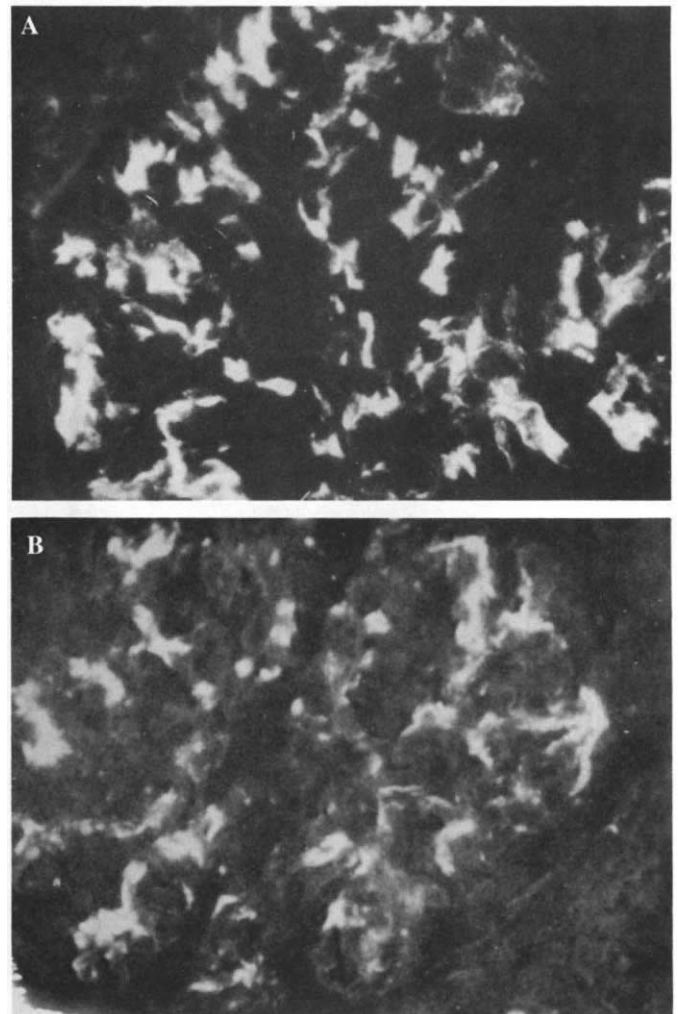scribed in such cases might be related to antigen-antibody complex deposition, occurring as a consequence of immune responses against the infectious agents which complicate HIV infection. This is possible because of the polyclonal B-cell activation leading to increased immunoglobulin production in patients with HIV infection [30]. Pathologic findings have been interpreted as consistent with postinfectious glomerulonephritis, perhaps a consequence of the infectious complications of AIDS [12]. Alternatively, HIV-related circulating immune complexes are common in HIV infected patients at all stages of disease [20–22, 31–40] and have been shown to play a role in the pathogenesis of immune-mediated thrombocytopenia in HIV-infected patients [41]. Deposition of such CICs may play a specific pathogenic role in mediating glomerulonephritis associated with HIV infection [42]. We recently described two patients with HIV infection and IgA nephropathy in whom CICs composed of IgA idiotypic antibodies reactive with anti-HIV IgG or IgM antibodies were isolated. Eluates of
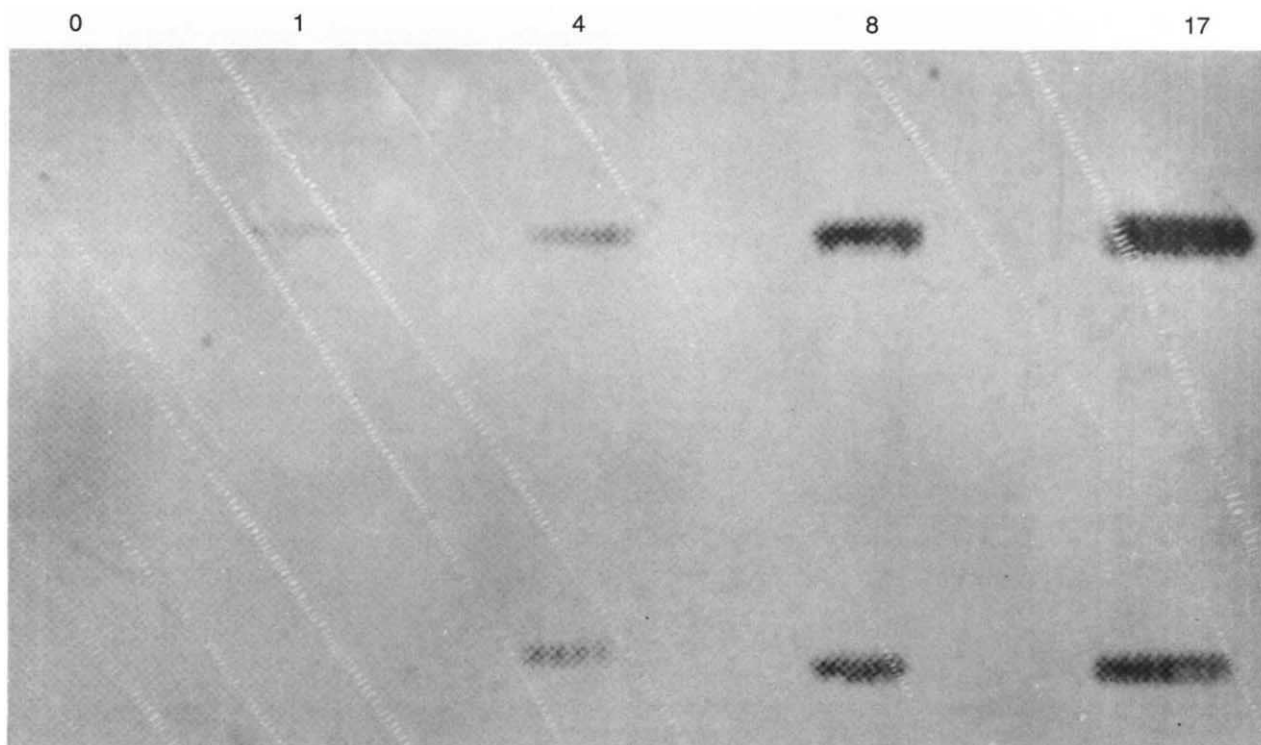
Fig. 6. *Agarose gel separation of kidney eluate over time (0, 1, 4, 8, and 17 hours after incubation of the eluted solution).* Samples were applied on Whatman 3MM filter paper squares and allowed to migrate into the 2MM gel for 5 minutes before separation. Antibody migrates toward the top and antigen towards the bottom of the figure. At 1 hour, a faint antibody line, but no antigen is seen. At 4, 8, and 17 hours after incubation, both antibody and antigen appear in increasing amounts. Example taken from Patient 2.

renal biopsy tissue in one patient contained an identical immune complex [16].

In the present study, the light and electron microscopic findings in the patients are consistent with immune complex mediated glomerulonephritis [42–44]. We demonstrated the presence of circulating antigen-antibody complexes of HIV p24/anti-HIV p24 IgA and an identical complex which could be eluted from renal tissue with an appearance typical of immune complex-mediated glomerulonephritis, in the first patient, whose glomerulus showed evidence of IgA deposition. An HIV gp120/anti-HIV gp120 IgG circulating complex was present in the second patient, and this complex could be recovered from the renal biopsy eluate even though IgG was found only in the tubules and not in the single available glomerulus. In Patient 3, we demonstrated the presence of two circulating immune complexes, one composed of an IgG antibody complexed to HIV p24 antigen, and the other composed of an IgM anti-antibody complexed to the anti-HIV p24 IgG. Analysis of the renal biopsy eluate revealed the presence of only the IgG/HIV p24 complex. In the fourth patient an IgG/HIV p24 antigen complex could be demonstrated in the plasma, but only the HIV antigen and complement could be recovered from the renal biopsy eluate, even though the glomeruli in this biopsy demonstrated a typical pathological appearance consistent with immune complex-mediated glomerulonephritis. All patients' biopsies had evidence of microcystic tubular dilatation, and three of the four had tubular reticular structures noted on electron

microscopy. In addition, HIV-related antigens were detected in all four patients' urine and in glomerular cells in the eluted biopsy tissue. The latter finding was confirmed by our ability to demonstrate the presence of HIV gag genome in renal parenchyma in all four patients but not in renal tissue of other patients with renal disease in the absence of HIV infection.

The differences in the immunoglobulins detected in the elution and immunofluorescence studies in two of the patients may be due to differences in the sensitivities of these techniques. Analyses of eluates involve dissociation of deposited and *in situ* complexes, while immunofluorescence can only detect surface immune reactants, leaving others masked by steric hindrance [45].

The role of viruses in the pathogenesis of immune complex glomerulonephritis has been appreciated for some time. The several possible pathogenic mechanisms involved in viral induced glomerulonephritis have recently been reviewed [42]. The presence of circulating antigen-antibody complexes, consisting of HIV p24 bound to anti-p24 IgA antibody and anti-p24 IgG antibody, or HIV gp120 bound to anti-gp120 IgG antibody and elution of complexes, composed of the same material, from renal tissue from the first three patients in higher concentrations compared to plasma suggests that one mechanism which may be responsible for the renal disease in our patients is the deposition or trapping of such complexes. The increased detection of antibody and antigen over time (Fig. 6) and the results of enrichment studies, moreover, strengthen the likelihood that
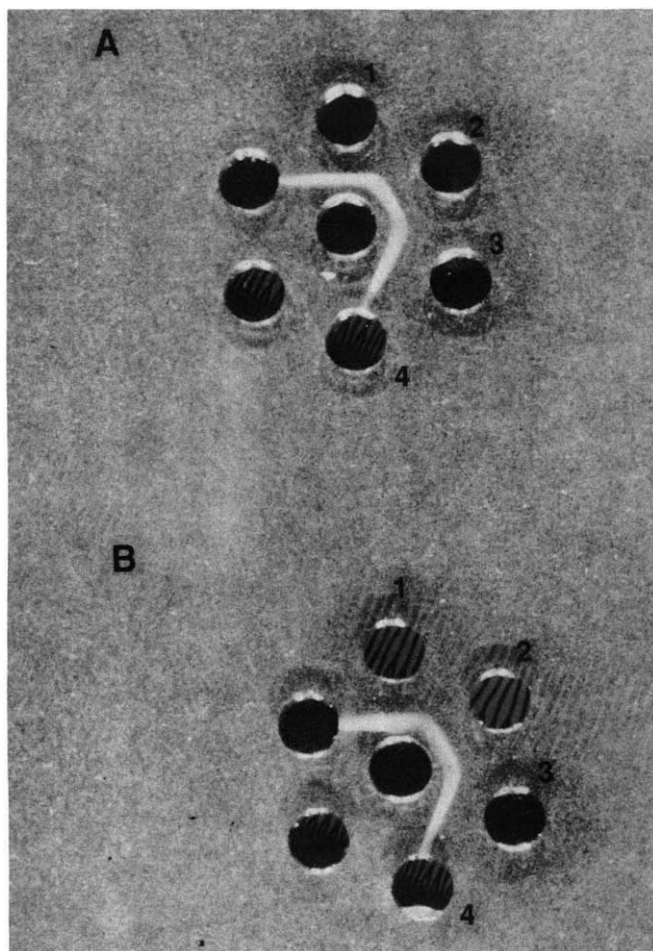
Fig. 7. *Immunodiffusion analysis of immune complex components isolated from the circulation and renal biopsy material.* **A.** Antibody from the CIC placed in the central well reacted with urinary antigen (well 1), CIC antigen (well 2), and antigen eluted from the renal biopsy tissue (well 3), but not with soluble kidney extract (well 4). The other two wells contain PBS. **B.** Antibody from the eluate reacts in an identical manner. Example taken from Patient 2.

this represents true elution of tissue deposited immune complexes. Our data highlight the association between the histological and immunological findings, but, although suggestive of causality, do not prove that immune complexes caused the disease.

Alternatively, such disease may be associated with *in situ* immune complex formation [42–44]. Cohen et al demonstrated proviral HIV DNA in tubular and glomerular epithelial cells in two of the four specimens of tissue from HIV infected patients with glomerulonephritis using *in vitro* DNA hybridization techniques [46]. They also found such markers in patients with AIDS without clinically obvious renal disease. However, they were only able to demonstrate the presence of either HIV core (p17, p24) or envelope (gp41, gp120) protein antigens in tubules in one of their four patients with immune complex glomerulonephritis, but were unable to demonstrate the presence of such antigens in the glomeruli. Although the reason(s) for this discrepancy is unclear, it is possible that the minute amounts of antigen present in the glomeruli were below the detection limits

of the assay system used by these investigators. Concentration of such substances in tubules or identification by specific elution techniques may enhance the sensitivity of such analyses. In our study, we were able to detect the presence of intraglomerular antigen only following a tenfold increase in the detection limits of our immunofluorescence technique by applying laser enhanced microscopy coupled to computer-assisted image analysis.

The role of cellular incorporation of HIV genome products in the development of renal disease is unknown. Preliminary evidence suggests HIV may infect renal tissue, specifically mesangial and endothelial cells, *in vitro* [47]) as well as epithelial cells [46]. It is possible that viral infection itself or alteration of cellular proteins might lead to subsequent attachment of circulating anti-HIV antibodies or complexes (in antibody excess) to an implanted or transformed antigen, presuming there are free combining sites available to the antibody [42]. We recently demonstrated the ubiquitous presence of HIV genome in glomerular and tubular tissue from HIV infected patients with and without renal disease [18]. The presence of HIV genome in renal cells, whether a result of infection of renal cells or as a consequence of its presence in infiltrating immune cells would seem to provide the necessary conditions for the implantation or expression of an antigen which might initiate an *in situ* mechanism of renal immune complex disease pathogenesis [42]. The nephritogenic antigen might be an HIV gene product in renal tissue or a transformed cell or cell protein. The data presented in this paper are consistent with the hypothesis that a combination of both pathogenic mechanisms may be involved in the development of renal disease in our patients.

Renal parenchymal viral infection itself, however, may be crucial to the pathogenesis of specific immune complex renal disease. The development of glomerulonephritis may be dependent on the renal parenchymal incorporation of human immunodeficiency viral antigens [16, 18, 46] or other viral agents [13, 48, 49], although renal cellular HIV infection alone may not be a sufficient condition for disease expression. Our recent studies suggest that a triggering mechanism may be critical to the expression of renal disease in an HIV-infected patient [18]. Hepatitis B virus X protein, for example, has been shown to transactivate HIV gene expression *in vitro* [48]. Renal cellular responses, therefore, particularly if altered by viral infection, may be important determinants of the expression of disease [2, 42, 50]. Our present finding of HIV antigens in glomerular cells in human biopsy tissue is consistent with pathological [46] and *in vitro* studies [47], and strengthens the causal association of HIV infection and glomerulonephritis. Alternatively, the finding of HIV gene products in renal tissue could simply be markers of HIV infection, or indicators of the severity or stage of infection. The pathogenic significance of a positive PCR still remains to be established.

Similarly, the role of genetic [51, 52], immunologic and other host responses [52] may be crucial in determining renal pathologic outcomes. It is of interest that a substantial proportion of the polyclonal immunoglobulin response in HIV infected patients is comprised of IgA, and that IgA-containing immune complexes are prevalent in patients with HIV infection and AIDS [31–40]. These immune responses may partially explain the increasing prevalence of IgA nephropathy seen in the HIV infected population [14–17, 51]. Likewise, gp120-anti-gp120
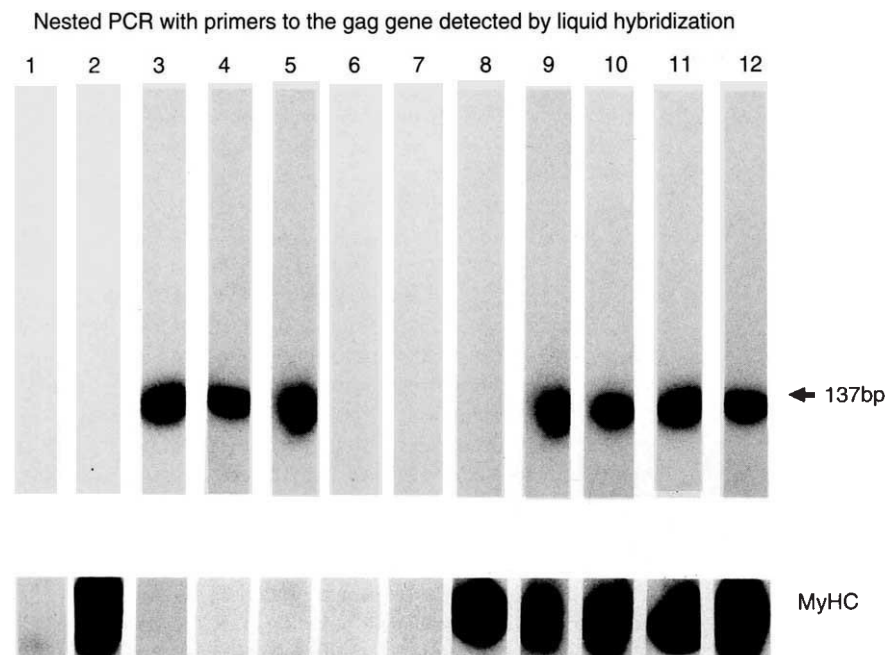
Nested PCR with primers to the gag gene detected by liquid hybridization

Fig. 8. *Amplification of HIV-1 gag gene and human cardiac myosin heavy chain gene (MyHC) by nested PCR.* Autoradiograms from liquid hybridization using SK19 for gag gene and MyHC30 for MyHC gene as probes. Size markers are restriction fragments of phage X 174 digested with HaeIII restriction endonuclease. bp: base pair length of the amplified fragment. Lane 1: reagent control (without DNA template) showing no amplification of HIV-1 gag gene or MyHC gene. Lane 2: sample containing DNA known to be negative for HIV-1 gag gene (negative control) showing amplification of MyHC gene but no amplification of HIV-1 gag target sequences. Lanes 3-7: Positive control: tenfold serial dilutions in decreasing concentrations (10^2 to 10^0 copies of a plasmid containing a 9 kb fragment of the HIV-1 genome). Dilutions from one hundred copies to one copy show amplification of HIV-1 gag gene in lanes 3 to 5; there was no amplification below 1 copy (lanes 6 and 7) of the HIV-1 gag gene or of the MyHC gene. Lane 8: DNA from renal biopsy tissue from an HIV negative patient with glomerulonephritis shows no amplification of the HIV-1 gag gene; there was amplification of the MyHC gene. Lane 9: DNA from renal biopsy tissue from Patient 1 shows amplification of HIV-1 gag gene and the MyHC gene. Lane 10: DNA from renal biopsy tissue from Patient 2 showing amplification of HIV-1 gag gene and MyHC gene. Lane 11: DNA from renal biopsy from Patient 3 showing amplification of HIV-1 gag gene and MyHC gene target sequences. Lane 12: DNA from renal biopsy from Patient 4 showing amplification of HIV-1 gag gene and MyHC gene target sequences.

antibody immune complexes have been shown to modulate immune cell function, perhaps contributing to nephrogenicity at the tissue level [53]. It is possible that certain specific immune responses, perhaps to specific modes of antigen presentation or specific CICs are more likely to provoke an ongoing renal inflammatory response. Additionally, the role of concurrent or intercurrent viral infection in affecting renal responses remains to be determined [2, 48, 49].

Finally, certain investigators have suggested that racial or ethnic background may be an important determinant of outcome in patients with renal disease in the presence of HIV infection [1–3, 52]. The work of Nochy et al [19] has been instructive in this regard. These workers demonstrated a relatively high prevalence of immune complex disease in both Black and White patients with HIV infection and renal disease. Although there was a 52% prevalence of glomerulonephritis in White patients, there was a substantial, 21% prevalence of glomerulonephritis in Black patients. The majority in this latter group were felt by the investigators to have immune-complex glomerulonephritis coexistent with focal glomerulosclerosis. Only one of these patients was an intravenous drug user. In contrast, six of the sixteen White patients with glomerulonephritis in their series used intravenous drugs. The majority were homosexual, in contrast to the Black patients. The HIV infected population treated at George Washington University

Hospital is roughly equally distributed between Black and White patients (52). The patients in the present series were all black, with homosexuality or bisexuality, or sexual transmission rather than drug use as a risk factor for the development of HIV infection. It may be that the type of antigen or its presentation or the presence of intravenous drug use, an independent risk factor for the development of focal glomerulosclerosis in the absence of HIV infection [54], may play critical roles in determining renal histologic outcomes in specific subgroups of patients with HIV infection who develop renal disease. The finding of advanced sclerosis in HIV-infected patients with glomerulonephritis may be a consequence of final common pathways leading to a fibrotic response, whether initiating factors originally incited an inflammatory response, or an increase in renal matrix production [54]. In HIV associated renal diseases, it is likely that cytokine mediated mechanisms [55, 56], associated with the common renal interstitial inflammatory cellular infiltrative response, or with renal or systemic HIV infection, may ultimately mediate fibrotic responses, regardless of the initial histologic pattern.

A substantial proportion of HIV-infected patients with nephrotic syndrome may have glomerulonephritis [18, 19]. Nochy et al [19] have emphasized that a relatively large proportion of patients may have coexistent focal glomerulosclerosis and immune complex disease. The paucity of reported cases of

glomerulonephritis in HIV infected patients may be a function of sample selection, since patients with asymptomatic urinary abnormalities, transient, resolved acute renal failure, or stable, advanced renal failure and nephrotic syndrome and HIV infection may not be biopsied as frequently as uninfected patients with similar clinical features, because of the reluctance to treat with immunosuppressive medications [13, 16]. The clinical presentation of three of our four patients was similar to that reported as typical for classic HIV-associated nephropathy. Renal biopsy is important in determining the histologic diagnosis in patients with HIV infection and renal disease [12, 13, 18, 19]. Patient 3 had a spontaneous improvement in renal function over two months. Patient 2 had a transient beneficial response to steroid therapy. Steroid therapy has also been used in another patient with HIV infection and renal disease with salutary effects [57]. The role of such therapy could be evaluated in specific controlled trials in HIV infected patients with renal disease with defined clinical and histologic parameters.

We have demonstrated renal disease associated with HIV immune complexes and incorporation of both HIV genomic material and core antigen in renal tissue. Circulating immune complex deposition and *in situ* mechanisms of immune-mediated renal disease may underlie the pathogenesis of glomerulonephritis in HIV-infected patients. The term "HIV nephropathy" should not be limited to a single differential diagnostic possibility, such as focal glomerulosclerosis, without clear evidence of its pathogenesis.

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