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Experimental chronic renal ischemia: Morphologic and immunologic studies

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Experimental chronic renal ischemia: Morphologic and immunologic study. Although unilateral clamping of the renal artery to induce chronic ischemia of the kidney tissue has been utilized in several animal species, the resultant morphologic, ultrastructural and immunologic changes have never been well characterized. Moreover, the pathogenesis of these changes, as well as their roles in causing or facilitating the development of chronic tubulointerstitial nephritis have not been known. To examine some of these issues, male Sprague-Dawley rats were subjected to unilateral stenosis of the left main renal artery for 28 days. Stenotic and contralateral kidneys of experimental animals and kidneys from sham-operated controls were subjected to: (1) light microscopic, electron microscopic and immunofluorescent studies; (2) morphometric quantitation of the structural changes; (3) staining for actin, epithelial membrane antigen, keratin, and vimentin by immunoperoxidase technique; (4) staining for complex glycoproteins by a panel of 13 lectins; and (5) phenotyping and quantitation of the interstitial inflammatory infiltrates by monoclonal antibodies, using immunoperoxidase technique. The results reveal that: (1) The ischemic kidney tissue displays marked tubulointerstitial damages including abundant interstitial chronic inflammatory infiltrates, with good preservation of glomerular structure, which is consistent with the standard criteria of chronic tubulointerstitial nephritis. (2) The antigenic profile of the ischemic tubular epithelium displayed marked alterations including a neo-expression of vimentin and keratin, as well as a loss of endogenous avidin binding activity, Ia antigen and several complex surface glycoproteins detectable by lectins. (3) Neither electron dense deposits nor immunoglobulins are detectable in the kidneys from experimental or control animals. (4) Tubulitis, defined as infiltration of tubular epithelium by inflammatory cells, was present in up to 42.2% of tubular cross sections of the ischemic kidneys. (5) The interstitial inflammatory infiltrates were composed of B lymphocytes, T helper lymphocytes, and macrophages whereas the T non-helper lymphocytes were scanty, a phenotypic pattern similar to that of several other experimental rat models of chronic tubulointerstitial nephritis. It is concluded that: (1) In the Sprague-Dawley rats, ischemia alone can cause a constellation of changes fulfilling the accepted features of chronic interstitial nephritis; (2) ischemia alters the antigenic profile of the tubular epithelium and thereby may initiate a cell mediated immune response, accounting for the observed tubulitis and interstitial inflammation; and (3) ischemia may well be the final common pathway for chronic tubulointerstitial nephritis of diverse etiologies.

Chronic tubulointerstitial nephritis (CTIN) is characterized morphologically by tubular atrophy, interstitial fibrosis and interstitial inflammation of variable severity. CTIN may be of secondary or primary nature. In the former, the chronic tubulointerstitial damage is considered to be secondary to glomerular or vascular disease, whereas in the latter it is thought to occur de novo [1]. Some degree of CTIN is present in virtually every renal disease in advanced stage, and may well be functionally important [2]. Actually, regardless of the nature of the primary renal disease, the decline in glomerular filtration rate is known to correlate better with tubulointerstitial than with glomerular or vascular changes [3, 4].

The pathogenesis of CTIN is poorly understood [1]. It has been speculated that in CTIN, at least in its secondary form, ischemia, decreased peritubular blood flow, glomerulosclerosis, and interstitial inflammation may be interrelated in a complex manner [5, 6]. In this general scheme, the mechanisms by which ischemia cause tubular atrophy, interstitial fibrosis and interstitial inflammation have not been elucidated.

Unilateral clamping of the renal artery to induce chronic ischemia of the kidney tissue has been utilized in several experimental models of hypertension [7–12]. However, most such studies have focused exclusively on various physiologic aspects of the resultant changes in hemodynamics and renal function, almost to the total neglect of the morphologic, ultrastructural and immunologic aspects of the ischemia-induced changes in the tubulointerstitial compartment [7–12]. In this study the tubulointerstitial changes of the kidneys in Sprague-Dawley rats with unilateral renal artery stenosis were evaluated by light microscopy, electron microscopy, immunofluorescent and immunoperoxidase techniques. The interstitial inflammatory infiltrate, which was surprisingly abundant, was phenotyped by monoclonal antibodies. The results suggest that ischemia alone can cause CTIN, and provide some insight into the histogenesis of the observed lesions.

Methods

Experimental design

Male Sprague-Dawley rats weighing between 250 and 300 grams (60 to 70 days in age) were anesthetized with pentobarbital sodium (30 mg/kg i.p.) and were subjected to unilateral stenosis of the left main renal artery (RAS). Under the dissecting microscope, this renal artery was isolated and a blunted 26 gauge needle placed along its side. A ligature was then passed around the artery and the needle at two different locations approximately 0.9 cm apart. After the needle was removed, proper suturing and adequate pulsation of the renal artery between the two ligations were checked to ensure that stenosis,

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but not obstruction, was created. Stenosis of the renal artery was judged to be 90% or greater. Following recovery, the rats were maintained on a standard diet with free access to tap water. The control group included six normal, age- and weight-matched, sham-operated rats.

To insure that non-sterile surgery is not a cause of the renal lesion, sterile techniques were used for eight groups of rats each of which consisted of two experimental rats and one sham-operated, control rat. These groups were sacrificed at days 2, 5, 9, 14, 20, 27, 36, and 45 and the renal tissues were harvested only for light microscopic study. The renal changes in both experimental and control rats were similar to those in rats that underwent non-sterile surgery, confirming that these lesions were not related to infection.

To select the rats for detailed functional and immunologic studies, groups of three rats each were sacrificed at different intervals after RAS. It was found that in the stenotic kidney, subtle tubular changes, including focal necrosis and mild atrophy, were noted as early as three days after RAS; however, interstitial inflammation was not seen until nine days after RAS, peaked at about 28 days and slightly decreased thereafter. Tubular atrophy, thickening of tubular basement membrane, interstitial fibrosis, tubulointerstitial calcification and glomerulosclerosis gradually increased until the last days of observation (60 days). In the contralateral kidneys, (detailed in the **Results** section) focal lesions were first noted on day 14 and became more pronounced subsequently. These observations suggested that rats with 28 days of RAS were best suited for detailed studies.

Functional studies

For each animal, before sacrifice blood pressure was recorded by a Gould pressure monitor #SP1405 (Gould Instrument, Oxnard, California, USA). Inulin clearance of each kidney was measured by a method detailed elsewhere [13].

Morphologic studies

Tissue preparation. Under general anesthesia, the kidneys and the aorta were exposed by blunt dissection with cotton swabs. Complete exsanguination was achieved in a few minutes by transaction of the aorta coupled with gentle cardiac massage. The kidneys were then harvested, weighed separately and bisected coronally. One half of each kidney was snap-frozen and stored at −70°C until used. A thin strip of the other half was immediately fixed in 4% glutaraldehyde for electron microscopy and the rest was fixed in 10% buffered formalin for light microscopy.

Light microscopy (LM). Aside from routine examination of formalin-fixed, paraffin-embedded tissue sections stained by hematoxylin and eosin, periodic acid-Schiff (PAS) and Masson's trichrome techniques, the following features were morphometrically evaluated in each case: (1) number of sclerotic glomeruli expressed as a percent of the 40 to 100 glomeruli counted in each specimen; (2) mean diameter of cortical tubules [at least 50 cross-sections of tubules were measured, using a morphometric computer system (Software: Bioquant, R&M Biometric, Nashville, Tennessee; hardware: Compaq 286, Houston, Texas, USA)]; (3) mean glomerular diameter (at least 20 glomeruli, excluding the globally sclerotic one, each having a vascular pole, were used; measurement was made for the diameter connecting the vascular pole and the corresponding point on the Bowman's capsule); (4) percentage of tubular cross sections showing mononuclear, inflammatory cell infiltration between tubular cells (tubulitis); and (5) the concentration of interstitial inflammatory cells, expressed as the mean count of these cells in five cortical fields, each being delineated by a one cm^2 grid attached to the eyepiece; the ×40 objective lens of an American Optic Microscope was used for this study.

Immunofluorescent study (IF). Five micron sections of the snap-frozen tissues were stained with fluorescein isothiocyanate-labeled rabbit antibodies against rat IgG, IgM and C_3, respectively (dilution 1/50, Cappel Laboratories, Cochran, Pennsylvania, USA).

Electron microscopy (EM). Both cortical and medullary tissues were subjected to EM study, using a standard technique. To avoid potentially misleading artifacts associated with immersion fixation, perfusion-fixed renal tissue from two additional experimental animals were also examined.

Immunohistochemistry. To test the hypothesis that the antigenic profile of renal tubules is altered by ischemia, sections from both formalin fixed and frozen tissues were stained for keratin (AE_1/AE_3 antibody, dilution 1/50, Hybridtech, San Diego, California; and CAM 5.2 antibody, prediluted, Becton Dickinson, Mountain View, California, USA), for actin (HHF35 antibody, Enzo Co., 1:1280 dilution, New York, New York), for vimentin (dilution 1/50, Dako, Carpinteria, California, USA) and epithelial membrane antigen (dilution 1/50, Dako). A standard avidin-biotin-peroxidase-complex technique was used for these antibodies [14].

Lectin histochemistry. To test the hypothesis that ischemia may be associated with a profound alteration in the structure of the surface glycoprotein of tubular cells, staining of renal tissue with various lectins was performed. Lectins are proteins of non-immunologic origin derived from plants; they can bind specifically to carbohydrate groups and have been known to be specific markers for glycoconjugates including those in the kidney tissue [15]. The details of the two-step biotin-peroxidase staining technique, the specificities and the optimal dilutions of the lectins used in this study were reported elsewhere [15], and are summarized in Table 1.

Phenotyping of the inflammatory cells

The sources, specificities, and dilutions of the primary monoclonal antibodies used in this study are listed in Table 2 [16–20]. Tissue sections were stained using a modified avidin-biotin peroxidase technique as follows [14]: four-micron thick sections were cut from the frozen kidney tissue, dried for one hour, fixed in acetone for seven minutes, and dried again for another hour. Blocking of endogenous avidin binding activity was achieved by incubating the sections with the avidin solution for ten minutes followed by biotin solution for ten minutes (Avidin-Biotin Blocking Kit, Vector Laboratory, Burlingame, California, USA). Sections were consecutively incubated with primary antibodies (Table 2) for one hour, and with secondary antibodies [Horse anti-mouse Ig (ABC Kit, Vector Laboratory) diluted in PBS containing 20% rat serum] for 45 minutes. Each of these steps was preceded by three washes in PBS, pH 7.4. The color reaction was developed by an incubation of ten minutes in 50 mg% diaminobenzidine (DAB) in Tris buffer (pH 7.5) activated

Table 1. Immunohistochemical and lectin histochemical profile of proximal tubules

	Controls	Stenotic kidneys	Contralateral kidneys: Atrophic areas	Contralateral kidneys: Non-atrophic areas
Epithelial membrane antigen	–	–	–	–
Actin	–	–	–	–
Keratin	–	+	+	–
Vimentin	–	+++	+++	–
Ia	++	–	–	++
EABA	++	–	–	++
Sophora japonica	–	–	–	–
Ulex europaeus I	–	–	–	–
Tritium vulgaris	++	++	++	++
Canavalia ensiformis	++	++	++	++
Phaseolus vulgaris leukoagglutinin	+++	+++	+++	+++
Phaseolus vulgaris erythroagglutinin	+++	+++	+	+++
Pisum sativum	+	+	+	+
Lens culinaris	++	+	+	++
Arachis hypogaea	++	+	+	++
Ricinus communis I	+++	+	+	+++
Bandeiraea simplicifolia I	+++	+	+	+++
Dolichos biflorus	+++	–	–	+++
Glycine max	+++	–	–	+++

See reference 16 for details on the lectins used in this study; tubular staining was semi-quantitated on a – to +++ scale (– = no staining; + = mild; ++ = moderate; +++ = marked); EABA = endogenous avidin binding activity.

by one drop of H_2O_2. Sections were dehydrated, counterstained with methyl green or hematoxylin and mounted.

Sections of spleen, thymus, and lymph nodes from normal rats were examined by the above technique to confirm the specificities of the primary antibodies. Controls for the techniques included sections treated with DAB only, sections treated with the avidin-biotin complex and DAB, and sections treated with secondary antibodies diluted in PBS with and without normal rat serum, followed by the avidin-biotin complex.

Each phenotype was quantitated by counting (×20 objective lens, American Optic Microscope) the number of interstitial, positively stained cells within five fields, each being delineated by a one cm^2 grid attached to the eyepiece. Each phenotype was also expressed as the percentage of the total number of cells, regardless of phenotypes. The latter method of quantitation was done to facilitate comparison of phenotypes from different experimental groups, with a clear understanding that the denominator, due to the known overlapping of antibody specificities, does not represent the actual number of interstitial inflammatory cells. An effort was made to use the same area in consecutive sections for quantitation. Quantitation was performed separately for stenotic kidney, atrophic and non-atrophic cortex of the contralateral kidney, and control kidney.

Statistics

Statistical comparisons were made using analysis of variance and the method of least significant difference for multigroup comparisons.

Results

Functional studies

The mean blood pressures of the experimental animals ranged from 106 to 136 mm Hg (mean 120.66 ± 4.1) and that of the controls from 103 to 108 mm Hg (mean 105.66 ± 2.34). The clearances of the stenotic kidneys were too low to measure. The clearance of the contralateral kidneys and control kidneys ranged from 829 to 3522 μl/mn (mean: 3002.25 ± 561.66), and from 1344.2 to 2309.7 μl/mn (mean 1840.3 ± 140.7). When the clearance is expressed as μl/g of kidney tissue, the values for the contralateral kidneys and for control kidneys were 1693.6 μl/g ± 121.6 and 1662.7 ± 375.5 μl/g, respectively.

Morphometric studies

The morphometric data are summarized in Table 3. The means of the wet renal weights, glomerular diameters, tubular diameters and the percentages of non-sclerotic glomeruli of the stenotic kidneys were significantly less than those of the contralateral sides. Interstitial inflammation of the stenotic kidneys, and of the focal lesional areas of the contralateral kidneys was significantly more severe than that of the intact areas of the contralateral kidneys and of control kidneys.

Light microscopy

There was a distinct difference between the stenotic kidneys which were atrophied and the contralateral kidneys which were hypertrophied.

The tubules of the stenotic kidneys displayed severe changes including diffuse atrophy, focal thickening of the tubular basement membrane, loss of brush border and tubular "simplification" in which different tubular segments lost their specific features and assumed a uniform, undifferentiated appearance (Figs. 1 and 2). There was tubular cell necrosis (Fig. 2), focal tubular calcification and focal tubular dilatation, most pronounced at the corticomedullary junction. There were focal tubular casts consisting of homogeneous PAS positive material with or without necrotic tubular cells (Fig. 2). Up to 49% (mean 42.2 ± 3.1) of tubular cross sections showed tubulitis in which mononuclear inflammatory cells (averaging 1 to 2 per tubular cross section) were present between tubular cells (Fig. 2). The interstitium displayed mild, focal fibrosis and severe inflammation consisting exclusively of mononuclear cells without plasma cells, neutrophils or eosinophils. Although the inflammation was diffuse, it was most pronounced at the corticomedullary junction where tubular cast were most numerous. Interstitial Tamm-Horsfall protein was not observed. The structure of the glomeruli was surprisingly well preserved and showed only moderate reduction in size, and a mild degree of focal segmental mesangial sclerosis, thickening and collapse of glomerular capillaries (Fig. 1). The small arteries and arterioles displayed mild focal intimal thickening.

The contralateral kidneys showed rare foci of tubulointerstitial damage. Each foci of involvement affected an average of twenty adjoining tubules. The tubulointerstitial changes in these foci were similar to those in the stenotic kidneys, except that the degree of interstitial inflammation was significantly less (Fig. 3, Table 4). Most of these foci did not contain glomeruli, but when a rare glomerulus was identified, it was either normal

Table 2. Antibodies used in phenotyping of inflammatory cells

Clone	Sources	CD	Specificity	Dilution	Refs.
OX1	Sera-Lab (Westbury, New York)	CD45	Leukocyte-common antigen on all hematopoietic cells except erythrocytes	Undiluted supernatant	[16]
W3/13	Sera-Lab	CD2	T lymphocytes, thymocytes, some plasma cells and stem cells	Undiluted supernatant	[18]
W3/25	Sera-Lab	CD4	T helper cells, most thymocytes	Undiluted supernatant	[17, 18]
OX8	Sera-Lab	CD8	T non-helper cells, most thymocytes, some natural killer cells	Undiluted supernatant	[17, 18]
OX4	Sera-Lab	NA	Ia marker present on B cells, dendritic cells	Undiluted supernatant	[19]
ED1	Serotec (Kidlington, Oxford, England)	NA	Macrophages, monocytes, dendritic cells	1/600 of ascitic fluid	[20]

NA = not available.

Table 3. Comparative morphometric data for control and experimental rats[a]

	Controls ($N = 6$)	Stenotic kidneys ($N = 6$)	Contralateral kidneys ($N = 6$)	
			Atrophic areas	Non-atrophic areas
Weight (mg)[b]	1096 ± 20	419 ± 22	1794 ± 63	
% of globally sclerotic glomeruli[c]	0.5 ± 0.2	14.3 ± 5.0	ND	1.3 ± 0.4
Glomerular diameters (μm)[d]	87.6 ± 0.9	68.0 ± 2.8	ND	90.4 ± 0.1
Cortical tubular diameters (μm)[e]	42.5 ± 0.1	32.3 ± 0.9	30 ± 1.1	45.6 ± 0.1
% of cortical tubular cross sections with tubulitis[f,g]	0 ± 0	42.2 ± 3.1	25.6 ± 5.3	0 ± 0
Interstitial inflammation[g,h]	43.6 ± 3.1	1936.1 ± 167.2	673.6 ± 36.1	61.1 ± 4.7

ND = not done because glomeruli are usually not present in these areas.

[a] Data are expressed as mean ± SEM

[b] $P = 0.0000001$, controls vs. stenotic kidneys vs. contralateral kidneys

[c] $P < 0.007$; stenotic kidney vs. controls or contralateral kidneys

[d] $P < 0.0002$; stenotic kidney vs. controls or contralateral kidneys

[e] $P < 0.000012$; stenotic kidney or atrophic area of contralateral kidneys vs. controls or non-atrophic area of the contralateral kidneys

[f] $P = 0.0000113$; atrophic area of the contralateral kidneys vs. non-atrophic areas or controls

[g] $P = 0.0000001$; stenotic kidneys vs. non-atrophic areas of the contralateral kidney or controls

[h] The interstitial inflammation is expressed as the number of inflammatory cells in 5 random fields each delineated by a 1 cm^2 reticule attached to the eyepiece

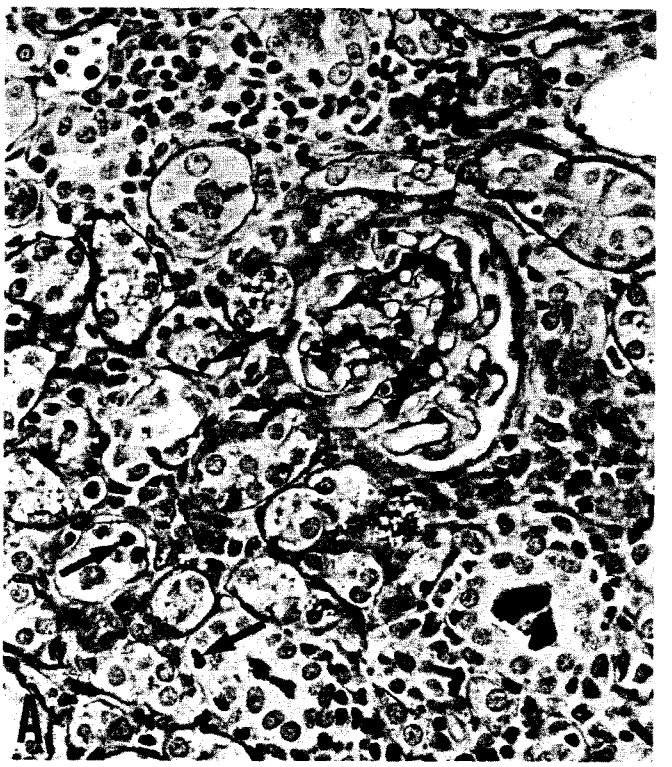

Fig. 1. *Stenotic kidney.* The tubules are atrophic and display simplification of epithelium, thickened basement membrane and tubulitis (arrows indicate inflammatory cells within tubular epithelium). There is interstitial chronic inflammation. A glomerulus displays segmental, mild mesangial sclerosis (periodic acid-Schiff, × 1478).

or showed only mild changes similar to those of the stenotic kidneys. The renal tissue surrounding the foci of tubulointerstitial damage was normal and displayed no significant changes. The blood vessels in, or near, or outside the atrophic areas showed focal intimal fibrosis, not significantly different from one another and from the vessels in the stenotic kidneys.

The control rats sham-operated with or without sterile technique did not show any significant renal lesions; specifically,

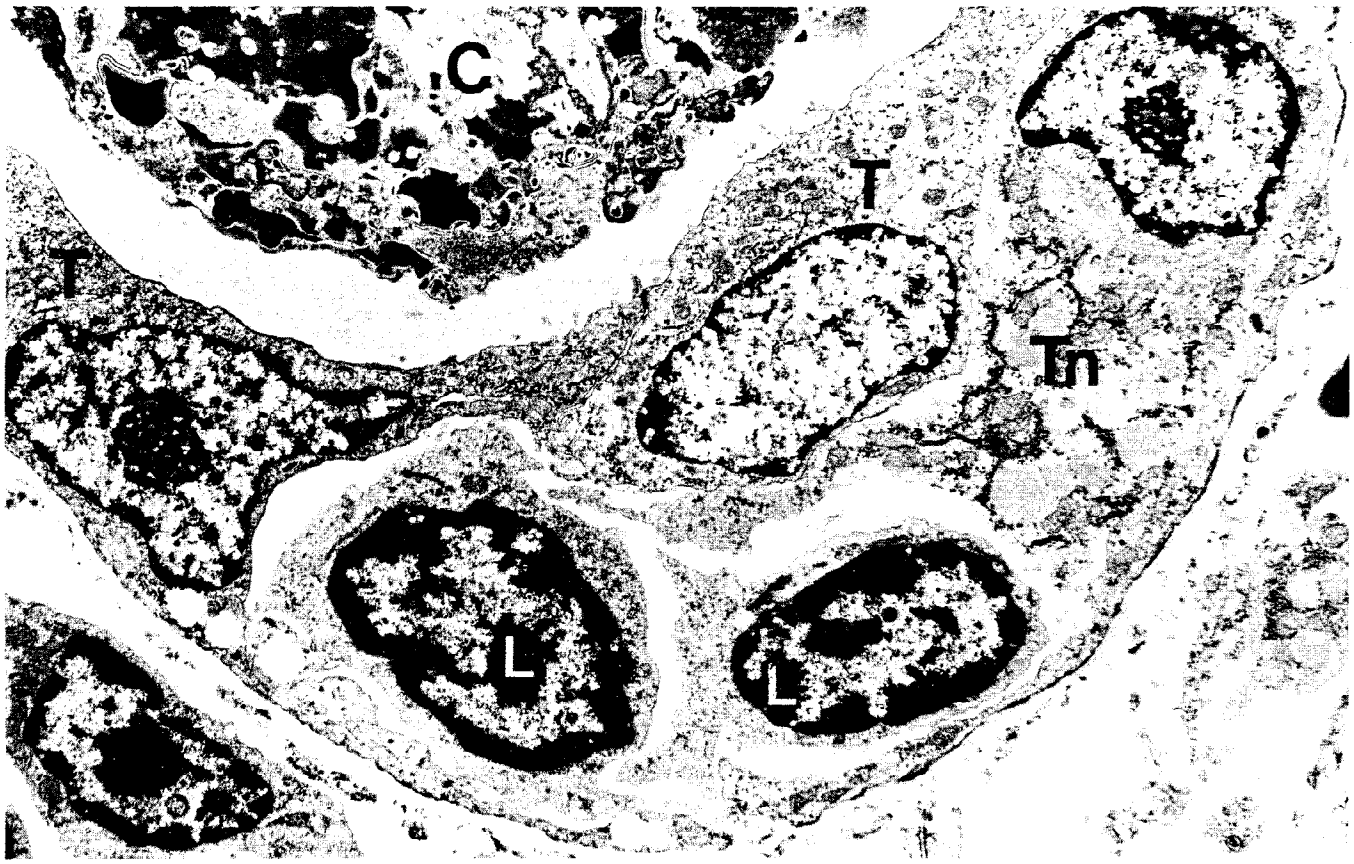

Fig. 2. *Stenotic kidney.* The structure of atrophic tubular cells (T) (probably from proximal tubules) becomes "simplified" as evidenced by a loss of brush border, a loss of basolateral infoldings and a scanty number of organelles. Atrophic tubules also show features of tubulitis characterized by the presence of lymphocytes (L) between tubular cells. One tubular cell (Tn) displays features of cellular damage including a loss of cell membrane. The tubular cast (C) is composed of cell debris (electron microscopy, × 15,400).

interstitial inflammation or tubular atrophy was virtually absent.

Immunofluorescent studies

In the control kidneys and in the non-atrophic areas of the contralateral kidneys of the experimental rats, focal, linear staining of proximal tubular basement membrane or Bowman's capsule for C_3 was noted, but staining for IgG or IgM showed negative results. The stenotic kidneys showed no deposits of C_3, IgG or IgM in any of the components of renal parenchyma.

Electron microscopy

The most prominent changes were present in the atrophic tubules of both kidneys, where most tubular cells assumed an undifferentiated appearance characterized by a cell membrane without basal lateral infoldings or brush borders, scanty organelles and loss of cell polarization (Fig. 2). Tubulitis was common, but the nature of the inflammatory cells participating in this process could not be elucidated by EM study (Fig. 2). Tubular cells in contact with mononuclear cells occasionally showed degenerative features such as focal loss of cell membrane, or disruption of organelles (Fig. 2). However, these features were also seen in some tubular cells without such contact. Some tubules showed reduplicated tubular basement membrane, the space between which was occupied by mononuclear inflammatory cells. Electron dense deposits were never observed in any location.

Immunoperoxidase studies

The results are summarized in Table 1. Epithelial membrane antigen was not identified in kidney tissues from either control or experimental animals. Smooth muscle cells in blood vessel walls and mesangial cells of both control and experimental kidneys were equally stained for actin, whereas the tubules of neither group stained for actin. Several atrophic tubules in the stenotic kidneys of experimental animal showed a weak staining for keratin (Fig. 4A), whereas intact tubules in the contralateral kidneys or kidneys from control animals did not show any staining for keratin (Fig. 4B). Although vimentin was not seen in any tubules in the control kidneys or in the normal tubules of the contralateral kidneys of experimental animals from ischemic and contralateral kidneys (Fig. 5A), it was strongly expressed in atrophic tubules (Fig. 5B). There was also strong global diffuse staining for vimentins of some glomerular cells (probably visceral epithelial cells), which did not vary among different groups (Fig. 5A and B). The atrophic tubules displayed a complete loss of Ia antigen, which was clearly identified in normal proximal tubules (Fig. 6A and B), and a significant

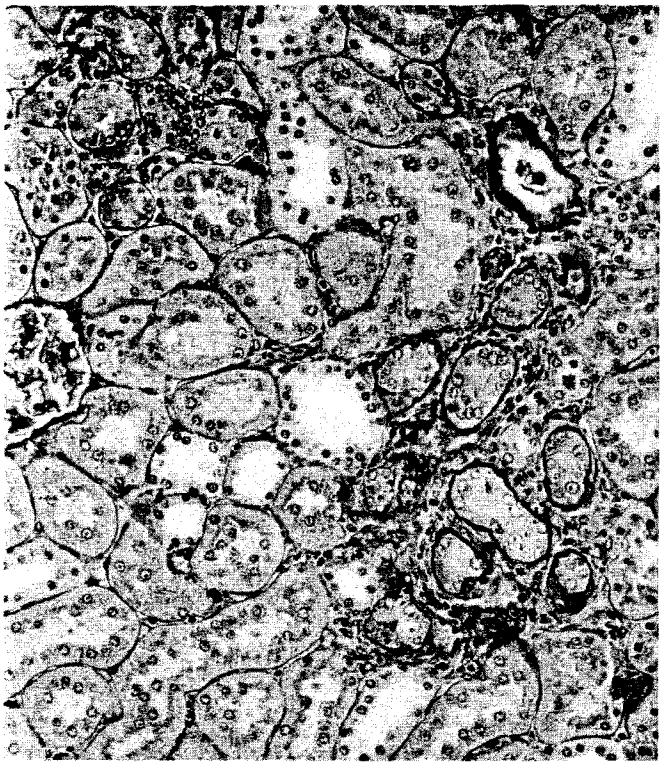

Fig. 3. *Contralateral kidney.* Two foci of tubular atrophy, interstitial fibrosis and interstitial inflammation (right and upper left) are surrounded by normal tissue (periodic acid-Schiff, × 739).

Table 4. Phenotypes of the interstitial inflammatory infiltrate[a]

	Controls ($N = 6$)	Stenotic kidneys ($N = 6$)	Contralateral kidneys ($N = 6$)	
			Atrophic areas	Non-atrophic areas
OX1[b]	52.3 ± 8.3 (34.6%)	654.3 ± 67.7 (23.8%)	183.0 ± 15.4 (23.2%)	75.6 ± 8.7 (21%)
OX4[c]	27.4 ± 1.4 (18.1%)	1008.5 ± 124.6 (36.8%)	182.0 ± 10.4 (23%)	59.9 ± 4.2 (16.6%)
W3/13[d]	20.9 ± 2.5 (13.8%)	161.4 ± 15.3 (5.8%)	113.8 ± 12.9 (14.4%)	59.4 ± 3.0 (16.5%)
W3/25[e]	39.1 ± 5.4 (25.9%)	695.1 ± 74.8 (25.3%)	232.6 ± 18.7 (29.6%)	141.7 ± 2.8 (27%)
OX8[f]	3.4 ± 0.8 (2.2%)	76.5 ± 8.5 (2.7%)	17.2 ± 2.0 (2.2%)	9.9 ± 1.5 (2.7%)
ED1[g]	7.7 ± 0.7 (5.1%)	142.7 ± 30.4 (5.2%)	57.1 ± 10.9 (7.2%)	12.7 ± 1.8 (3.5%)

[a] Each phenotype is expressed as mean ± SEM of the counts for all kidneys. The count for each kidney is the total number of positively stained cells in 5 fields each delineated by a 1 cm^2 reticule attached to the eyepiece. Each phenotype is also expressed as % of the total number of cells (see text for details of quantitative technique).

[b] $P \leq 0.0000001$; stenotic kidneys vs. controls and contralateral kidneys

[c] $P \leq 0.0000002$; stenotic kidneys vs. controls and contralateral kidneys

[d] $P \leq 0.0000006$; stenotic kidneys vs. controls and non-atrophic areas of contralateral kidneys

[e] $P \leq 0.000000$; stenotic kidneys vs. controls and contralateral kidneys

[f] $P \leq 0.0000001$; stenotic kidneys vs. controls and contralateral kidneys

[g] $P \leq 0.0000112$; stenotic kidneys vs. controls and contralateral kidneys

decrease in the endogenous avidin-binding activity as compared to that of normal proximal tubules.

Lectin histochemistry

The results of these studies are summarized in Table 1. As compared to intact proximal tubules, the staining of atrophic proximal tubules by the different lectins used was either unchanged, significantly decreased, or completely negative. The pattern of unchanged staining was noted for four lectins (*Triticum vulgaris, Concanavalin ensiformis, Phaseolus vulgaris* leukoagglutinin and erythroagglutinin). The pattern of decreased staining was noted for five lectins (*Pisum sativum, Lens culinaris, Arachis hypogaea, Ricinus communis I and Bandeiraea simplicifolia I*). Complete loss of staining was noted for *Dolichos biflorus* and *Glycine max* (Fig. 7A and B), which have been known to be specific markers for glycoconjugates having oligosaccharide D-galactose-N-acetylglucosamine. There was no situation in which a lectin histochemical staining was positive in atrophic tubules but failed to stain intact tubules.

Phenotyping of the interstitial inflammatory infiltrates

The results are summarized in Table 4 and illustrated in Figures 8 and 9. All the positive and negative controls yielded appropriate results. The staining clearly showed that the interstitium of the stenotic kidneys showed diffuse infiltration of inflammatory cells stained by antibody OX_1 [for common leukocyte antigen (CLA), a marker for leukocytes], OX_4 (for Ia antigen), W3/13 (mostly for T cells), W3/25 (mostly for T helper cells), OX8 (mostly for T non-helper cells), and ED_1 (for macrophages). Significant differences in the percentage of each phenotype were noted, thus the OX_4^+ cells were most numerous, followed closely by the W3/25$^+$ cells and the OX_1^+ cells, whereas the $OX8^+$ cells were the least numerous (Fig. 9A-E). Although the infiltrate was more pronounced at the corticomedullary junction, there were no significant phenotypic differences in the infiltrates among different areas of the kidney.

As seen in Table 2, there is some overlapping in the specificities of the utilized antibodies. Thus, although the most numerous phenotype is OX_4^+ cells, it was not possible to assess accurately the contribution of B lymphocytes to this population, because OX_4 antibody recognizes Ia antigen localized not only on the surface of B cell but also on dendritic cells, macrophages, and some activated T cells [19]. Moreover, the total number of OX_4^+ cells (mostly B cells) and OX 3/13 (mostly T cells) exceeded the number of $OX1^+$ cell (all leukocytes) by a wide margin. So were the total numbers of W3/15$^+$ cells (mostly T helper cells) and $OX8^+$ cells (mostly T non-helper cells) when compared with W3/13$^+$ cells (T cells). These interpretational difficulties, which have been encountered by other investigators using similar antibodies [22–28], do not detract from the observation that stenosis of the renal artery can cause severe interstitial inflammation characterized by a mixture of cells including T helper cells, B cells, macrophages and a paucity of cytotoxic T cells.

As a rule, the stenotic kidney showed the highest density of

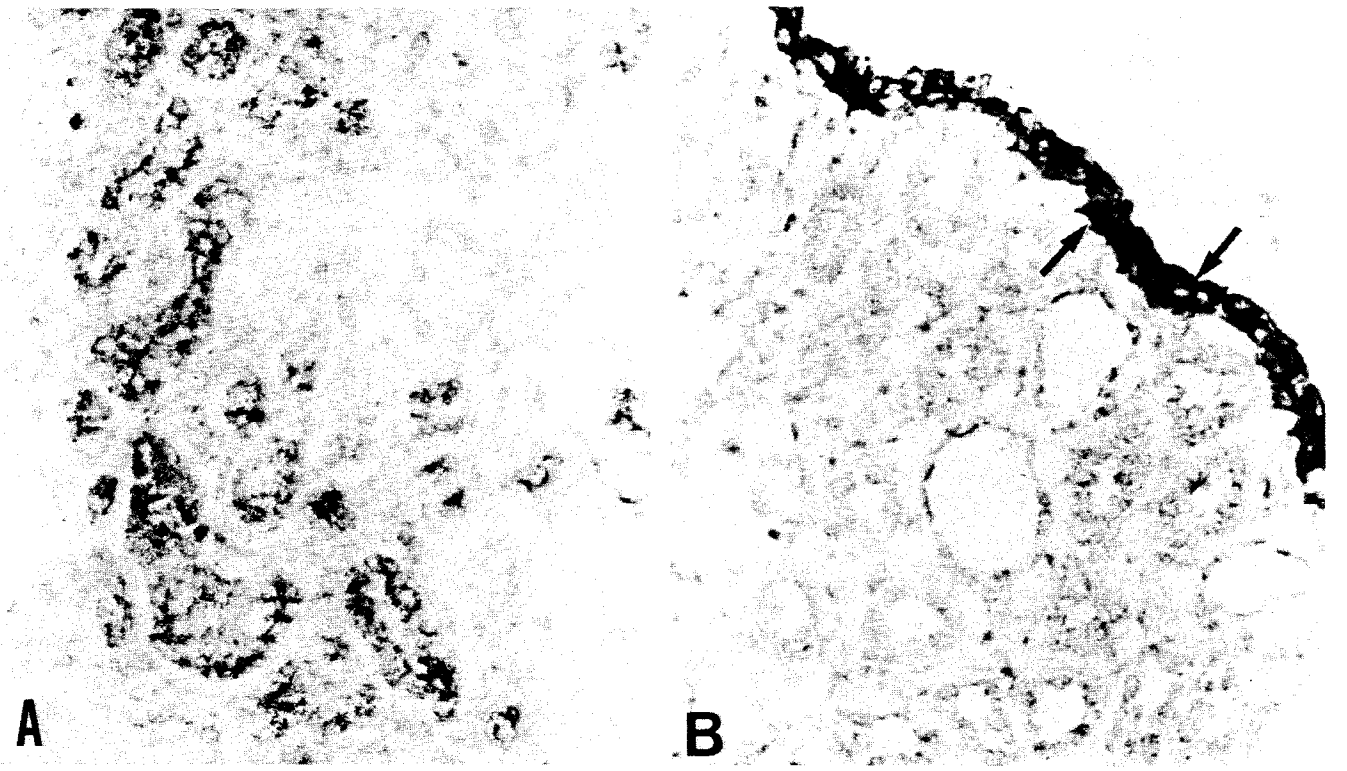

Fig. 4. A. *Atrophic tubules of the stenotic kidney display a weak staining for keratin (immunoperoxidase on fresh frozen tissue, × 1,478).* **B.** Intact tubules of the contralateral kidney show a negative result; however, a strong positive staining is noted for the transitional epithelium of renal pelvis (arrows) (immunoperoxidase on fresh frozen tissue, × 1,478).

interstitial inflammatory infiltrates, followed by the atrophic areas and the non-atrophic areas of the contralateral kidneys, and the control kidneys, respectively. The percentage of the ED_1^+ cells, $OX8^+$ cells, and $W3/25^+$ cells, respectively, were roughly similar for all four groups of lesions. However, different percentages were noted for other phenotypes; thus, the percentage of OX_4^+ cells in the stenotic kidneys was significantly higher than those of other types of lesions, whereas the percentage of the $OX3/13^+$ cells in these kidneys was the lowest.

Discussion

Although experimental renal artery stenosis has been created in several animal species, including the rat, for the study of hypertension, careful morphologic studies of the resulting structural lesions have not been carried out [7–12]. This benign neglect is surprising, considering the widespread belief that ischemic damage is one of the final common pathways of evolution for several renal diseases of diverse initial etiologies [2, 5, 6]. The failure to adequately characterize the structural changes induced by ischemia may well account for the poor understanding of the mechanism by which ischemia causes renal damage.

Unilateral renal artery stenosis in the rat model utilized in this study resulted in well-established hypertension, and diffuse tubulointerstitial changes in the stenotic kidneys, as well as focal tubulointerstitial damage and compensatory hypertrophy in the contralateral kidneys. These changes have been described in humans with unilateral renal artery stenosis due to atherosclerosis or vascular dysplasia [8–10]. Although the diffuse tubulointerstitial lesion of the stenotic kidney is clearly related to ischemia, the pathogenesis of the focal lesion of the contralateral kidney is intriguing. We have speculated that this lesion is also of ischemic nature for the following reasons. (1) Although this lesion is small and focal, its morphological features, including the interstitial lymphocyte phenotypes and antigen profile of the involved tubules, are almost identical to those of the ischemic lesion seen in the stenotic kidneys. (2) This lesion is morphologically similar to those seen in human unilateral renal arterial stenosis, which is thought to be at least partially caused by ischemia secondary to hypertension-induced vascular changes [8–10]. In that aspect, it is noted that examination of several organs of the experimental animals (lung, heart, thymus, muscle and liver) did not show the degree of focal interstitial infiltrate and parenchymal atrophy that was seen in the kidneys. The changes in the stenotic kidneys of the rats closely simulate those observed in human kidneys with unilateral renal arterial stenosis, both sharing some distinctive features, including diffuse tubular atrophy and tubular simplification, with a disproportionally mild degree of interstitial fibrosis, and a surprisingly good preservation of glomerular structures [10]. However, the kidneys in this experimental model displayed a marked interstitial inflammation which has been only infrequently noted in the human counterpart [10].

The renal lesions in our model are not related to several factors discussed below. Although the possibility of ascending infection cannot be completely ruled out without urine culture,

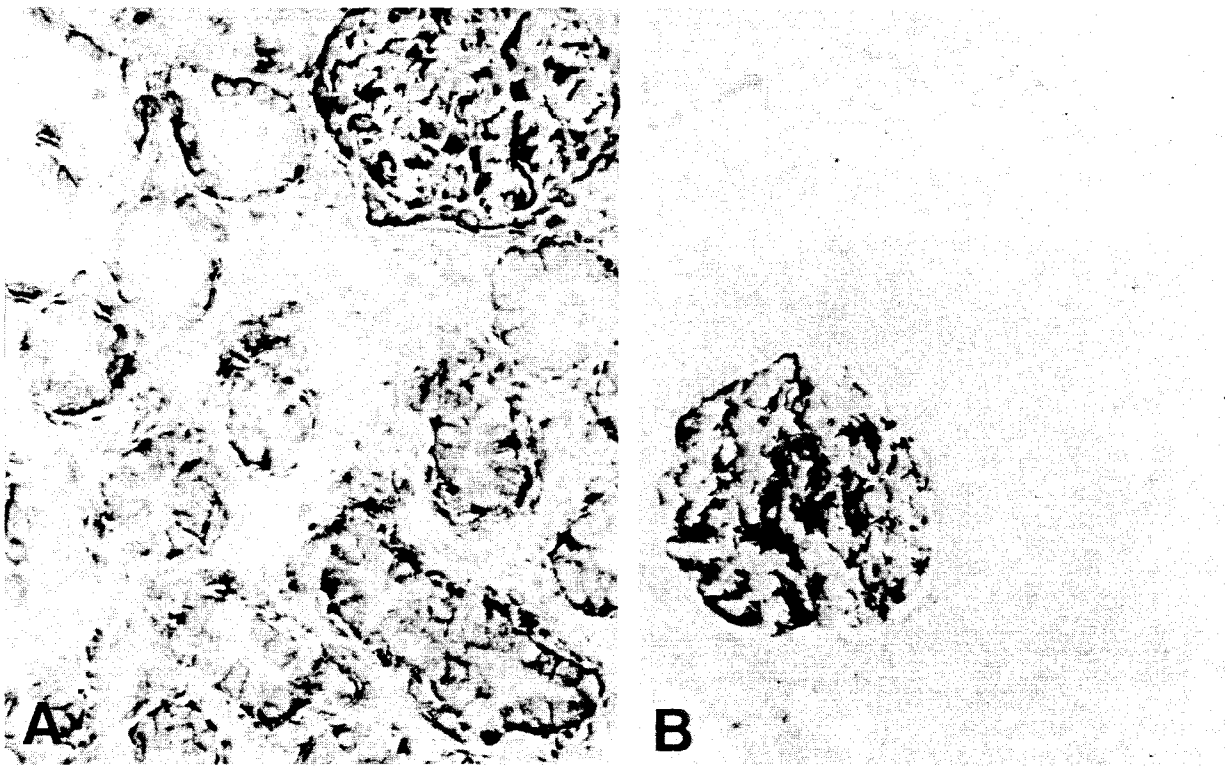

Fig. 5. A. *Atrophic tubules of the stenotic kidneys display a strong staining for vimentin; staining of glomerular cells, most probably visceral epithelial cells, is also noted (immunoperoxidase, × 1,478).* **B.** Intact tubules of the contralateral kidney show a negative result, whereas glomerular staining similar to that of the stenotic kidney is present (immunoperoxidase, × 1,478).

this potential cause of interstitial inflammation is most unlikely in this model because: (1) significant inflammation was never observed in the non-atrophic areas of the contralateral kidneys or in renal pelvis of either side, and (2) plasma cells and neutrophils which would suggest an infectious etiology, at least in early stage, were not a component of the inflammatory infiltrate. The possibility of physical manipulation of the kidney or infection acquired during surgery as the causes of the tubulointerstitial changes can be ruled out by the observation that the same renal lesions were observed in experimental rats that underwent either sterile or non-sterile surgery, and that the control sham-operated animal, regardless of surgical technique, did not express these renal lesions. Although spontaneous nephropathy has been well documented in Sprague-Dawley rats, it is seen exclusively in aged animals (older than 16 to 30 weeks) [29–32]. All the rats used in our study are young and the time of sacrifice were less than 90 days in age, moreover, the renal lesions induced in our model bear little morphological resemblance to those described in spontaneous nephropathy [29–33]. These above considerations suggest that at least in the rat, ischemia alone can be associated with severe CTIN. A similar ischemic mechanism may partially explain why significant interstitial lesions are frequently seen in advanced renal diseases of diverse etiologies [12].

Our study suggests that ischemia not only caused marked morphologic alterations of the tubules, but was also associated with profound changes in the antigenic profile of the tubules, especially the proximal tubules. These changes, which involve both the cytoskeletal and cell membrane proteins, include a neo-expression of vimentin and keratin, a loss or decrease of membrane glycoproteins, a loss of Ia-related antigen on cell surface and a loss of endogenous avidin binding activity. Vimentin, a member of the family of intermediate filaments, is expressed by embryonic renal tubular cells, but gradually disappears when the tubules become mature [23, 26, 34]. Keratin is another member of the intermediate filament family, and is composed of at least 19 subtypes, only some of which are present in a specific tissue. Our study showed that although vimentin and the subtypes of keratin detectable by the utilized antibodies were not present in normal tubules, they were clearly expressed by the atrophic tubules. Renal tubular cell membrane is rich in glycoproteins. These glycoproteins can be recognized by immunohistochemical staining with lectins, which are proteins derived from plants that bind to specific sequences of carbohydrates in the backbone of cellular glycoproteins. The lectin histochemical staining in this study demonstrates that atrophic tubules show a marked decrease or complete loss of some membrane glycoproteins. These changes, however, are not specific for ischemic damage, and at least some of them have been described in other conditions including tubular damage associated with adriamycin toxicity or protein overload and induced proteinuria [23, 26]. Whether the tubular injury precedes these antigenic changes or is consequent to it cannot be determined from the present study. Nevertheless, these changes may potentially initiate an immune

Fig. 6. A. *Intact proximal tubules (P) and some glomerular cells (arrows) of contralateral kidney show a strong staining for Ia antigen; distal tubules (D) are not stained (immunoperoxidase, × 1,478).* **B.** The atrophic tubules (t) of the stenotic kidney are not stained, whereas a strong staining is noted for interstitial inflammatory cells (immunoperoxidase, × 1,478).

reaction and account for the presence of interstitial inflammation [35].

Another interesting finding in this model is the presence of marked tubulitis. Although tubulitis has not been emphasized in any experimental models, it has been demonstrated repeatedly in renal allograft rejection, and less frequently in a variety of human renal diseases with a component of tubulointerstitial damage [36–38]. In renal allografts, the inflammatory cells in tubulitis have been thought to represent natural killer cells or sensitized cytotoxic T cells effecting cell-mediated rejection. In other renal diseases, the significance of tubulitis has not been elucidated [36–38]. In this model, the ubiquitous presence of tubulitis may be related to: (1) a profound alteration in the antigenic profile of tubular cells, which initiates a cell-mediated immune mechanism [29, 30]; (2) an ischemia-induced expression of chemotactic factors or adhesion molecules, which attracts the migration of lymphocytes [39–42]; or (3) a passive phenomenon associated with widespread interstitial inflammation.

Interstitial fibrosis is a defining feature of CTIN. It is has been described in association with ischemic damage in human kidneys [8, 10] and was observed in this experimental model. By what mechanism ischemia, or other forms of CTIN, causes interstitial fibrosis is not clear [8, 10]. In the final analysis, interstitial fibrosis, to a large extent, must be related to an increase in collagen synthesis by interstitial fibroblasts, which in turn are regulated by cytoactive peptides including platelet-derived growth factor, insulin-like growth factor, transforming growth factor, epidermal growth factor and fibroblastic growth factor [39, 40, 43–45]. Most of these factors are known to be released by inflammatory cells, and some of then have been localized in normal tubular epithelial cells as well [46–50]. Whether there is an over-expression of the peptide growth factors by damaged tubular cells is not known. Nevertheless, tubular cells have been shown to promote the proliferation of fibroblasts co-cultured with them in separate but communicating chambers, through an epithelial cell secretion of a mediator related to insulin-like growth factor [51]. Alternatively, infiltrating ischemia-induced inflammatory cells may account for the release of the growth promoting peptides.

Although severe interstitial inflammation was noted in our model, its histogenesis is not clear. The absence of electron dense deposits in EM studies, and of immune complex in IF studies in both glomerular and tubulointerstitial compartments in our study, a situation also noted in humans [8, 10], mitigates against a role of immune complex mechanism. However, the possibility of a cell-mediated mechanism can be raised and interstitial mononuclear cell phenotyping was done to assess this possibility. Similar phenotyping studies have been performed on rat kidneys in other models including cyclosporine nephrotoxicity [21], acute unilateral obstruction [22], interstitial nephritis due to protein overload proteinuria [26], autoimmune interstitial nephritis [24], 5/6 nephrectomy [25], acute and chronic aminonucleoside nephrosis [23], and experimental focal segmental sclerosis [28]. Although the staining techniques and the methods of cell quantitation in these studies are different

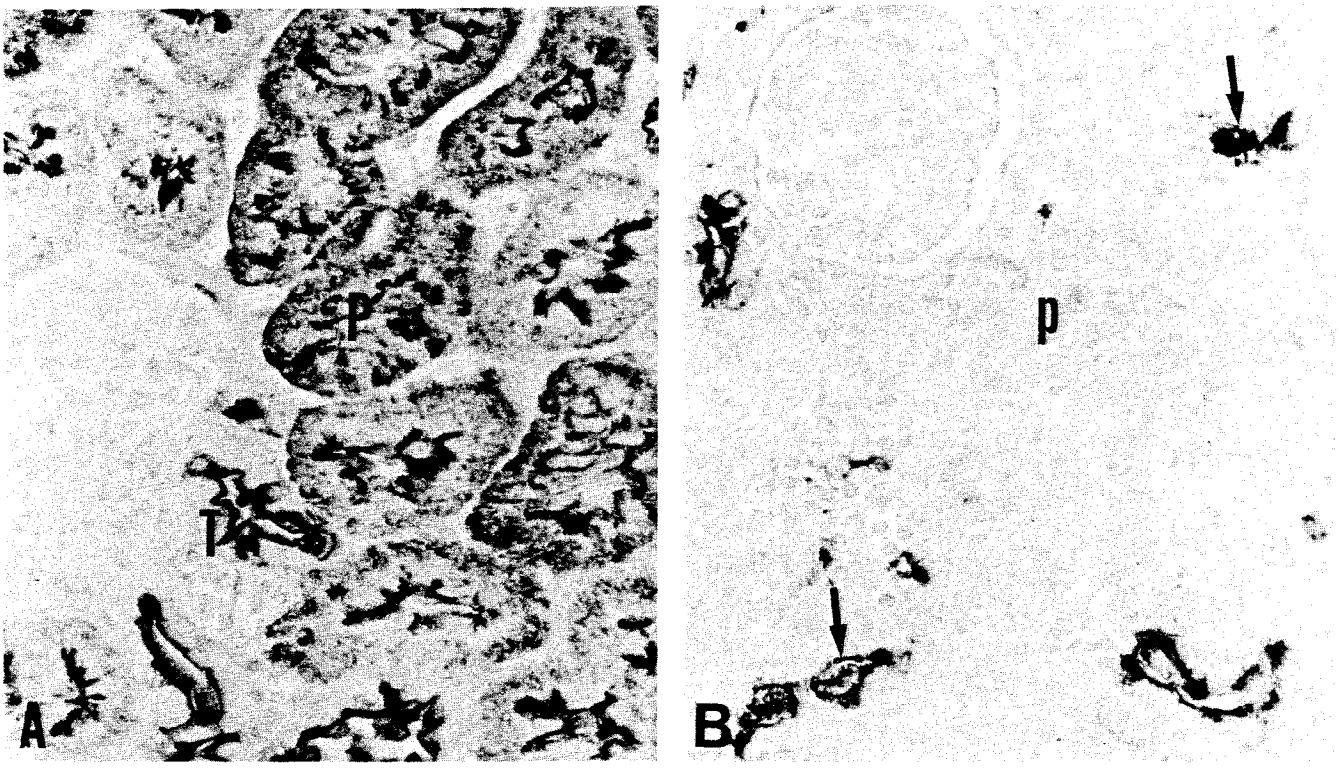

Fig. 7. A. *Both intact proximal (P) and distal or collecting tubules (T) of the contralateral kidneys are stained by Dolicho biflores but in different patterns (immunoperoxidase, × 1,478).* **B.** The atrophic proximal tubules (p) of the stenotic kidneys are not stained, however, the adjacent distal or collecting ducts with tubular casts (arrows) are stained positive (immunoperoxidase, × 1,478).

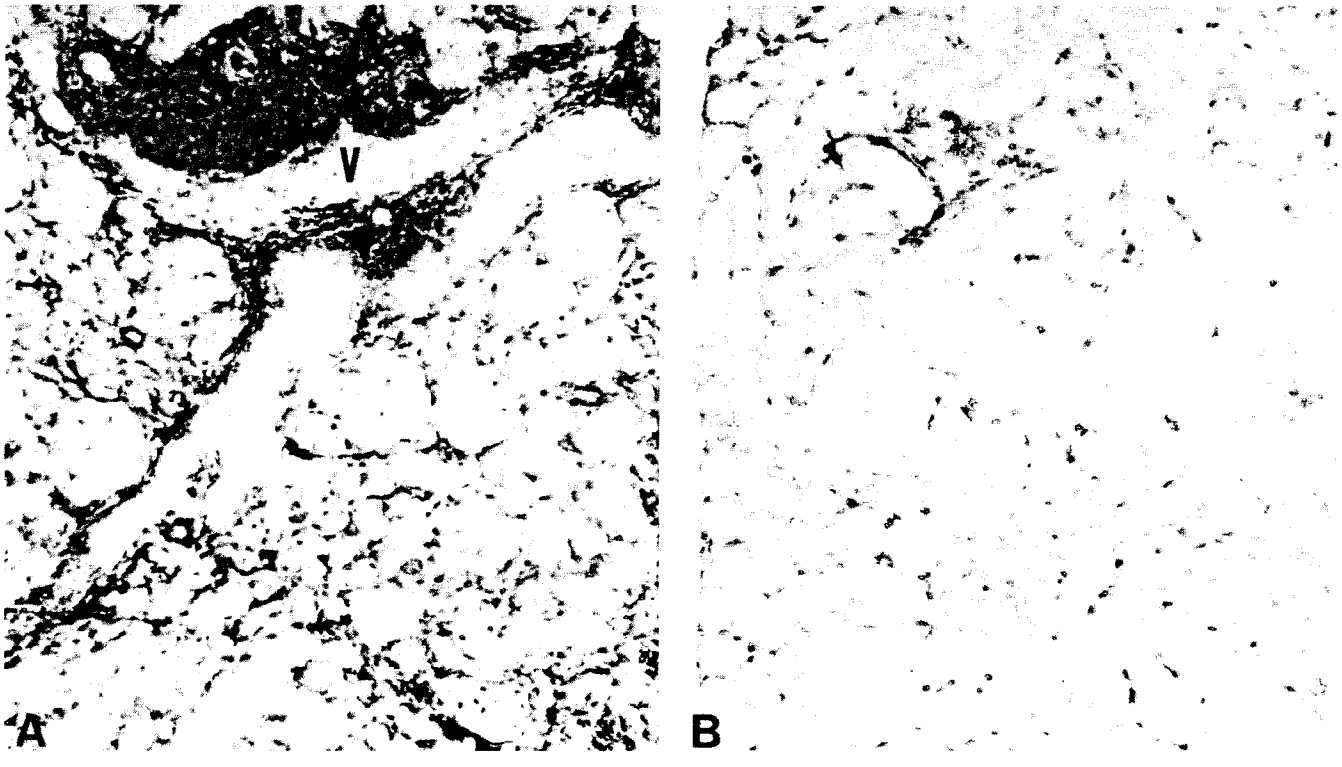

Fig. 8. A. *Stenotic kidney.* Interstitial inflammatory cells stained positive for leukocyte common antigen (LCA) are present, with a perivascular (V) accentuation (immunoperoxidase, × 739). **B.** Contralateral kidney. Several LCA positive cells are seen in two small foci of tubulointerstitial damage (upper left). These cells are scanty in the surrounding areas (immunoperoxidase, × 739).

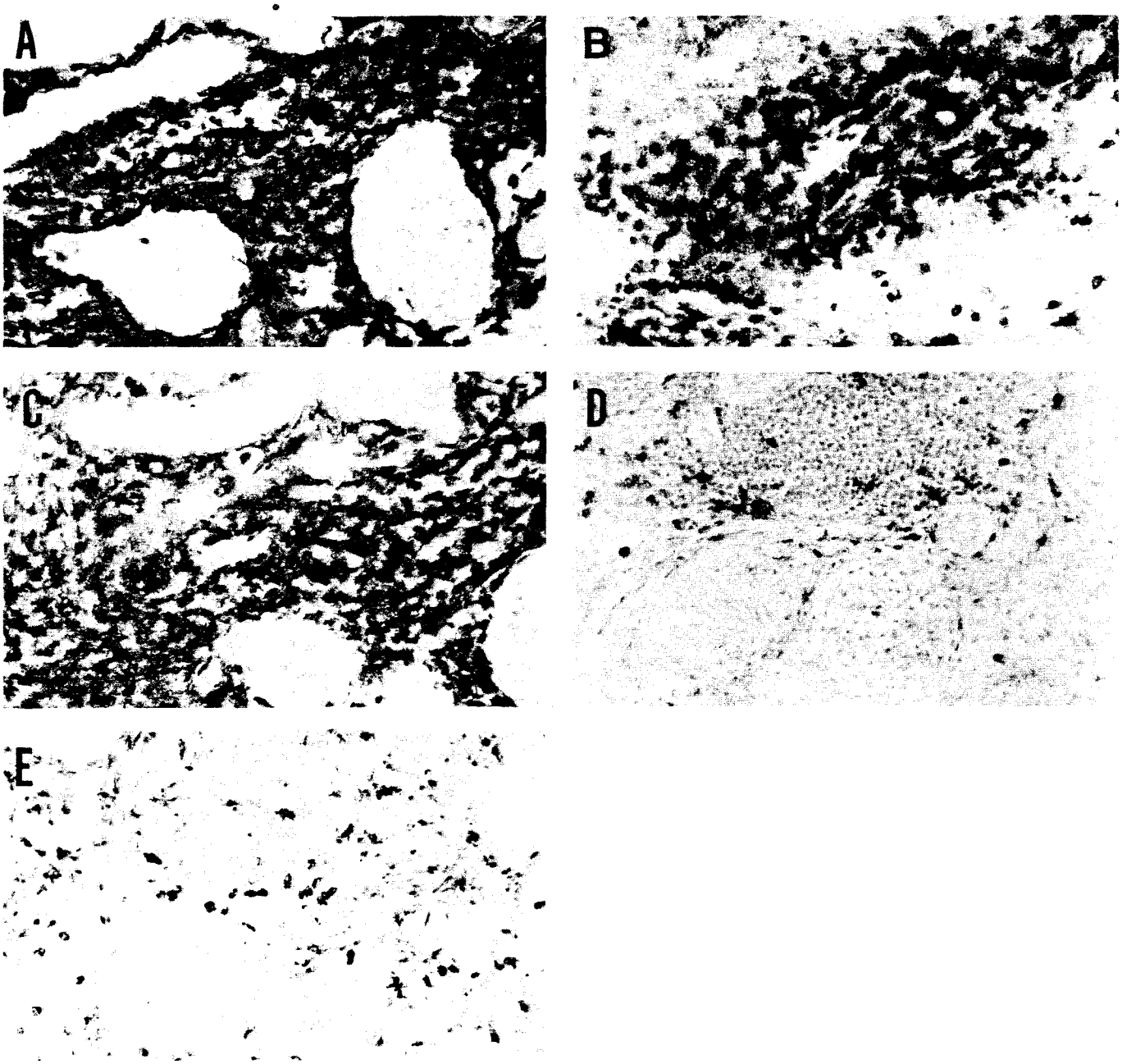

Fig. 9. **A.** *Stenotic kidney*. Consecutive sections of an area of interstitial inflammation stained for different markers of inflammatory cells. **A.** Most of the inflammatory cells are stained positive for Ia antigen (OX4 antibody). **B.** W3/13 antibody (mostly for T lymphocytes). **C.** W3/25 antibody (mostly for T helper cells). **D.** OX8 antibody (mostly for t non-helper cells). **E.** ED1 antibody (mostly for macrophages) (immunoperoxidase, × 739).

from those in our study, the same antibodies have been utilized [21–28]. When the results of these studies are compared to one another and to those of the present study, some phenotypic differences are noted; for example Gillum et al [22] noted that T helper and T non-helper cells represented the most and the least frequent phenotype, respectively, in a model of chronic cyclosporin nephrotoxicity. In contrast Eddy suggested that T non-helper cells were more numerous than T helper cells in interstitial nephritis induced by protein overload proteinuria. Careful review of data reported in these studies [21–28], however, has shown that the noted differences are due mainly to differences in interpretation of the specificity of the utilized antibodies. Indeed, when the raw numbers of each cell type stained positive by individual antibodies are compared, remarkable similarities are noted [21–28]. Thus, in all studies except one [22] the $W3/25^+$ cells and the $OX8^+$ cells represented the most and the least numerous phenotype, respectively, with the W3/13 and OX_1 phenotypes falling in between [21, 23–26]. Moreover, in the four studies including the current one, when the antibody OX_4 was used to identify B cells [21, 23, 26], a high percentage of interstitial inflammatory cells stained positive by this antibody. This is in sharp contrast to that of human

tubulointerstitial lesions where, without exception, B cells represent the smallest population [52–62]. These interpretational difficulties, common to all phenotypic studies of rat renal tissue, do not detract from the observation that there is remarkable uniformity of cell phenotypes which constitute the renal interstitial inflammatory infiltrates of diverse lesions.

The pathogenesis of the interstitial inflammation in ischemic disease, as well as whether the inflammatory cells reflect a migration of blood-borne cells to the kidneys or the proliferation of a resident population of cells, is not clear. Data obtained from this study and from the literature suggest the blood-borne inflammatory cells are recruited to the kidney through several possible mechanisms: (1) ischemia-induced changes of tubular antigenic profiles may initiate a cell-mediated immune reaction [34, 35]; (2) ischemic tubules may release inflammatory mediators, which in turn recruit inflammatory cells. It should be noted, however, that most inflammatory mediators known thus far are released by inflammatory cells, but not by parenchymal cells [39]; (3) overproduction of ammonium, known as a form of tubular adaptation in chronic tubular damage including that associated with ischemia [63], has the capability to activate the complement system, resulting in the activation of the C3-9 membrane attack complex [64, 65]. This complex, in turn, is known to be a powerful inflammatory mediator [40]. Although renal tissue from our model was not assessed for the presence of C3-9 complex, its presence has been documented in several forms of experimental or human CTIN [26, 66]; and (4) protein overload proteinuria in rat has been reported to be associated with interstitial inflammation, the phenotype of which was similar to that of our model [26]. The interstitial inflammation was thought to represent a reaction to tubular cell damage by tubular protein overload [26]. Although proteinuria was not evaluated in our model, proteinuria, at least of a mild degree, is noted to be a frequent feature of ischemic renal disease in humans [67]. From our model and other reported models of chronic tubulointerstitial damage, it appears that tubular damage, regardless of etiology, can initiate a reactive interstitial inflammation. Although this hypothesis needs further testing, it is supported by several observations: (1) Interstitial inflammation, though occasionally mild, is a constant accompaniment of tubular damage [2]. (2) Regardless of the primary diseases, the final morphologic changes of the tubule are rather non-specific but uniform [1, 2] including the ubiquitous neo-expression of vimentin [28, 27], so are the phenotypes of the accompanying interstitial inflammation [22–27, 51–62]; and (3) in fact, even in tubulointerstitial nephritis induced by injection of bovine tubular membrane antigen into Brown-Norway rats—an unequivocal example of antibody mediated CTIN—a late phase of mononuclear cell interstitial infiltration occurs, the phenotypes of which are similar to those of the present model [25].

In summary, unilateral artery stenosis in rats induced a CTIN characterized by a profound change in the morphology and the antigenic profile of tubular epithelium, and a severe interstitial inflammation. Phenotyping by monoclonal antibodies revealed that the inflammation was composed of T helper cells, B cells, macrophages and a scanty number of T non-helper cells. These phenotypes were remarkably similar to those of previously-reported tubulointerstitial nephritis of diverse etiologies. Ischemia may well be responsible for the changes frequently described in CTIN.

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