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Thrombotic Microangiopathy in the HIV-2-Infected Macaque

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Thrombotic microangiopathy (TMA) has been increasingly reported in human immunodeficiency virus (HIV)-infected humans over the past decade. The pathogenesis is unknown. We prospectively analyzed the renal pathology and function of 27 pigtailed macaques (*Macaca nemestrina*), infected intravenously with a virulent HIV-2 strain, HIV-2_{287}, in addition to that of four uninfected control macaques. Necropsies were performed between 12 hours and 28 days after infection. HIV-2 antigen was detectable in peripheral blood mononuclear cell (PBMC) cocultures in all animals after 10 days of HIV-2 infection; a rapid decline in $CD4^+$ PBMC (<350/μl) was seen in five of six animals 21 days and 28 days after infection. No macaque developed features of clinical AIDS. Typical lesions of human HIV-associated nephropathy were undetectable. Six of the 27 HIV-2-infected macaques demonstrated both histological TMA lesions (thrombi in glomerular capillary loops and small arteries, mesangiolysis) and ultrastructural lesions (mesangiolysis, subendothelial lucency, platelet thrombi in glomerular capillary lumina). Extrarenal thrombi were detected in the gastrointestinal and adrenal microvasculature of macaques that had developed renal TMA. None of the control animals demonstrated features of renal TMA at necropsy. In a retrospective analysis of kidneys obtained from 39 additional macaques infected with HIV-2_{287}, seven cases demonstrated TMA. *In situ* hybridization showed no detectable HIV-2 RNA in kidney sections of 65/66 HIV-2-infected macaques, including all 13 TMA cases. Expression of the chemokine receptor CXCR4, the putative coreceptor for HIV-2_{287}, was absent in intrinsic renal cells in all HIV-2-infected macaques. The HIV-2-infected macaque may be a useful model of human HIV-associated TMA. Our data do not support a role of direct HIV-2 infection of intrinsic renal cells as an underlying mechanism. *(Am J Pathol 1999, 155:649–661)*

Renal thrombotic microangiopathy (TMA) was first described in an AIDS patient by Boccia et al in 1984.[1] Subsequently TMA has been reported in several hundred HIV-infected humans worldwide and may be the second most common renal injury associated with HIV infection.[2–7] HIV-associated nephropathy (HIVAN), characterized by focal and segmental glomerulosclerosis (FSGS) and a collapsing glomerulopathy, has been identified as the single most common cause of chronic renal insufficiency in HIV-infected humans.[8–11] The TMA injury involves damage to the endothelial lining of glomerular capillaries and arterial microvessels, with activation of the plasma coagulation cascade, causing thromboses in these vessels.[12] Occlusive thrombi in the renal microvasculature, detachment of endothelial cells from the basement membrane, and mesangiolysis are typical findings in HIV-associated TMA. TMA presents clinically as hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP), depending on the presence of extrarenal manifestations, and patients may have features of thrombocytopenia, hemolytic anemia, and renal failure.[2,13]

The pathogenesis of HIV-associated TMA is poorly understood. A central question remaining is whether the virus directly infects intrinsic renal cells in the course of this disease.[2,14] To date, efforts to identify direct HIV infection of intrinsic renal cells in any disease process have been conflicting but generally have failed to provide convincing evidence of such infection. *In vitro* studies purportedly demonstrating such infection have used protocols in which viral transfection rather than infection seems to have occurred[15]; studies attempting direct infection of renal cells by viral isolates have generally failed to demonstrate productive infection.[16] *In vivo* studies of human renal biopsies of patients with HIVAN have provided some evidence of direct HIV infection,[17,18] but these studies are not convincing because of problems of reproducibility[19,20] or questions concerning possible contamination of studied renal cell populations by infiltrating infected leukocytes.[18]

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The situation concerning HIV infectivity is further complicated by limited knowledge concerning possible receptors for viral entry into specific renal cell types. Chemokine receptors have been identified as essential coreceptors for human immunodeficiency virus infection in mammalian cells.[21–23] The chemokine receptor CCR5 serves as the major coreceptor together with CD4 for macrophage-tropic strains of HIV-1,[24–28] whereas T lymphocyte-tropic strains of HIV-1 use the chemokine receptor CXCR4 as the principal coreceptor required for infection.[29,30] The expression of chemokine receptors in HIV-associated renal disease is unknown.

In this study, we report on a nonhuman primate model infected by a well-characterized strain of HIV-2, HIV-2_{287}.[31,32] Pigtailed macaques (*Macaca nemestrina*) infected by HIV-2_{287} predictably and rapidly develop clinical features characteristic of human HIV-1 infections. A significant proportion of these animals develop renal and systemic TMA that is morphologically similar to TMA as it occurs in humans. These lesions develop despite an apparent lack of HIV infection of renal parenchymal cells, although systemic infection of hematopoietic cells can be readily demonstrated. Additional studies to identify the presence of chemokine coreceptors needed to support cellular infection by HIV-1 in humans suggest the absence of these receptors on renal cells,[33,34] which may account for the difficulty in demonstrating the direct infection of such cells by strains of HIV-1. We believe the characterization of a model of HIV-associated TMA offers a major opportunity to understand this important disease process.

Study Design

Pigtailed macaque monkeys infected by HIV-2_{287}, recovered from macaque F89287 after a second passage of a human isolate HIV-2_{EHO}, develop a reliable and very reproducible immunodeficiency syndrome with many features of human HIV disease.

We prospectively analyzed the renal pathology and function of 27 pigtailed macaques, experimentally infected intravenously with a virulent HIV-2 strain, HIV-2_{287}. Necropsies were performed at 12 hours and at days 1, 2, 4, 6, 10, 14, 21, and 28 (n = 3, each time point) after infection. Renal pathology was evaluated at necropsy by light and electron microscopy. Renal function was assessed at necropsy by measurement of serum-creatinine, urea, and urinary protein excretion. Renal pathology and function were examined prospectively in four uninfected control macaques matched for age and sex. Furthermore, kidney tissues obtained from 39 pigtailed macaques experimentally infected with HIV-2_{287} that were involved in viral dissemination studies were analyzed retrospectively. HIV-2 RNA was detected in kidney tissues by an *in situ* hybridization technique. The expression of the HIV infection coreceptor CXCR4 in kidney tissues was analyzed by *in situ* hybridization.

Materials and Methods

Animals

Twenty-seven pigtailed macaques, *Macaca nemestrina* (ages 1.2–3.1 years, median 2.1 years; 11 male, 16 female) were inoculated intravenously with 50 $TCID_{50}$ (50% tissue culture infectious doses) HIV-2_{287}. All macaques involved in this study were colony-born and weighed between 2.1 and 4.5 kg (median 3.0 kg) at necropsy. Before inoculation, each animal was determined to be clinically healthy by physical examination and complete blood cell count. All animals in this study were screened and determined to be negative for simian immunodeficiency virus (SIV) and simian retrovirus (SRV) coinfection before HIV-2 inoculation. This was determined by antibody, cell culture, and polymerase chain reaction (PCR) assays. The macaques were euthanized at 12 hours and on day 1, 2, 4, 6, 10, 14, 21, 28 (n = 3, each time point) after inoculation. The animals were sedated with an intramuscular injection of ketamine-HCl (10 mg/kg body weight) followed by an intravenous overdose of pentabarbital. Complete necropsy examinations were performed on all macaques. All study protocols and procedures were reviewed and approved by the Washington Regional Primate Research Center and the University of Washington Animal Care and Use Committee.

Four uninfected, untreated, clinically healthy pigtailed macaques, *M. nemestrina* (ages 1.4–2.6 years; three male, one female) were euthanized and served as control animals.

Kidney tissue specimens were obtained from 39 additional macaques experimentally infected by HIV-2_{287}. These animals were involved in previous studies investigating HIV-2 dissemination kinetics and pathogenesis. Protocols of these studies have been published previously.[31,32] All 39 macaques (ages 0.3–6.4 years, median 1.9 years; 16 male, 23 female) were colony-born and weighed between 0.4 and 9.3 kg (median 2.9 kg) at necropsy. The macaques were euthanized between 63 and 556 days (median 165 days) after HIV-2 infection.

Virus

HIV-2_{287} was derived by serial passage of HIV-2_{EHO} (originally obtained from Dr. Luc Montagnier, Pasteur Institute) in *M. nemestrina*. Animal F87265 was inoculated intravenously with 10^6 TCID (tissue culture infective dose) cell-free HIV-2_{EHO} as well as 10^7 autologous peripheral blood mononuclear cells (PBMCs) infected *in vitro*. Ten milliliters of whole blood obtained from macaque F87265 44 weeks after infection was transfused into animal F89071, and then 10 ml of blood from animal F89071 at 20 weeks after infection was inoculated into F89287. The HIV-2_{287} challenge stock was derived from coculture of lymph node mononuclear cells of animal F89287 with fresh stimulated allogenic macaque PBMCs. Virus stocks were prepared as clarified supernatants (3000 × g for 20 minutes at 4°C) and aliquoted and stored at −80°C until use. In the prospective part of this study, all 27 macaques

were inoculated intravenously with 50 $TCID_{50}$ of the virus stock solution.

Detection of HIV-2-Infected PBMC

HIV-2 infected PBMC were detected by a quantitative coculture assay as described.[31,35] Briefly, freshly isolated macaque PBMCs or lymphocytes were serially diluted in triplets, starting with 10^6 cells, and cocultivated with fresh human CD8-positive T-cell-depleted phytohemagglutinin-activated PBMCs. Cultures were incubated for 14 days, and the presence of virus was detected using an HIV-2 p27 antigen capture assay. Titers were calculated as the maximum dilution of cells that gave positive cultures and reported as numbers of positive cells/10^6 PBMCs.

Hematological and Serological Parameters

Samples of serum, EDTA-plasma, and urine were obtained from each macaque before experimental euthanasia. Complete blood count, serum-creatinine, urea, and urinary protein excretion were measured using standard procedures. The CD4-positive T-lymphocyte subset was measured as described previously.[36] Briefly, lymphocytes were stained with PE-conjugated anti-CD4 antibody (LEU 3a; Becton-Dickinson, San Jose, CA) and analyzed with a flow cytometer (FACScan/FACSort; Becton-Dickinson).

Histopathology

Tissue samples of kidney, liver, lung, brain, adrenal gland, skin, gut, and lymph nodes were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and sectioned. Sections of kidney tissue were routinely stained with hematoxylin and eosin, periodic acid-Schiff (PAS), and Jones's silver methenamine reagents, respectively. Tissue sections of all other organs were routinely stained for hematoxylin and eosin, PAS, and elastic van Gieson, respectively.

Electron Microscopy

Tissue for electron microscopy was fixed in half-strength Karnovsky's solution (1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.0). After fixation, tissue was postfixed in 1% osmium tetroxide for 2 hours, dehydrated in graded ethanols, and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and examined with a Phillips 410 electron microscope (Phillips Export BV, Eindhoven, the Netherlands). Kidney tissue examination included at least two and usually three or more glomeruli per animal, as well as a survey of cortical and medullary interstitium, tubules, and blood vessels.

Molecular Probes

HIV-2

A 1.7-kb sequence of DNA coding for HIV-2 gp120 (*env*) (nucleotides 6480–8335 of the $HIV\text{-}2_{287}$ sequence) was subcloned into pCR II (Invitrogen) (kindly provided by Bristol-Myers Squibb, Seattle, WA), linearized with *Xho*I, and transcribed with Sp6 RNA polymerase for the antisense probe, or linearized with *Bam*HI and transcribed with T7 RNA polymerase for the sense probe. Detailed protocols for the transcription reaction and the characterization of the specificity of this riboprobe have been described previously.[33]

CXCR4

A 1.1-kb sequence of DNA coding for human CXCR4 was subcloned into pcDNAI/amp (Invitrogen) (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, originally provided by Dr. Nathaniel Landau),[24,37] linearized with *Hin*dIII, transcribed with Sp6 for the antisense probe or linearized with *Xba*I, and transcribed with T7 for the sense probe. Specificity of the CXCR4 antisense riboprobe has been demonstrated previously by Northern analysis and by *in situ* hybridization using a series of cell lines transfected with different members of the family of chemokine receptors.[34]

In Situ *Hybridization*

HIV-2 RNA and CXCR4 mRNA were detected in tissue sections with *in situ* hybridization techniques following protocols that we have previously used.[33,38,39] Four-micron sections of formalin-fixed, paraffin-embedded tissue samples were rehydrated through xylene and graded ethanols, washed with 0.5× standard saline citrate (SSC) (1× SSC = 150 mM NaCl, 15 mM Na citrate, pH 7.0), and digested with proteinase K (5 μg/ml; Sigma) in Tris buffer for 30 minutes at 37°C. Several 0.5× SSC washes were followed by prehybridization for 2 hours in 100 μl of prehybridization buffer (0.3 mol/L NaCl, 20 mmol/L Tris (pH 8.0), 5 mmol/L EDTA, 1× Denhardt's solution, 10% dextran sulfate, 10 mmol/L dithiothreitol). The hybridizations were started by adding 500,000 cpm of ^{35}S-labeled riboprobe in 50 μl of prehybridization buffer and allowed to proceed overnight at 50°C. After hybridization, sections were washed with 0.5× SSC, and treated with RNase A (20 μg/ml, 30 minutes at 37°C), washed in 2× SSC (2 × 2 minutes), followed by three high-stringency washes in 0.1× SSC/0.5% Tween 20 (Sigma) for 40 minutes each at 50°C and several 2× SSC washes. After the tissue was dehydrated and air-dried, it was dipped in NTB2 nuclear emulsion (Kodak, Rochester, NY) and exposed in the dark at 4°C for 2 weeks (HIV-2) or 4 weeks (CXCR4). After developing, the sections were counterstained with hematoxylin and eosin, dehydrated, and coverslipped.

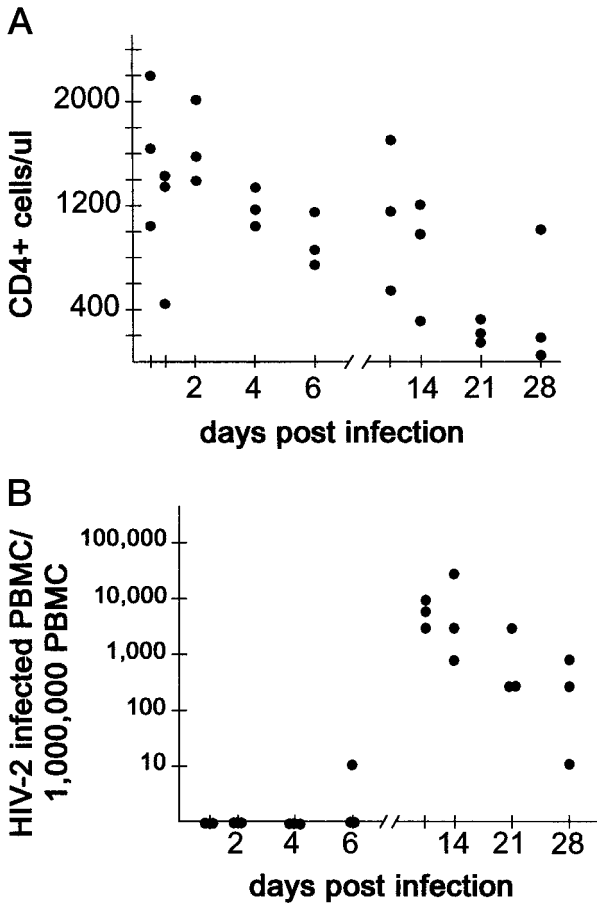

Figure 1. HIV-2-infected macaques develop an immune deficiency syndrome. HIV-2_{287} infection was associated with a rapid decline in CD4-positive PBMC (**A**) and high virus load (**B**). Necropsy blood samples were analyzed for CD4-positive cells (**A**) and number of infected cells/10^6 PBMC (**B**) (for details see Materials and Methods). Graphics illustrate the individual data of each animal at necropsy.

Results

HIV-2 Infection Induces an Immunodeficiency Syndrome in Pigtailed Macaques

Infection with HIV-2_{287} in this animal model results in a rapid and predictable decline in CD4-positive cells in the peripheral blood lymphocyte population. Blood samples obtained at necropsy demonstrated a decrease in CD4-positive cells below 350 cells/μl in five of six animals 21 days and 28 days after infection (Figure 1A). HIV-2-infected PBMCs were detectable in PBMC cocultures in 12/12 animals after 10 days of infection (Figure 1B). None of the 27 macaques involved in this study developed other features of clinical AIDS.

HIV-2-Infected Macaques Develop Renal TMA

Pathological examinations of renal tissue from the 27 macaques acutely infected with HIV-2 revealed TMA lesions in six animals. Renal TMA was detectable in two out of three animals as early as 12 hours after HIV-2 infection, and four out of nine macaques had developed TMA after 14 days of infection. None of the macaques euthanized between 1 and 10 days after HIV-2 infection demonstrated features of TMA at necropsy. Table 1 summarizes the histological and ultrastructural features of all 27 macaques.

By light microscopy five macaques showed the presence of thrombotic microangiopathy. The histological diagnosis was based on the presence of discrete thrombi within glomerular capillary loops and small arterioles and the presence of mesangiolysis (Figure 2, A and B). These lesions were identical to those observed in human HIV-associated TMA (Figure 2C). Whereas four animals had focal TMA lesions involving approximately 10–25% of the glomeruli available for examination, one animal showed a very global pattern of TMA involving more than 60% of the glomeruli. As indicated in Table 1, several macaques revealed variable degrees of interstitial inflammation. These lesions characteristically consisted of discrete foci of accumulations of mononuclear inflammatory cells, principally lymphocytes and macrophages. Tubular atrophy in these areas was typically minimal to mild. Areas of inflammation generally comprised less than 5% of the renal parenchyma examined histologically in each affected animal.

Inflammatory cell infiltrates were generally absent at sites of TMA lesions. Typical pathological characteristics of human HIVAN were undetectable in all 27 macaques. Specifically, we did not identify focal and segmental glomerulosclerosis (FSGS), hyperplasia or proliferation of visceral epithelial cells, or features of tubulointerstitial injury other than the variable, mild interstitial inflammation in some of the HIV-2-infected animals.

Ultrastructural examination confirmed the diagnosis of TMA in all five cases. A frequent ultrastructural finding in the glomeruli of all animals with histological appearance of TMA was areas of mesangiolysis with accumulation of electron-lucent material admixed with cellular debris (Figure 3A). Another frequent finding in some glomeruli of all TMA cases was detachment of endothelial cells from the glomerular basement membranes, with subendothelial accumulation of electron-lucent material (Figure 3B). All animals with the histological TMA diagnosis showed variable degrees of intracapillary thrombi composed of amorphous material, platelets, cellular debris, and, in some cases, fibrin (Figure 3C). Visceral epithelial cells were generally well preserved and did not display features of retraction and/or prominent effacement of foot processes. However, at sites of severe injury disruption of the podocyte network was identified (Figure 3C). Immune complex-type electron-dense deposits were undetectable in all observed glomeruli. Electron microscopic examination of renal tissue was performed in two additional cases that showed no significant pathological abnormalities by light microscopy but revealed mild proteinuria at necropsy (Table 1 and Figure 5B). One animal (K95267, Table 1) had clear features of TMA ultrastructurally, including mesangiolysis, endothelial detachment from basement membranes with accumulation of subendothelial electron-lucent material, and platelet thrombi in glomerular capillary loops. However, the second proteinuric macaque showed neither histological nor ultrastructural

Table 1. Pathologic Features in Macaques Acutely Infected with HIV-2 and in Uninfected Control Macaques

Time p.i.	Animal	Glomerular and vascular histology	Renal interstitial inflammation	Electron microscopy	Extrarenal TMA
HIV-2 infected					
12 hours	K95267	nspa	Very focal	TMA	No
	J95248	nspa	Very focal	n.d.	No
	K96161	TMA (focal)	Mild, focal	TMA	Gut, periadrenal
1 day	J96226	nspa	No	n.d.	No
	K94386	nspa	Mild, focal	n.d.	No
	J96064	nspa	No	n.d.	No
2 days	J96279	nspa	No	n.d.	No
	J95271	nspa	Very focal	n.d.	No
	J94456	nspa	No	n.d.	No
4 days	J95314	nspa	No	n.d.	No
	J95227	nspa	Focal	n.d.	No
	J95263	nspa	No	n.d.	No
6 days	F95276	nspa	No	n.d.	No
	K95280	nspa	No	n.d.	No
	F95265	nspa	No	n.d.	No
10 days	J96140	nspa	Mild, focal	n.d.	No
	J96173	nspa	No	n.d.	No
	M96111	nspa	No	n.d.	No
14 days	F94434	Focal mesangiolysis	Mild, focal	TMA	Periadrenal
	T96156	nspa	Very focal	n.d.	No
	K96183	nspa	Focal	n.d.	No
21 days	F95269	TMA (global)	No	TMA	Gut, adrenal
	J96174	nspa	No	nspa	No
	M96162	nspa*	No	nspa*	No
28 days	K95061	TMA (focal)	No	TMA	Gut, periadrenal
	J95284	TMA (focal)	No	TMA	Gut
	T96194	nspa	No	n.d.	No
Uninfected					
	J96083	nspa	Very focal	nspa	n.d.
	M95311	nspa	No	nspa	n.d.
	J96086	nspa	Mild, focal	nspa*	n.d.
	F96092	nspa	Very focal	nspa*	n.d.

*See text.
nspa, No specific pathologic abnormalities; n.d., not defined; p.i., postinfection.

abnormalities. Visceral epithelial cells and foot processes were well preserved in both cases.

One further case (M96162, Table 1) was indeterminate for TMA. This animal lacked unequivocal microthrombi on histological and ultrastructural examination, but had some features that were suggestive of such a process, including the histological appearance of congested, ischemic glomeruli and the ultrastructural presence of a thrombus in one small artery.

None of the four uninfected control macaques demonstrated histological or ultrastructural features of renal TMA (Table 1). Histological examination revealed variable degrees of interstitial inflammation (focal accumulation of mononuclear inflammatory cells) in three of four uninfected control macaques. Ultrastructural examination did not detect features of mesangiolysis, endothelial cell injury, or thrombi in glomerular capillary lumina in any of the four uninfected control animals. One animal (J96086, Table 1) demonstrated ultrastructural features of a glomerular basement abnormality. Thickening and lamination of the basement membrane similar to those seen in human hereditary nephritis/Alport's syndrome were present without any additional identifiable abnormalities. Immune complex-type electron-dense deposits were undetectable. One animal (F96092, Table 1) demonstrated predominantely mesangial immune complex-type electron-dense deposits with focal small accumulations. Similar deposits were occasionally present in subendothelial portions of peripheral capillary walls and rarely present in intramembranous and subepithelial locations. No additional identifiable ultrastructural abnormalities were present. Endothelial cells, mesangial cells, and visceral epithelial cells were well preserved in all cases. Microvascular thrombi remained undetectable.

TMA Is a Systemic Disease in HIV-2-Infected Macaques

We analyzed tissue sections of liver, lung, brain, adrenal gland, skin, and gut (including stomach, duodenum, ileum, jejunum, and colon) for the presence of microthrombi in three animals with diagnosed renal TMA. Focal microthrombi were detected in the gastrointestinal submucosa, the adrenal gland, and in the periadrenal connective tissue in these animals that had developed renal TMA. Microthrombi were undetectable in all other organs. We subsequently analyzed tissue sections of gut and adrenal (n = 4 tissue sections per animal) of all 27 macaques acutely infected with HIV-2. The analysis was performed on PAS-stained tissue sections by an observer unaware of the presence of renal TMA in these animals.

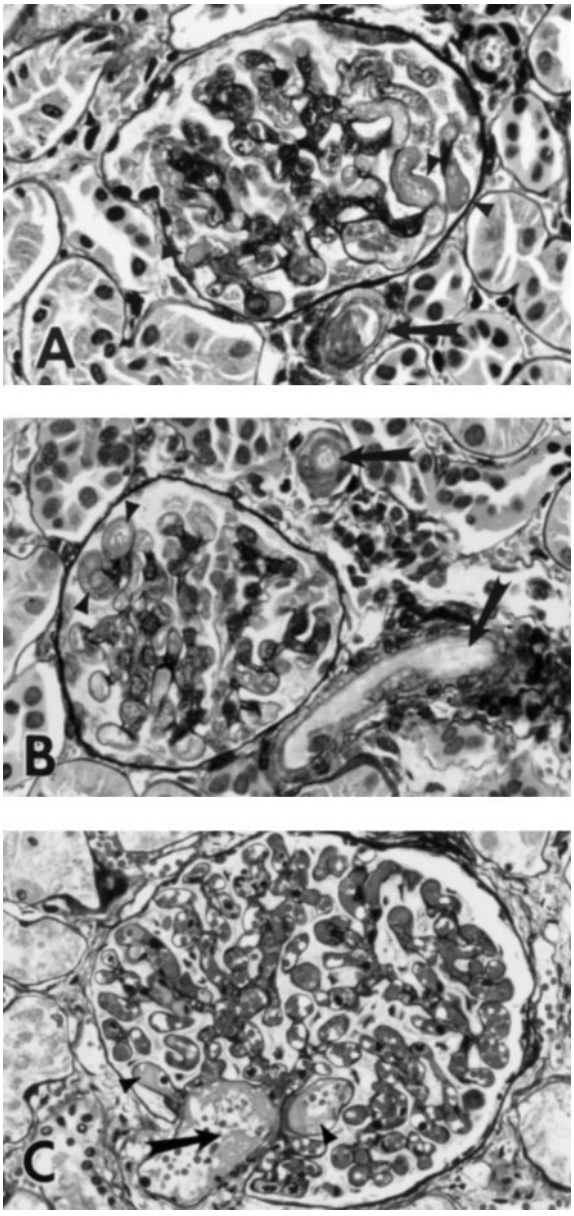

Figure 2. Histological appearance of renal TMA. A and B: Characteristic lesions of TMA in HIV-2-infected macaques included discrete thrombi within glomerular capillary loops (arrowheads) and small arterioles (arrows). The tubulointerstitium showed no specific pathological abnormalities. Inflammatory cell infiltrates were generally absent at sites of TMA lesions. C: Kidney from an HIV-infected human patient dying of TMA demonstrates identical lesions of thrombi in hilar arterioles (arrow) and glomerular capillaries (arrowhead). Silver methenamine staining. Original magnification A–C, ×400.

Extrarenal thrombi were identified in five of 27 macaques (Figure 4, Table 1). Four animals had developed focal microthrombi in the gastrointestinal submucosa, three in the periadrenal connective tissue, and one in the adrenal medulla. The lesions were generally focal, involving less than 5% of the analyzed tissue, and inflammatory cell infiltrates were absent at sites of the thrombi. All five animals with extrarenal thrombi had diagnosed renal TMA lesions. Extrarenal microthrombi were undetectable in all animals that had not developed renal TMA.

HIV-2-Infected Macaques Show No Clinical Manifestation of Renal TMA

None of the 27 macaques that had been infected with HIV-2_{287} had serological evidence of renal failure at necropsy. Figure 5 illustrates the renal functional parameters of the six macaques acutely infected with HIV-2 that had developed renal TMA. At necropsy all six animals were clinically healthy and indistinguishable from the animals without TMA. Serum BUN (Figure 5A) and creatinine levels (data not shown) showed no significant difference in HIV-2-infected macaques that had developed TMA compared to HIV-2-infected macaques without TMA or uninfected, normal control macaques (Figure 5A). However, three animals had developed a mild proteinuria at necropsy (74–111 mg protein/dl; Figure 5B). Whereas two of these three HIV-2-infected macaques demonstrated the presence of renal TMA, the third proteinuric macaque (J96174, Table 1) showed no pathological abnormalities by either histological or ultrastructural examination.

Comparison of the hematological parameters in the HIV-2-infected macaques that had developed TMA with HIV-2-infected macaques that had not developed TMA showed no significant correlation between the incidence of TMA and the red blood cell count (Figure 6A), platelet count (Figure 6B), white blood cell count (data not shown), or CD4 cell count (data not shown), respectively. None of the 27 HIV-2-infected animals had evidence of thrombocytopenia at the time of necropsy (Figure 6B).

The four uninfected control macaques were clinically healthy at necropsy. None of the control animals demonstrated renal functional alterations at the time of necropsy (Figure 5).

Retrospective Analysis of Kidneys from HIV-2-Infected Macaques

In the second component of this study, we retrospectively examined renal tissue samples obtained from 39 additional macaques that had been infected with HIV-2_{287}. Seven of these 39 animals demonstrated typical histological and ultrastructural features of renal TMA lesions. Table 2 summarizes the clinical and hematological data of the seven macaques that had developed renal TMA. All seven macaques had a focal distribution pattern of the TMA lesions involving 10–25% of the glomeruli. As in the macaques acutely infected with HIV-2, lesions characteristic of human HIVAN remained undetectable. Tubulointerstitial inflammatory infiltrates were detectable in some specimens, but these lesions were generally mild, were of focal distribution, and were not associated with the TMA lesions of affected glomeruli or blood vessels.

In this group one animal had histological features of a focal crescentic glomerulonephritis and a focal vasculitis. No lesions characteristic of TMA were identifiable in this animal. The tubulointerstitium showed no pathological abnormalities. By electron microscopy crescentic lesions were undetectable in several analyzed tissue samples obtained from this animal. Ultrastructural analysis showed an increased number of circulating mononuclear cells

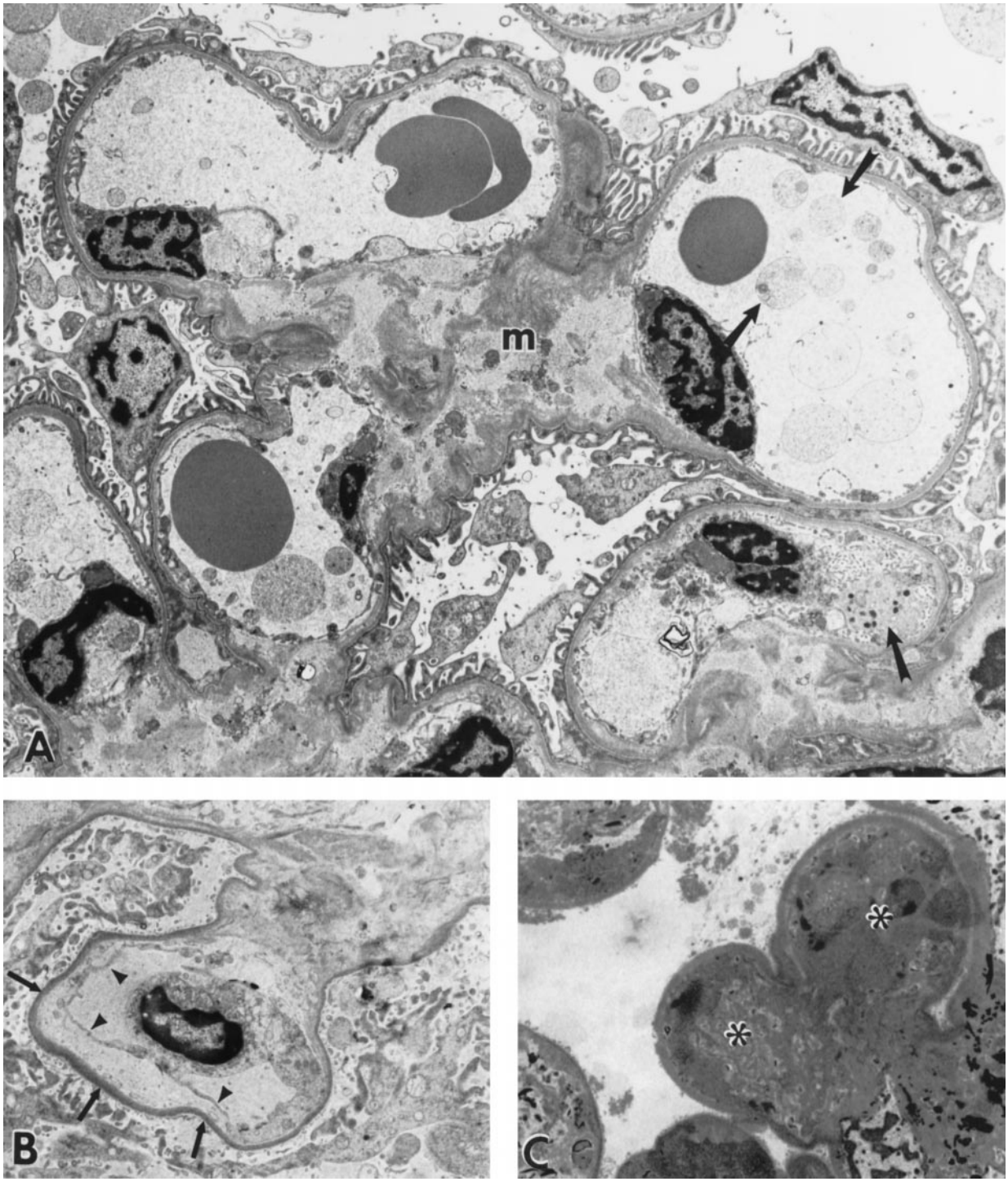

Figure 3. Ultrastructural appearance of renal TMA. A: Frequent ultrastructural findings were areas of mesangiolysis (m) with accumulation of electron-lucent material mixed with cellular debris. Several platelets (arrows), many of which are degranulated, can be seen within the capillary loops. B: Detachment of the endothelial cell (arrowheads) from the basement membrane (arrows), with subendothelial accumulation of electron-lucent material. C: All animals with the histological TMA diagnosis showed variable degrees of intracapillary thrombi (*) composed of amorphous material, platelets, cellular debris, and, in some cases, fibrin. Original magnification A–C, ×1600.

within glomerular capillary lumina. Glomerular endothelial cells, visceral epithelial cells, and mesangial cells demonstrated normal morphological features. Immune complex-type electron-dense deposits were absent in all analyzed glomeruli of this case.

Serum and urine samples obtained at necropsy were additionally available in three of seven macaques with renal TMA in the retrospectively analyzed group. No decrease in renal function or elevation of urinary protein excretion was demonstrable in these three animals.

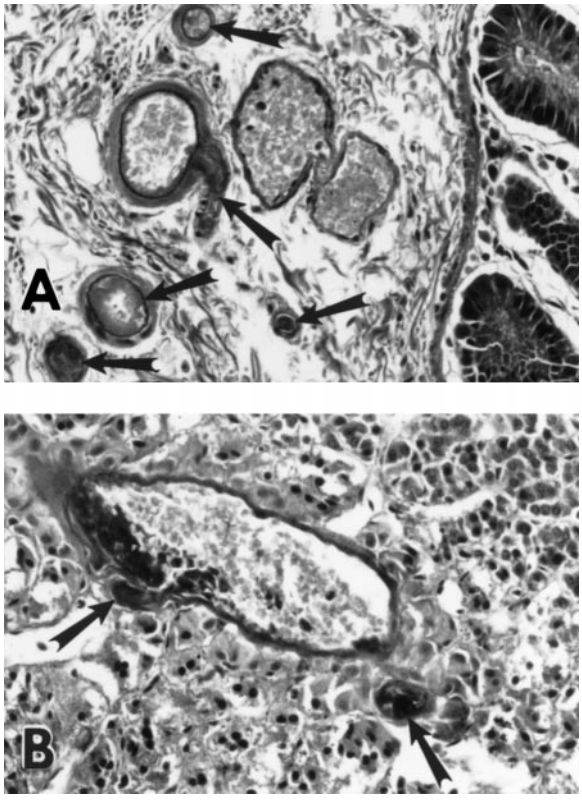

Figure 4. TMA is a systemic disease in HIV-2-infected macaques. A and B: Different cases. Focal microthrombi (arrows) were detected in the gastrointestinal submucosa (A), in the adrenal medulla (B), and in the periadrenal connective tissue (data not shown) in some animals that had developed renal lesions. Elastic van Gieson staining. Original magnification A and B, ×400.

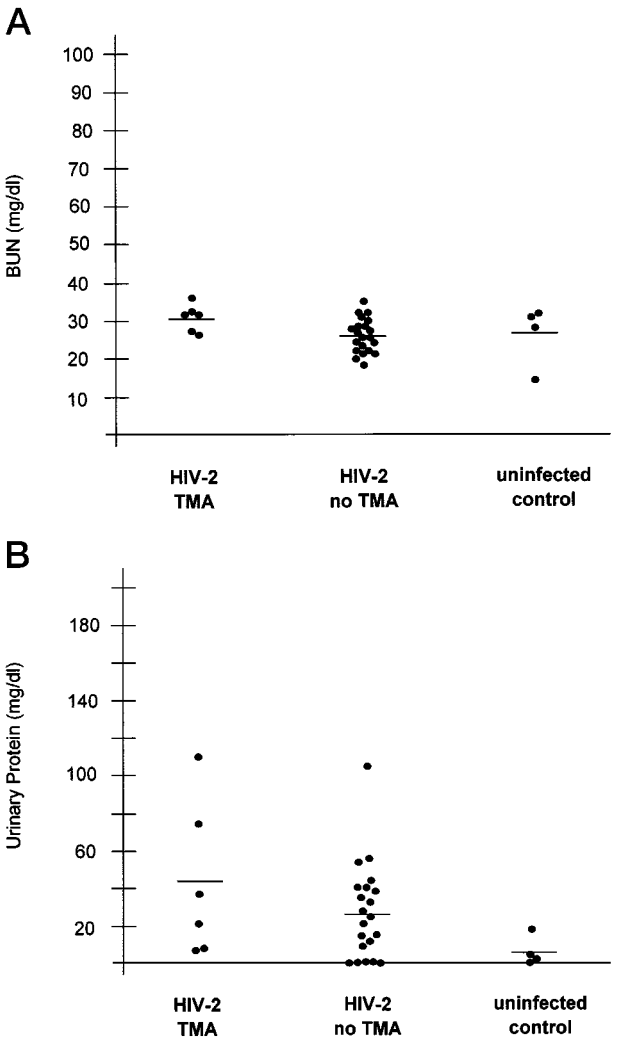

Figure 5. Renal functional parameters in HIV-2-infected and uninfected macaques. Serum and urine samples were obtained immediately before euthanasia. Blood urea nitrogen (BUN) (A) and urinary protein excretion (B) were measured using standard procedures. The graphic illustrates the individual data of each animal; the bar represents the mean value. No significant differences in renal functional parameters were demostrable between the different animal groups. HIV-2 TMA: HIV-2-infected macaques that had developed TMA ($n = 6$); HIV-2 no TMA: HIV-2-infected macaques that had no detectable TMA lesions at necropsy ($n = 21$); uninfected control: normal, uninfected macaques, matched for age and sex ($n = 4$).

HIV-2 RNA Is Undetectable in Kidneys of HIV-2-Infected Macaques

Direct infection of renal parenchymal cells has been implicated in pathogenetic mechanisms underlying HIV-associated renal disease. We analyzed sections of renal tissue samples from all 66 HIV-2-infected macaques for the presence of HIV-2 RNA by *in situ* hybridization. HIV-2 RNA was completely absent in kidney tissue sections of 65/66 HIV-2-infected macaques. All 13 macaques that had developed renal TMA lesions showed no detectable HIV-2 RNA by this technique (Figure 7C). In one animal acutely infected with HIV-2 (K96183, Table 1), a few HIV-2-infected cells were demonstrable (Figure 7D). HIV-2 RNA expression in this animal was localized to infiltrating mononuclear leukocytes at sites of mild, focal tubulointerstitial inflammation (Figure 7D). HIV-2 RNA expression was completely absent in renal parenchymal cells of the glomerular, tubular, or vascular compartments.

By the same technical approach, HIV-2-infected cells were identified in tissue samples of lymphoid organs (lymph nodes, spleen) of HIV-2-infected macaques. Figure 7A illustrates several HIV-2 RNA-expressing mononuclear cells within a mesenteric lymph node obtained from a macaque 10 days after intravenous HIV-2 infection. HIV-2-infected cells were detected in lymph node tissue samples of all macaques after 10 days of HIV-2 infection (Eitner et al, manuscript in preparation).

Chemokine Receptor CXCR4 Expression in Kidneys of HIV-2-Infected Macaques

Chemokine receptors have been identified as coreceptors for HIV infection in mammalian cells. Our preliminary results indicate that HIV-2_{287} preferentially utilizes the chemokine receptor CXCR4, rather than CCR5, as coreceptor (M.-A. Rey-Cuille and S.-L. Hu, personal observations). We studied the cellular sites of synthesis of CXCR4 in kidneys of all 27 macaques acutely infected with HIV-2_{287}, in the seven additional macaques that had developed TMA, and in four uninfected normal macaques. The

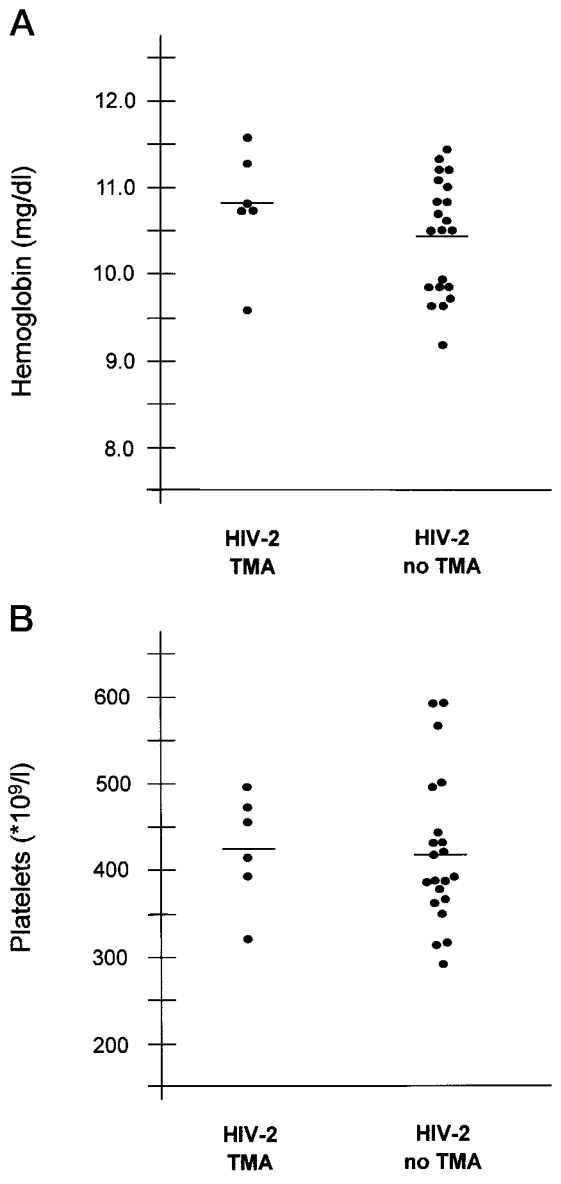

Figure 6. Hematological parameters in HIV-2-infected macaques. EDTA-plasma samples were obtained immediately before euthanasia. Hemoglobin (**A**) and platelet counts (**B**) were measured using standard procedures. The graphic illustrates the individual data of each animal; the bar represents the mean value. No significant differences in red blood cell counts or platelet counts were demostrable between the different animal groups. HIV-2 TMA: HIV-2-infected macaques that had developed TMA ($n = 6$); HIV-2 no TMA: HIV-2-infected macaques that had no detectable TMA lesions at necropsy ($n = 21$).

CXCR4 antisense probe used in this study was originally generated against human CXCR4. We were able to show that this riboprobe also detects nonhuman primate CXCR4. *In situ* hybridization demonstrated the presence of CXCR4-expressing cells in lymph node tissue from HIV-2-infected macaques (Figure 8A) and from uninfected macaques (data not shown). Numerous CXCR4 mRNA-expressing lymphocytes were detectable in the macaque lymph nodes (Figure 8A). Several of the analyzed kidneys showed the presence of a few CXCR4 mRNA-expressing cells by *in situ* hybridization. Those cells appeared to be leukocytes and were localized in the interstitium at sites of focal mononuclear cell infiltration (Figure 8D). By *in situ* hybridization CXCR4 mRNA expression was completely absent in intrinsic renal cells of the glomerular, tubular, interstitial, or vascular compartments (Figure 8C). Endothelial cells of the renal vasculature showed no detectable CXCR4 mRNA hybridization signal. Chemokine receptor CXCR4 expression was undetectable at sites of TMA lesions in these animals.

Discussion

TMA has frequently been reported in HIV-infected humans and is now considered to be the second most common renal injury in patients with HIV infection.[2–7] HIV-associated TMA lesions are characterized by occlusive thrombi in glomerular capillaries and small arteries, with no evidence of immune complex deposition as an underlying etiology.[40] We are the first to report the presence of renal TMA lesions in an animal model of HIV infection. Our findings indicate the potential utility of the HIV-2-infected macaque as a model for human HIV-associated TMA.

We recognize that the best animal model for human HIV infections would be one in which HIV-1 infects and induces an AIDS-like disease. Previous investigation has shown that chimpanzees are susceptible to infection with HIV-1, but to date only one animal has progressed to clinical AIDS.[41] In addition, availability, financial considerations, and the protected status of chimpanzees as an endangered species make the chimpanzee an unsuitable candidate for extensive animal model development. Similarly, although pigtailed macaques (*M. nemestrina*) are susceptible to HIV-1 infection, the infection appears to be of limited duration, with low virus levels and re-

Table 2. Thrombotic Microangiopathy (Seven Cases) in a Series of 39 HIV-2-Infected Macaques

	Clinical data					Hematology at necropsy			
Animal	Age (years)	Sex	Time p.i. (days)	Dose ($TCID_{50}$)	Route	Hb (mg/dl)	WBC (*10^9/L)	Platelets (*10^9/L)	CD4+ (*10^6/L)
1	1.3	f	108	10	iv	6.5	2.2	302	87
2	1.4	f	141	0.1	iv	13.7	8.9	661	2342
3	4.5	m	307	100	iv	13.3	9.8	229	23
4	1.3	f	161	1	iv	12.4	8.9	405	262
5	1.2	f	167	1	iv	12.5	4.1	410	1706
6	4.1	f	134	100	iv	11.3	12.1	525	88
7	2.2	f	111	100	vag	12.1	7.9	393	1872

p.i., postinfection; $TCID_{50}$, 50% tissue culture infectious dose; iv, intravenous; vag, vaginal.

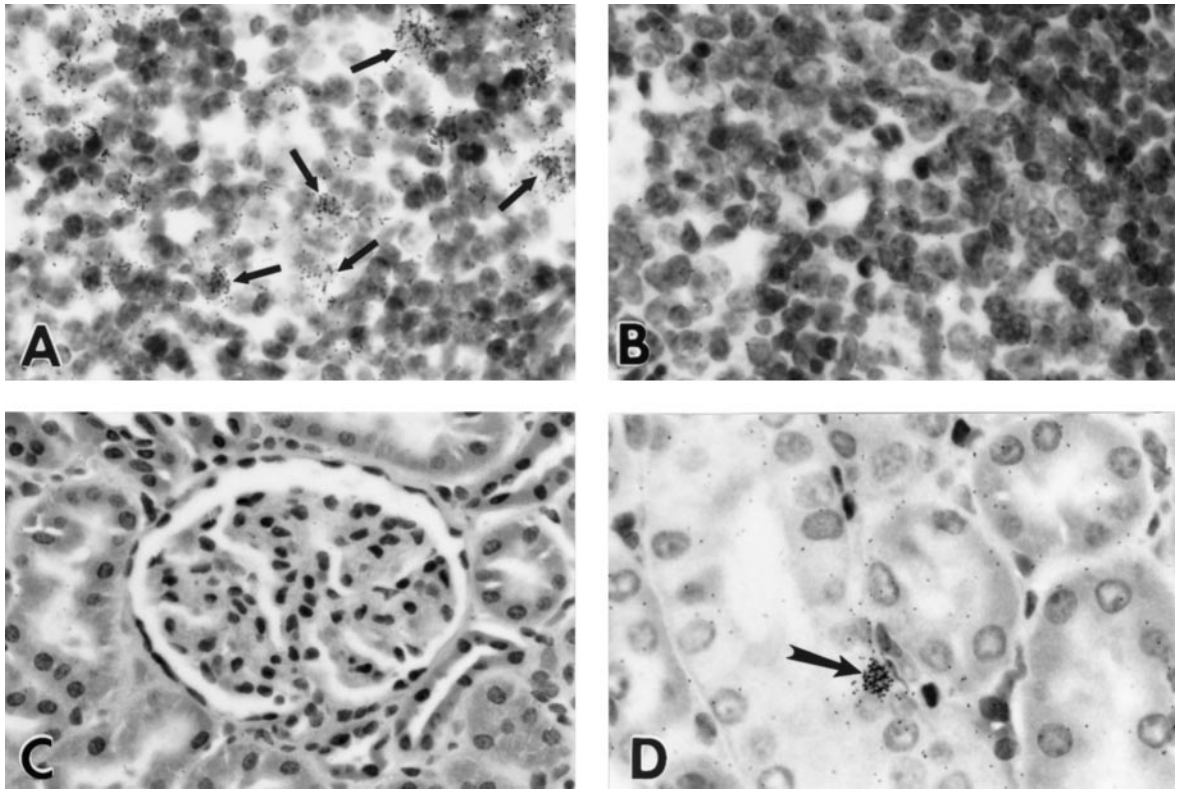

Figure 7. *In situ* hybridization for HIV-2 RNA expression in tissue sections. A and B: Mesenteric lymph node obtained from a macaque 10 days after intravenous HIV-2 infection. A: *In situ* hybridization for HIV-2_{287} gp120 RNA detected numerous HIV-2-infected cells in a section of this mesenteric lymph node. Examples of HIV-2-infected cells are indicated by arrows. B: The hybridization signal is absent on the same HIV-2-infected mesenteric lymph node, with substitution of a sense probe in a hybridization procedure that is otherwise identical to that used for A. C: HIV-2 RNA was completely absent in kidney tissues of 65/66 HIV-2-infected macaques, including all 13 TMA cases, one of which is illustrated here. The hematoxylin and eosin counterstain used for the *in situ* hybridization procedure has a limited sensitivity in the identification of microvascular thrombi compared to silver methenamine staining, as illustrated in Figure 2. Silver methenamine staining is not suitable as counterstaining for *in situ* hybridization procedures. D: In one animal very few HIV-2-infected cells were demonstrable (arrow). HIV-2 RNA expression was localized to infiltrating mononuclear leukocytes in the peritubular space and was completely absent in intrinsic renal cells. Original magnification A, B, D, ×1000; C, ×600.

stricted pathogenic features.[36,42] We have now demonstrated that one strain of HIV-2, HIV-2_{287}, originally derived from a human AIDS patient, is not only readily infectious for *M. nemestrina* but also highly pathogenic, progressing to a clinical AIDS syndrome within an accelerated time frame of 6–12 months.[31,32] Although HIV-2 is closely related to simian immunodeficiency virus (SIV), the HIV-2 viruses remain distinct from all known strains of SIV,[43,44] thereby presenting a unique opportunity for the study of a macaque model of human AIDS, using a virus adapted from a known human pathogen.

Evaluation of the renal pathology of 66 pigtailed macaques infected with HIV-2_{287} demonstrated TMA in 13 animals. One additional HIV-2-infected animal had features of a focal vasculitis and crescentic glomerulonephritis. Characteristics of human HIVAN (FSGS, hyperplasia/proliferation of visceral epithelial cells, tubular microcysts) were undetectable in all animals. The TMA lesions in the HIV-2-infected macaques were virtually identical with TMA lesions that can be seen in some HIV-infected humans. Interestingly, none of the HIV-2-infected macaques with TMA had developed clinical features typical for the manifestation of this disease in humans. Neither renal failure nor thrombocytopenia, frequently detectable in HIV-associated TMA in humans, was seen in the macaques with the pathological diagnosis of TMA. The subclinical course of TMA in this animal model could be explained by the scheduled euthanasia of the animals relatively early after HIV-2 infection. None of the macaques involved in this study had to be euthanized because of the development of severe AIDS manifestations.

Several hundred cases of HIV-associated TMA have been reported in the literature, with particularly increased recognition during the last several years (reviewed in[2–7]). Compared to several million HIV-1-infected humans worldwide, the percentage of TMA-affected HIV-1-positive patients might seem low. However, some authors consider HIV-associated TMA to be among the most common forms of TMA currently affecting adults,[7] with HIV-associated TMA accounting for up to 30% of hospitalized cases of TMA affecting adults in some institutions.[2] We hypothesize that TMA lesions might be underdiagnosed in HIV-infected humans, presumably because of a lack of suggestive clinical symptoms that would lead to renal biopsies in this patient group.

Because of the design of this study, we cannot completely rule out the presence of preexisting TMA lesions in these animals independent of the HIV-2 infection. However, nephropathological examinations of four uninfected

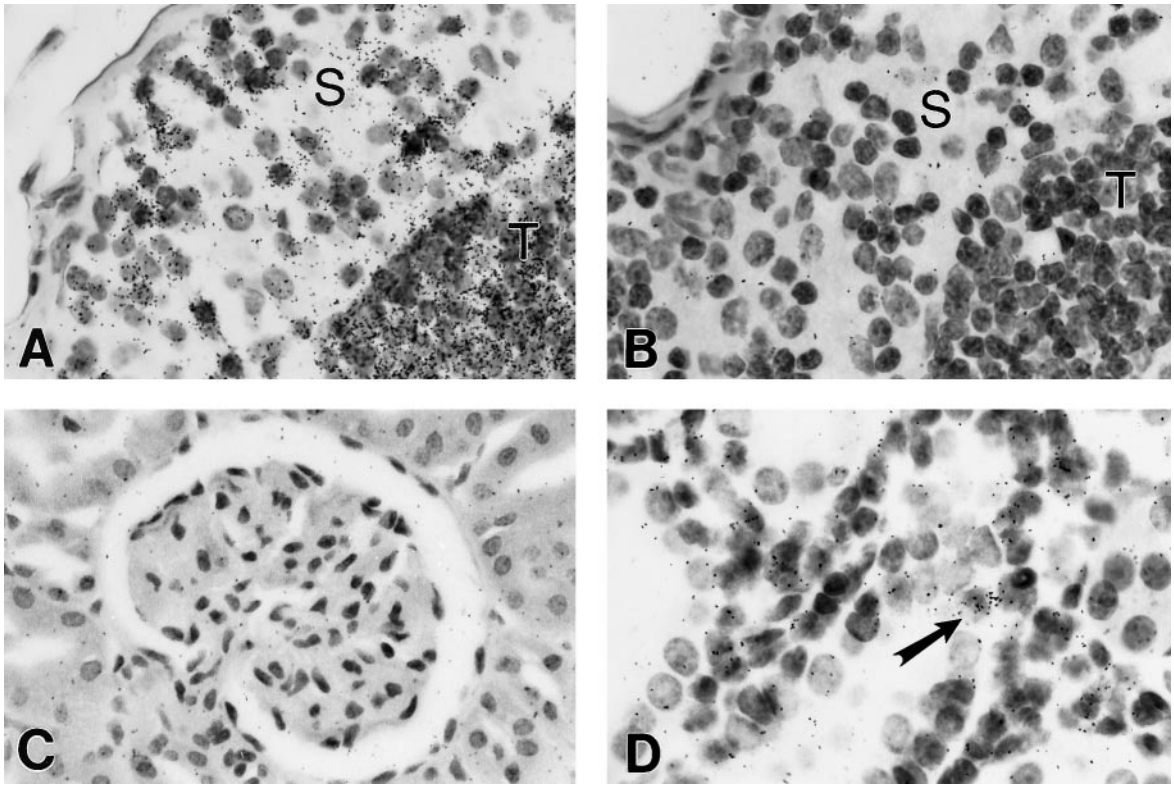

Figure 8. *In situ* hybridization for chemokine receptor CXCR4 mRNA expression. A and B: Mesenteric lymph node obtained from a macaque 10 days after intravenous HIV-2 infection (same case as shown in Figure 7, A and B). A: Numerous CXCR4 mRNA-expressing cells are demonstrable within the subcapsular sinus (S) and the adjacent T-lymphocyte rich outer cortex (T). B: No hybridization signal of the control CXCR4 sense riboprobe is detectable on the same lymph node. C: CXCR4 mRNA expression is completely absent in intrinsic renal cells of the glomerular, tubular, and vascular compartments in all kidneys obtained from HIV-2-infected macaques, including all 13 TMA cases, one of which is illustrated here. The hematoxylin and eosin counterstain used for the *in situ* hybridization procedure has a limited sensitivity in the identification of microvascular thrombi compared to silver methenamine staining, as illustrated in Figure 2. Silver methenamine staining is not suitable as counterstaining for *in situ* hybridization procedures. D: CXCR4 mRNA-expressing cells were rarely detectable in some of the analyzed kidneys. CXCR4 mRNA expression was restricted to a small number of leukocytes (arrow) at sites of focal interstitial mononuclear cell infiltration. Original magnification A, B, D, ×1000; C, ×600.

control macaques involved in this study did not reveal TMA lesions, indicating that these lesions are not commonly encountered in macaque populations. Further historical controls in our laboratory included additional uninfected normal macaques ($n = 5$), HIV-1-infected macaques ($n = 9$), SIV_{Mne}-infected macaques ($n = 83$), and SRV-infected macaques ($n = 40$). TMA lesions similar to those seen in the HIV-2-infected macaques were not identified in any of these 137 animals (Ref. 45 and our unpublished observations), indicating that TMA is a consequence of infection with certain retroviral species, such as HIV-2_{287} in this case, and not a preexisting condition. These findings further indicate that induction of an immunodeficiency state by such retroviral infections does not provide a sufficient basis for the development of TMA.

It is noteworthy that TMA lesions can be identified in some macaques as early as 12 hours after intravenous HIV-2 infection. At this time point HIV-2-infected cells were undetectable in PBMCs, in lymphoid tissues, and in the kidney. The presence of viral proteins capable of inducing TMA in the kidney without renal infection might be one explanation for the high incidence of TMA 12 hours after experimental intravenous HIV-2_{287} infection. Because of the design of this study, we cannot exclude the possibility that TMA developed in numerous animals very early after experimental infection, and that after an initial recovery, only a few animals continued to exhibit the disease. Future studies, including the inoculation of macaques with inactivated, noninfectious HIV-2_{287} preparations, might help in the further characterization of mechanisms involved in the pathogenesis of HIV-associated TMA.

TMA can be seen in macaques after intravenous and mucosal infection. Most animals that developed TMA in the present study had been infected intravenously, but this might reflect the fact that the majority of macaques involved in this study had been infected intravenously.

Finally, as all 13 macaques that had developed TMA were colony-born animals, we sought to determine whether a common familiar background might be associated with the TMA development in these animals independently of the HIV-2 infection, as sometimes happens in human kidneys with familial TMA because of abnormalities of clotting proteins. Macaques with TMA lesions could not be identified as members of one inbred family. Comparison of all known ancestors (up to five generations) showed that although some animals were related to the same grandparents, as expected for colony-born animals, several macaques clearly demonstrated absent

familial relationships with all other animals that had developed TMA.

Pathogenic mechanisms leading to endothelial cell injury and platelet deposition in the microvasculature of HIV-associated TMA are poorly understood.[2,11,14] Several studies have suggested the potential for HIV infection of renal tissue.[2,11] Direct endothelial cell infection by HIV-1 has been reported both *in vivo* and *in vitro* by several groups (reviewed in Ref. 2), but direct evidence for a pathogenic role of this infection in TMA is lacking. Published data on HIV infection of endothelial cells in HIV-associated TMA is very limited. Del Arco et al detected HIV-1 p24 antigen by immunohistochemistry in some bone marrow endothelial cells from a single case of thrombotic thrombocytopenic purpura in a woman infected with HIV.[46] However, HIV-1 immunostaining in this case was not associated with any evidence of local tissue damage in the analyzed bone marrow specimen. The presence of HIV-1 protein or RNA has never been reported in renal microvasculature endothelial cells of HIV-1-infected humans that developed TMA.

In the present study we sought to detect HIV-2 RNA in kidney sections of HIV-2-infected macaques by *in situ* hybridization. Renal expression of HIV-2 RNA was absent in all 13 animals that had developed TMA lesions. Furthermore, HIV-2 RNA was not detectable in renal parenchymal cells comprising the glomerular, tubular, or vascular compartments of all 66 HIV-2-infected macaques. A few HIV-2 RNA-expressing infiltrating leukocytes were detectable in a single animal at sites of focal interstitial inflammation. Despite the presence of mild, focal interstitial inflammation in several additional HIV-2-infected macaques, HIV-2 RNA remained undetectable in these cases. (Our *in situ* hybridization procedures detected numerous HIV-2 RNA-expressing cells in PBMCs or lymphoid tissues of the infected macaques.) We recognize that the *in situ* hybridization technique without PCR amplification has a limited sensitivity and that there are likely to be some infected cells in which the number of RNA copies is below the threshold of our detection system. However, *in situ* hybridization offers the important advantage of clearly colocalizing the RNA expression to cellular phenotypes.

Chemokine receptors have been shown to serve as coreceptors together with CD4 for HIV-1 and HIV-2 strains. Some HIV-2 isolates have been described that use CXCR4 for virus entry in a CD4-independent fashion.[47,48] This raises the possibility that HIV may be able to infect CD4-negative cells that express CXCR4 or perhaps other chemokine receptors. Some preliminary data indicate that HIV-2_{287} is most likely a CXCR4-tropic virus (M.-A. Rey-Cuille and S.-L. Hu, personal observations), although we cannot completely rule out the possibility that it is also using some unknown monkey receptor. In this study, CXCR4 expression was undetectable in intrinsic renal cells of HIV-2-infected macaques.

Two recent studies demonstrated chemokine receptor CXCR4 expression in endothelial cells.[49,50] CXCR4 mRNA and protein expression was detected in cultured endothelial cells isolated from human umbilical veins, human coronary arteries, human brain microvasculature, bovine aorta, bovine pulmonary arteries, and the endothelial cell layer in rabbit thoracic aortic tissue.[49,50] Both studies showed identical CXCR4 expression patterns on the transcript and protein levels, indicating that CXCR4 mRNA is subsequently translated into protein. We have previously reported that CXCR4 mRNA was undetectable by *in situ* hybridization in renal microvascular endothelial cells in normal, mature human kidney and in human allograft nephrectomies undergoing severe allograft rejection.[34] In the present study, CXCR4 mRNA expression was undetectable in endothelial cells of the renal vasculature in uninfected and HIV-2-infected macaques. This observation may provide additional evidence against a role for direct HIV-2 infection of intrinsic renal cells as a proximate cause of injury in this disease model. However, we cannot rule out the possibility that other chemokine receptors that could act as coreceptors for HIV-2 infection might be expressed on renal vascular endothelial cells.

The HIV-2_{287} *M. nemestrina* model may be useful in the further study of the pathogenesis of HIV-associated TMA lesions. This study does not support a role for productive viral infection of intrinsic renal cells in the pathogenesis of this disease. We believe that our failure to detect specific viral RNA and chemokine receptor CXCR4 mRNA in renal parenchyma of macaques infected with HIV-2 provides additional insight into the difficulty encountered in attempts to demonstrate direct infection of intrinsic renal cells by immunodeficiency viruses. The most attractive hypothesis remaining to explain the high incidence of TMA in both human and nonhuman primates infected by HIV involves injury to the endothelium, with particular susceptibility of the glomerular and arteriolar vascular beds. Studies to determine whether the endothelium is damaged or assumes a procoagulant phenotype in HIV-associated TMA are currently under way.

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