Kidney International, Vol. 59 (2001), pp. 941–949

Role of CD8$^+$ cells in the progression of murine adriamycin nephropathy

YANG WANG, YI PING WANG, YUET-CHING TAY, and DAVID C.H. HARRIS

Department of Renal Medicine, University of Sydney at Westmead Hospital, Westmead, New South Wales, Australia

Role of CD8$^+$ cells in the progression of murine adriamycin nephropathy.

Background. Many studies have shown that interstitial inflammation in human and experimental renal disease is characterized by T-cell infiltration, but published data on the involvement of inflammatory cell subsets in progressive tubulointerstitial lesions are often conflicting. A previous study suggested a role for cytotoxic T lymphocytes in the damaging effect of CD4$^+$ T-cell depletion in murine adriamycin (ADR) nephropathy, a model of focal segmental glomerulosclerosis (FSGS), and tubulointerstitial inflammation. The aim of this study was to investigate the role of CD8$^+$ cells in this model.

Methods. Male BALB/c mice were treated with five intraperitoneal injections of anti-CD8 monoclonal antibody (mAb), beginning from five days after ADR treatment, when overt proteinuria was established. Seven mice in each of groups A (ADR + mAb), B (ADR only), and C (saline treated, age matched) were sacrificed at week 6. Changes in renal function and histopathological features were assessed. Tubulointerstitial inflammation and glomerular inflammation were examined immunohistochemically.

Results. mAb treatment reduced CD8$^+$ cell levels to <2% of normal in spleen. Proteinuria in group A was no different from that in group B at week 6, but was markedly higher than in group C. Creatinine clearance was significantly ameliorated by anti-CD8 treatment (71.8 ± 4.9 μL/min vs. 29.2 ± 2.8 in group B and 81.9 ± 3.7 in group C). Morphometric analysis showed less FSGS in group A compared with group B (6.5 ± 1.9 vs. 13.0 ± 2.8, $P < 0.001$), as well as less tubular atrophy (indicated by increased ratio of tubule cell height to tubular diameter, 0.25 ± 0.24 in group A vs. 0.04 ± 0.02 in group B, $P < 0.05$). CD8 depletion also reduced interstitial expansion (6.3 ± 2.2% vs. 16.4 ± 3.1 in group B, $P < 0.001$) and fibrosis ($P < 0.01$). Macrophage infiltration in tubulointerstitium was less in group A than in group B ($P = 0.052$). The number of interstitial CD4$^+$ cells appeared to increase after anti-CD8 treatment, but was not statistically different between groups A and B.

Conclusion. Anti-CD8 treatment protects against renal functional and structural injury in this murine model of chronic proteinuric renal disease.

Key words: focal segmental glomerulosclerosis, interstitial damage, proteinuria, lymphocyte, natural killer cell, macrophage, cytokine.

Received for publication June 23, 2000
and in revised form September 18, 2000
Accepted for publication September 22, 2000

© 2001 by the International Society of Nephrology

Studies in several models of inflammatory renal disease have demonstrated that sensitized T cells were an important component of the renal lesion [1–4] and could passively transfer disease to tolerant hosts [5, 6]. Specifically, CD8$^+$ T cells have been shown to play an important effector role in various types of immune-mediated renal disease [5, 7–9]. Penny, Boyd, and Hall demonstrated that renal injury in Heymann nephritis is mediated by CD8$^+$ cytotoxic T-lymphocytes (CTL) [8]. Furthermore, CD8$^+$ T cells were regarded as the ultimate effector T cells of glomerular injury in nephrotoxic serum nephritis [9] and interstitial damage in antitubular basement membrane (TBM) disease [7]. However, to the best of our knowledge, no study has examined the role of CD8$^+$ cells in the progression of adriamycin (ADR)-nephropathy.

Previous studies in our laboratory have suggested a pathogenetic role for CD8$^+$ cells in ADR nephropathy in BALB/c mice, a robust model of human focal segmental glomerulosclerosis (FSGS), and one usually regarded as nonimmunological [10]. The prominence of CD4$^+$ and CD8$^+$ cells in the tubulointerstitium in this model suggested that these cells might play an important part in the pathogenesis of the interstitial lesion of ADR nephropathy. In vivo CD4$^+$ cell depletion aggravated the histologic and functional damage and was associated with an increase in interstitial CD8$^+$ cells and macrophages (unpublished data). The results raised the possibility that interstitial CD8$^+$ cells might play a key role in the progression of chronic renal disease in mice with ADR-induced nephrosis.

In the current study, to test the hypothesis that interstitial CD8$^+$ cell inflammation modulates the expression of chronic proteinuric renal disease, we determined the effect of in vivo depletion of CD8$^+$ cells on the functional and pathological progression of established murine ADR nephropathy.

METHODS

Animals

BALB/c mice (supplied by the Animal Care Facility, Westmead Hospital, NSW, Australia) were bred and main-

tained free of specific pathogens and under constant temperature (22°C) and humidity, on a 12-hour light/dark cycle in the Animal House of Westmead Hospital. They were given acidified water and commercial mouse chow (protein 18.9%; Glen Forrest Stockfeeders, Glen Forrest, WA, Australia) ad libitum. Seven- to eight-week-old (20 to 25 g body wt) male mice were used in all groups at the commencement of the experiments.

Mice were divided into three groups. Group C was the negative control group in which mice were treated with isotonic saline. Group B was the positive control treated with ADR (doxorubicin hydrochloride; Pharmacia & Upjohn Pty Ltd., Perth, Australia) by a single injection of 10 to 11 mg/kg body weight via the tail vein of each nonanesthetized mouse. Group A was the experimental group in which mice were treated with ADR plus anti-CD8 monoclonal antibody (mAb). All control and treated mice were housed individually in one plastic cage after treatment. Samples from seven mice in each group were obtained.

Monoclonal antibody administration

Mice in group A were injected intraperitoneally with 500 μg rat anti-mouse CD8 mAb (hybridoma 53-6.7) per mouse each time [11–13]. Mice were inoculated commencing on day 5 after administration of ADR, when overt proteinuria had already appeared. Mice were then reinoculated intraperitoneally with the same volume of mAb on days 6, 7, 21, and 35, to maintain the depleted state until sacrifice on day 42. On the same days, equal volumes of rat immunoglobulin (Sigma Chemical Company, St. Louis, MO, USA) were given to negative and positive control mice. The extent of $CD8^+$ cell depletion was assessed by fluorescence-activated cell sorter (FACS). Depletion was invariably >98% during entire experimental period.

Tissue preparation

All mice were sacrificed at day 42 after the day of ADR treatment (day 0). Prior to sacrifice, animals were anesthetized using a mixture of ketamine and xylazine (ketamine, 100 mg/kg body weight; xylazine, 8 mg/kg body weight) intraperitoneally. After cardiac puncture for blood sample collection, animals were completely exsanguinated via the abdominal aorta before kidney and spleen harvesting to minimize the number of circulating inflammatory cells remaining in the tissue. Kidney weight and body weight were measured immediately. All kidney and spleen specimens were processed without delay. Each kidney was divided into two pieces. One piece was fixed in 10% neutral-buffered formalin solution and embedded in paraffin. For immunohistochemical (IHC) staining, the other piece was frozen in a plastic mold.

FACS analysis

Antibodies used for flow cytometry are listed in Table 1. Spleen cells from individual mice were obtained at day 42. All of the following steps were performed on ice or at 4°C to prevent capping off of membrane molecules, unless stated otherwise. Spleen was crushed using a syringe plunger, and splenocytes were separated in RPMI 1640 medium (GIBCO BRL Life Technologies Inc., Grand Island, New York, USA) with 10% (vol/vol) fetal calf serum (FCS; Trace Biosciences Pty Ltd., Castle Hill, NSW, Australia). Suspensions were then separated by centrifugation at $200 \times g$ for five minutes in a Laboratory 4K15 Centrifuge (Quantum Scientific Pty Ltd., Milton, QLD, Australia). After lysing red blood cells using lysing buffer [150 mmol/L ammonium chloride, 10 mmol/L potassium carbonate, 0.1 mmol/L Na_4-ethylenediaminetetraacetic acid (EDTA), pH 7.5], the cells was resuspended in 2 mL of RPMI 1640 medium with 10% fetal calf serum (FCS) and 1% NaN_3. After staining with 1% trypan blue dye (Sigma Chemical Company), the number of viable cells was determined using a counting chamber. Cell suspensions containing 3×10^5 splenocytes were placed in Falcon FACS tubes (Becton Dickinson, Lincoln Park, NJ, USA), incubated with 20% normal goat serum (Sigma) to block the cell surface Fc receptors, and then incubated with 2 μL of the relevant antibodies for 30 minutes in the dark. After washing twice, cells were fixed with 2% (wt/vol) paraformaldehyde and stored in the dark at 4°C until analysis. Meanwhile, isotype control antibody was used to obtain background fluorescence values.

The samples were analyzed using Becton Dickinson Immunocytometry Systems Version 1.1 (Becton Dickinson, San Jose, CA, USA). Data were collected on 10,000 cells and are shown as contour diagrams.

Histology

Paraffin embedded kidney was sectioned at 4 micron (μ) onto poly-L-lysine (Sigma)-coated slides. The slides were stained with periodic acid-Schiff (PAS), hematoxylin and eosin (HE), and Masson's trichrome. The PAS stain was used to evaluate glomerulosclerosis. The HE stain was used to enumerate nuclei in glomeruli and cellular infiltration in interstitial areas. Masson's trichrome was used to evaluate fibrosis in tubulointerstitium.

Morphometric studies were performed quantitatively using Optimas image analysis system (Optimas Corporation, Seattle, WA, USA). Parameters evaluated were glomerular surface area, glomerular capillary area, mesangial matrix area, number of nuclei per glomerulus, interstitial volume, tubular diameter, and tubule cell height. Glomerulosclerosis was defined as the percentage increase in relative mesangial matrix area (PAS-positive area within the glomerulus divided by the glomerular capillary area, magnification ×400) [14]. The degree of

Table 1. Antibodies used in FACS and IHC staining

Clone	CD-antigen	Cell specificities	Sources
H129.19	Rat anti-mouse L3T4	$CD4^+$ cells	Sigma
53-6.7	Rat anti-mouse Ly-2	$CD8^+$ cells	PharMingen, Inc.
M3/84	Rat anti-mouse Mac-3	Macrophages	PharMingen, Inc.
RA3-6B2	Rat anti-mouse CD45R/B220	Pan-B cells	PharMingen, Inc.
R35-95	Rat IgG_2a, κ	Isotype standard	PharMingen, Inc.

interstitial expansion was quantitated using the relative interstitial volume on PAS-stained sections. Tubular atrophy was defined as the presence of dilated tubules with cellular degeneration (low cell height and absence of brush border) and thickened, wrinkled basement membrane. To quantitate tubular atrophy, the cross-sectional tubular diameter and tubule cell height were measured, as described previously [15]. Mean values were calculated from each of 20 glomeruli or tubules or 10 random cortical regions per section, as described previously [10].

In each biopsy, a semiquantitative score was used to evaluate the degree of interstitial fibrosis in a blinded fashion on Masson's trichrome-stained sections. The cortical interstitium was defined as the peritubular space and included the TBM and peritubular capillaries, as described previously [15]. Interstitial fibrosis was defined as the area occupied by trichrome stain positive interstitium [16, 17]. A minimum of 10 consecutive fields at a magnification of ×400 were assessed and scored in each section. The degree of interstitial fibrosis was estimated by evaluating the percentage of interstitial fibrosis per field and was graded on a scale of 0 to 4 [18, 19]: 0 = normal; 0.5 = small focal areas of fibrosis; 1 = fibrosis involving less than 10% of the cortical interstitium; 2 = involvement of up to 25% of the cortical interstitium; 3 = involvement of up to 50 to 75% of the cortical interstitium; and 4 = extensive fibrosis involving more than 75% of the cortical interstitium.

Immunohistochemistry

Primary antibodies used in immunohistochemistry are also shown in Table 1. The secondary antibody was a biotinylated rabbit anti-rat immunoglobulin (Dako® Corporation, Carpinteria, CA, USA). For immunohistochemistry, a piece of transverse section from each kidney or spleen was placed in prelabeled Tissue-Tek® Specimen Mold (10 × 10 × 5 mm; Miles Inc., Elkhart, IN, USA), surrounded with OCT Compound (Sakura Finetek Inc., Torrance, CA, USA). The mold with tissue was carefully immersed into liquid nitrogen for about 20 seconds until the block solidified. Five-μ sections were cut at −22°C in a MICROM HM505E cryostat and immediately placed on double-coated slides. After air drying for 24 hours, cryostat sections were fixed in cold Zamboni's fixative [20] and acetone. The avidin-biotin complex (ABC) technique was used as follows. Endogenous peroxidase activity was blocked by incubating sections for 15 minutes in 0.3% (vol/vol) H_2O_2 solution in phosphate-buffered saline. Blocking of endogenous avidin binding activity was achieved by incubating the sections with Biotin Blocking System (Dako). The slides were then washed for five minutes in Tris-buffered saline [TBS; 0.8% Tris-HCl (Sigma) and 0.9% NaCl (Sigma), pH 7.6] and incubated for 15 minutes in blocking reagent [TBS + 10% (vol/vol) normal rabbit serum; Dako]. The incubation with relevant primary antibodies was carried out for one to two hours at room temperature (RT). Control cryostat sections were incubated at the same time with control antibody (normal rat immunoglobulin). After rinsing twice in TBS for five minutes each, sections were incubated with secondary antibodies for 30 minutes at RT. This was followed by rinsing in TBS and incubation with ABC complex (Zymed Laboratories, Inc., San Francisco, CA, USA) for 30 minutes; 3,3′-diaminobenzidine (DAB) substrate-chromogen solution (Dako) was applied for less than five minutes and then washed thoroughly in distilled water. Slides were counterstained with hematoxylin (Sigma), dehydrated, and cover slipped. All specimens were stained in duplicate. To avoid false negative staining, a section of spleen was placed and stained on every slide as a positive control. Positive immunoreactivity resulted in brown staining of the cell membrane. Normal nuclei and cytoplasm were stained lightly. For assessment of interstitial infiltration, the positively stained cells located in the tubulointerstitial area only were counted from five random cortical fields (magnification ×400) in each section, and the numbers were averaged for each field. Glomerular macrophage infiltration was evaluated as the number of macrophages per 10 glomerular cross-sections.

Renal function

Spontaneously voided urine of each animal was collected for 12 hours in a metabolic cage on the day before sacrifice, with access to water only. Urinary volume and protein were measured. Blood samples for serum albumin and creatinine were obtained by cardiac puncture just prior to exsanguination. Total urinary protein was quantitated using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) based on the Bradford dye-binding procedure [21], according to the manufacturer's instructions in a DU-68 Spectrophotom-

eter (Beckman Instruments Inc., Irvine, CA, USA), for which purified bovine serum albumin (BSA; Sigma) at concentration ranges of 0.2 to 1.4 mg/mL was used as a reference standard. Serum albumin and plasma and urinary creatinine were analyzed by the Institute of Clinical Pathology and Medical Research, Westmead Hospital, using a BM/Hitachi 747 analyzer (Tokyo, Japan). All samples were tested in duplicate.

Renal function was assessed by serial measurements of plasma and urinary creatinine. Creatinine clearance (C_{Cr}) was determined according to the conventional clearance formula, $C_{Cr} = (U_{Cr} \times V)/P_{Cr}$, where U_{Cr} is the concentration of creatinine in urine, P_{Cr} is the concentration of creatinine in plasma, and V is the urine flow rate in milliliters per minute.

Statistical analysis

All results are expressed as the group mean ± SD. Statistical analysis was performed using SPSS 8.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used for multiple comparisons. A P value of less than 0.05 was considered statistically significant.

RESULTS

Preliminary experiments in our laboratory using FACS analysis showed that a single injection of 500 μg 53-6.7 mAb in BALB/c mice resulted in a selective depletion of $CD8^+$ cells to less than 2% of total splenocytes within 48 hours of mAb infusion and that the elimination of $CD8^+$ cells could be maintained for more than three weeks. In a group of age-matched BALB/c mice treated with 53-6.7 mAb using the same dosage and time course as group A, no functional or histologic abnormalities were observed (data not shown). In the present study, analysis of splenocytes at completion of the experiment (6 weeks after ADR administration) showed that all mice treated with 53-6.7 mAb had a greatly reduced number of $CD8^+$ cells compared with mice treated with rat immunoglobulin (Fig. 1). In contrast, the number of $CD4^+$ T cells in these treated mice increased to about 50% above that of normal mice at week 6. This result is in agreement with that reported by Ghobrial et al [22].

All ADR-treated animals developed renal injury characterized by proteinuria, FSGS, tubular atrophy, and interstitial damage. Kidneys of mice in group A were hypertrophied, whereas those in group B were pale and atrophic compared with that of normal animals. The kidney weight was significantly higher in group A in comparison with other two groups (Table 2).

Overt proteinuria appeared about five days after the ADR administration and remained at a high level throughout the study period in both groups A and B, as evaluated by urinalysis. Blood and urine parameters at the end of experiment are summarized in Table 2. Urinary protein and serum albumin in group A were no different to that in group B at week 6. C_{Cr}, both uncorrected and when corrected for body weight, was restored almost to normal by $CD8^+$ cell depletion.

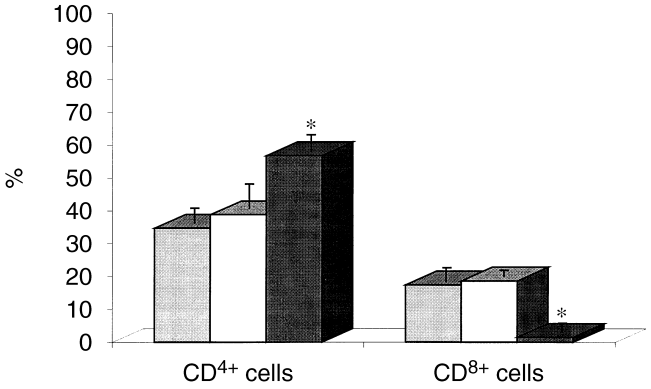

Fig. 1. $CD4^+$ and $CD8^+$ cell levels as a percentage of the total splenocyte population at week 6. Symbols are: (■) normal; (□) adriamycin (ADR); (■) ADR + anti-CD8. Values are expressed as mean ± SD. *$P < 0.05$ compared with other two groups.

$CD8^+$ cell depletion significantly alleviated the histologic manifestations of ADR nephropathy (Fig. 2). Light microscopic examination revealed that glomeruli and tubules were only mildly damaged at week 6 in group A mice, as compared with group B mice. Glomerular surface area was no different to control in group A mice, but was significantly diminished in group B mice, whose glomeruli had shrunken because of glomerulosclerosis. A few vacuoles were seen in some glomeruli of group A mice, but proliferation of mesangial matrix was mild. Morphometric analysis showed much less glomerulosclerosis in group A than in group B mice ($P < 0.001$), as well as more nuclei per glomerular cross section. Most tubules were intact in group A mice, and tubular atrophy was significantly alleviated by anti-CD8 treatment, in comparison with group B mice. Furthermore, treatment with anti-CD8 mAb reduced interstitial expansion in group A in comparison to group B mice ($P < 0.001$). Interstitial fibrosis, assessed semiquantitatively, was significantly less in group A than group B mice. Morphological data are summarized in Table 3.

At week 6, although the interstitial volume was significantly less in group A than that of group B, dense mononuclear cell infiltrates were found in the interstitium, consisting of macrophages and $CD4^+$ T cells. Almost no $CD8^+$ cells and rare B cells were found in sections from group A animals. There was numerically less macrophage infiltration in group A than in group B ($P = 0.052$), but still significantly more in both than in group C ($P < 0.001$). The number of $CD4^+$ cells appeared to increase in group A, in comparison to group B, but this was not statistically different. IHC staining showed macrophages but no $CD4^+$ cells in glomeruli (Fig. 3). IHC data are also summarized in Table 3.

Table 2. Kidney and body weights, and renal function at 6 weeks

	ADR + anti-CD8 (group A)	ADR (group B)	Negative control (group C)
Kidney weight *g*	$0.5 \pm 0.0^{a,f}$	0.3 ± 0.1	0.4 ± 0.1
Body weight *g*	22.7 ± 4.1	19.3 ± 3.9	23.2 ± 5.5
Renal function			
Urinary protein *mg/24 h*	10.4 ± 3.1^{e}	11.7 ± 3.9^{e}	1.1 ± 0.5
Serum albumin *g/L*	25.0 ± 5.2	23.8 ± 4.9	26.6 ± 2.2
C_{Cr} *μL/min*	$71.8 \pm 4.9^{a,f}$	29.2 ± 2.8^{e}	81.9 ± 3.7
C_{Cr}/kidney weight *μL/min/g*	$141.6 \pm 8.7^{a,b}$	115.9 ± 14.6^{c}	242.7 ± 72.0
C_{Cr}/body weight *μL/min/g*	$3.2 \pm 0.4^{a,f}$	1.5 ± 0.1^{e}	3.6 ± 0.7

Values are expressed as means ± SD. Abbreviation is C_{Cr}, creatinine clearance.
[a] $P < 0.05$ compared with age-matched negative control (group C)
[b] $P < 0.05$ compared with ADR-treated positive control (group B)
[c] $P < 0.01$ compared with age-matched negative control (group C)
[d] $P < 0.01$ compared with ADR-treated positive control (group B)
[e] $P < 0.001$ compared with age-matched negative control (group C)
[f] $P < 0.001$ compared with ADR-treated positive control (group B)

DISCUSSION

The most significant observation to emerge from our study is that in vivo depletion of $CD8^+$ cells by the administration of anti-CD8 mAb protected mice from renal functional impairment and dramatically reduced structural injury, including FSGS, tubular atrophy, and interstitial expansion in mice with established ADR nephropathy. Although the number of inflammatory cells in interstitium was only mildly reduced in anti-CD8–treated mice in comparison with mice treated with ADR alone, the interstitial volume was significantly less. This is most likely due to reduction of tubulointerstitial fibrosis. It is also noteworthy that despite marked alleviation of histologic and functional damage, proteinuria remained significantly elevated in CD8-depleted mice throughout the experiment. The object of this study was to examine the effect of CD8 depletion on established ADR nephropathy, and the persistence of proteinuria permitted an examination of inflammatory events independent of proteinuria.

Focal segmental glomerulosclerosis is a hallmark of several nonimmunological models of renal disease [23–27], among which ADR-induced nephropathy is considered to be an excellent analogue of human FSGS [10, 15, 28–31]. Interstitial infiltration with a variety of inflammatory cell subtypes is a prominent feature of human and experimental FSGS and correlates well with the degree of renal insufficiency [32]. However, the role of each inflammatory cell component of interstitial infiltration is still poorly understood. In our previous studies, $CD4^+$ cells, $CD8^+$ cells, and macrophages were shown to be prominent components of interstitial inflammation in murine ADR nephropathy [10]. Amore et al suggested that the noxious effect of ADR might be decreased by T-lymphocyte suppression and even more by the additional inhibition of mononuclear cell function [33]. They hypothesized that an imbalance of T-lymphocyte subsets, induced by ADR, might be the first step in the pathogenetic cascade, leading to appearance of proteinuria and that ADR nephropathy was at least partially immune mediated. Our previous studies showed that in vivo $CD4^+$ cell depletion aggravated histologic and functional damage in ADR nephropathy, suggesting that $CD4^+$ T cells did not mediate renal injury, but in contrast, had a protective effect in this model. Moreover, the damaging effect of CD4 depletion was accompanied by an increase in $CD8^+$ cells in the renal interstitium. Lysing of native cells by cytotoxic $CD8^+$ T cells appears to be an important cause of organ dysfunction in many situations. Intrinsic kidney cells such as proximal tubular epithelial cells express major histocompatibility complex (MHC) class I molecules and therefore can be presumably lyzed by $CD8^+$ cells [32]. These considerations prompted us to examine the effects of $CD8^+$ cells depletion in vivo in murine ADR nephropathy.

Most $CD8^+$ cells have suppressor or cytotoxic functions. Most $CD8^+$ cells are CTLs, but some may be natural killer (NK) cells [34, 35]. There are several mechanisms that might explain the role of $CD8^+$ CTL in renal injury. First, $CD8^+$ CTLs are thought to promote renal damage through recognition of MHC class I alloantigens and cell-mediated cytotoxicity. CTLs recognize antigen peptides presented on MHC class I and lyze the cells bearing this antigen. Two well-described mechanisms of cell-mediated cytotoxicity have been defined at the molecular level, the perforin-dependent pathway, and the Fas/Fas ligand (FasL) pathway. Both of them can result in target cell apoptosis. Second, $CD8^+$ T cells produce various cytotoxic cytokines. As NK cells are known to cause target-cell lysis in the MHC-unrestricted reactions [34, 36], these cells may also be important mediators of renal injury. Like T cells, NK cells exert cell-mediated cytotoxicity, and produce an array of cytokines and chemokines.

The production of cytokines, such as interferon-γ (IFN-γ), tumor necrosis factor (TNF), and lymphotoxin,

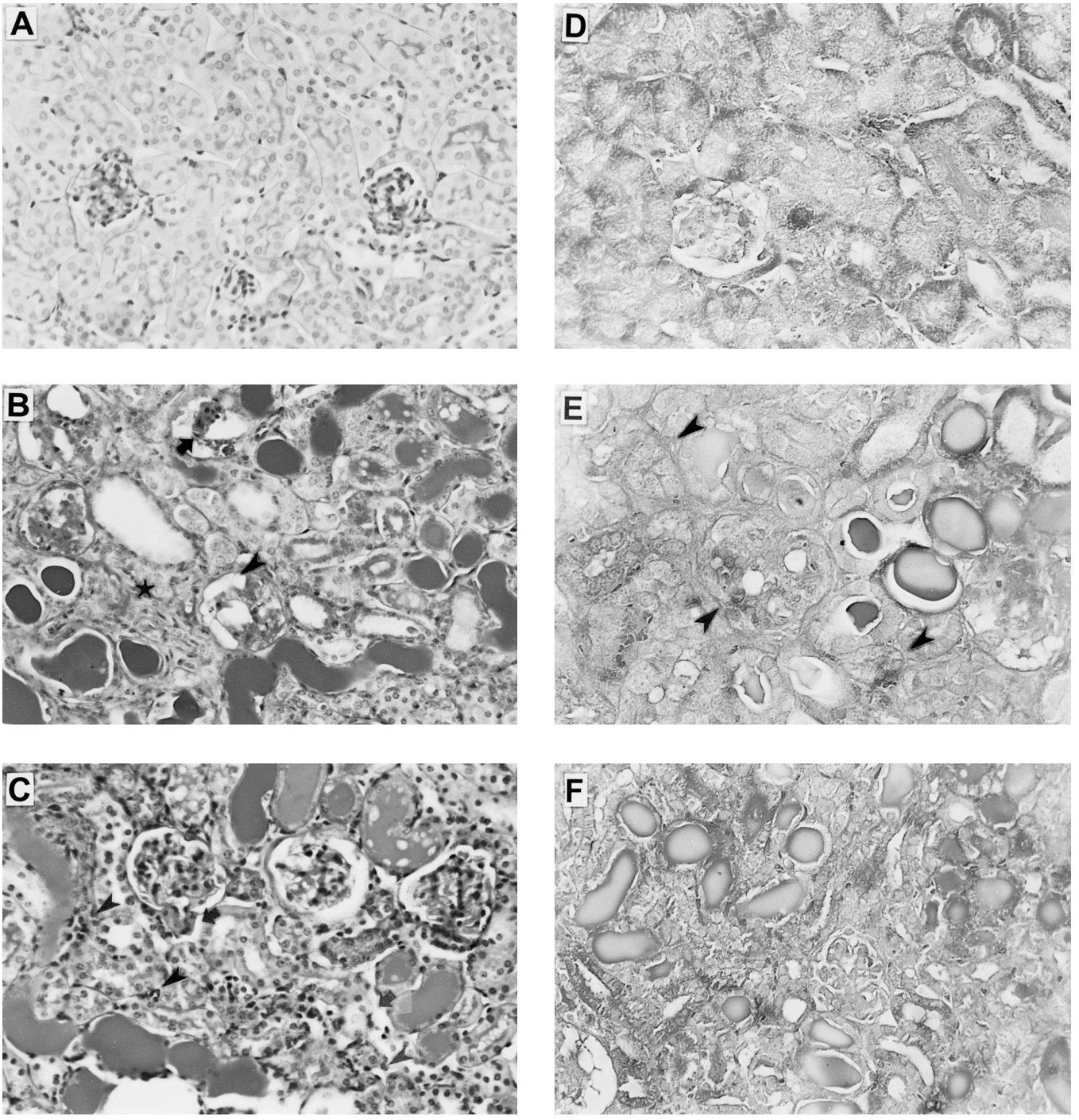

Fig. 2. Representative sections showing morphological changes as stained by PAS (*A–C*) and by Masson's trichrome (*D–F*). Magnification ×400. (A and D) Saline-treated control mice show normal glomeruli, tubules, and interstitium. (B and E) ADR-treated mice show glomerular sclerosis and vacuolization (B, arrowhead), tubular collapse and interstitial expansion (B, star), and numerous hyaline and cellular (B, arrow) casts at week 6. In addition, there is obvious interstitial fibrosis (E, arrowheads). (C and F) ADR mice treated with anti-CD8 show mild glomerular sclerosis and mild tubular dilation and atrophy at week 6, despite hyaline casts indicating proteinuria. However, interstitial cellular infiltration (C, arrowheads) is widespread, and the fissures between tubules (C, arrows) reveal the edema but not fibrosis in interstitium. There is also less collagen deposition in the interstitium (F) than that of mice treated with ADR only (E).

Table 3. Renal morphology and immunohistochemistry at 6 weeks

	ADR + anti-CD8 (group A)	ADR (group B)	Negative control (group C)
Glomerular changes			
GSA μm^2	8387 ± 1758[d]	5706 ± 1670[c]	7450 ± 666
GS %	6.5 ± 1.9[e,f]	13.0 ± 2.8[e]	0
N of nuclei per glomerulus	34.8 ± 3.7[e,f]	24.2 ± 4.9[e]	45.6 ± 3.1
Glomerular macrophages *cells/10 gcs*	1.4 ± 1.6[d]	4.8 ± 2.6[e]	0.5 ± 0.7
Tubulointerstitial morphology			
Tubular diameter *μm*	50.3 ± 22.9[b]	93.5 ± 47.1[c]	38.2 ± 4.7
Tubule cell height *μm*	9.7 ± 5.5[c,d]	3.5 ± 1.0[e]	15.9 ± 4.0
Interstitial volume %	6.3 ± 2.2[e,f]	16.4 ± 3.1[e]	0.4 ± 0.1
Interstitial fibrosis (0–4+)	1.5 ± 0.5[d,e]	2.3 ± 0.8[e]	0
Interstitial immunohistochemistry *cell number/400× field*			
$CD4^+$ cells	156.0 ± 39.1[e]	148.1 ± 19.6[e]	1.8 ± 1.5
$CD8^+$ cells	0.5 ± 0.7[f]	63.3 ± 9.3[e]	0.2 ± 0.5
Macrophages	48.3 ± 13.6[e]	59.8 ± 11.4[e]	8.4 ± 3.6
B cells	1.7 ± 2.2	2.6 ± 1.1	2.1 ± 3.6
Total	206.4 ± 13.9[e]	273.8 ± 10.3[e]	12.5 ± 2.3

Values are expressed as means ± SD. Abbreviations are: GSA, glomerular surface area; GS, glomerulosclerosis; gcs, glomerular cross section.
[a] $P < 0.05$ compared with age-matched negative control (group C)
[b] $P < 0.05$ compared with ADR-treated positive control (group B)
[c] $P < 0.01$ compared with age-matched negative control (group C)
[d] $P < 0.01$ compared with ADR-treated positive control (group B)
[e] $P < 0.001$ compared with age-matched negative control (group C)
[f] $P < 0.001$ compared with ADR-treated positive control (group B)

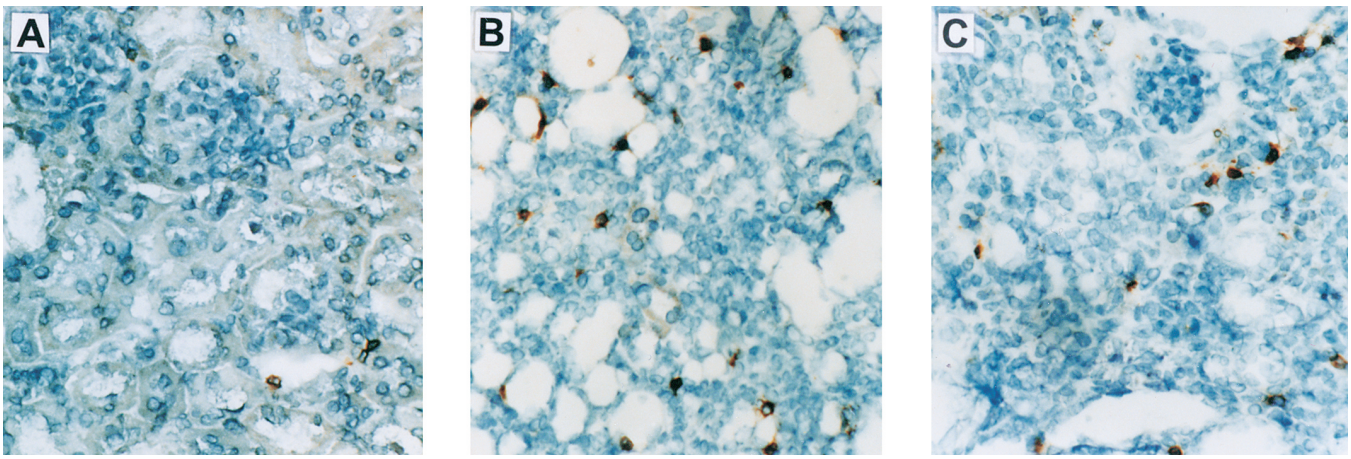

Fig. 3. Photomicrographs of immunohistochemical (IHC) sections from saline-treated control mice (*A*), ADR-treated mice (*B*), and mice treated with ADR plus anti-CD8 (*C*). Sections are stained by antimouse Mac-3 mAb (macrophage). Magnification ×400.

by $CD8^+$ cells is consistent with their direct cytotoxic function. IFN-γ plays a role in macrophage activation and induction of MHC class I and class II molecules and can thus contribute to cell-mediated renal injury [37, 38]. In a murine model of anti-TBM disease, Meyers and Kelly showed that some of the $CD8^+$ effector cells that cause a delayed-type hypersensitivity (DTH) lesion expressed large amounts of transforming growth factor-β (TGF-β) [39]. TGF-β is the most potent stimulator of collagen, fibronectin, and proteoglycan production in fibroblasts [40]. Inhibition of TGF-β using antibody or natural neutralizer (decorin) can effectively reduce extracellular matrix deposition in mesangial proliferative glomerulonephritis [41, 42], among other models of renal disease.

In the present study, there was a decrease in the number of macrophages in the glomeruli of mice treated with ADR plus anti-CD8. This raises the possibility that macrophages cause damage in ADR nephropathy. Macrophages play a role as antigen-presenting cells. They can internalize, modify, and present extracellular molecules as immunogenic fragments in combination with MHC class I and class II molecules on their cell surface to allow recognition by effector cells. Moreover, macrophages synthesize and secrete several cytokines that are capable of causing glomerular damage. For example,

interleukin-1 and TNF-α can regulate the function of native glomerular cells, such as mesangial cell proliferation, and may cause alterations in glomerular blood flow and glomerular ultrafiltration [43, 44]. Macrophages are also an abundant source of TGF-β, which is likely to be involved in the progression to sclerosis in glomerulonephritis [41]. The mitigation of glomerulosclerosis correlated well with a reduction of glomerular infiltration with macrophages in the present experiment. Thus, macrophages are likely to be important effectors of injury in this model.

Other plausible explanations of renal injury, apart from the contribution of $CD8^+$ cells and macrophages, should be considered. It is possible that proteinuria per se could be the cause of renal injury. Although Bertani et al suggested in 1986 that urinary protein itself has an intrinsic toxicity and could contribute to progressive renal disease [45], many researchers have equated this toxic effect with associated inflammatory events. Several publications have shown the association between urinary protein and tubulointerstitial infiltration of inflammatory cells [46–48]. For example, maneuvers that decrease proteinuria, such as dietary protein restriction, also decrease interstitial inflammation (abstract; Eddy et al, *Kidney Int* 37:412, 1990). Previous studies from our group showed that urinary protein up-regulated gene expression and secretion of monocyte chemoattractant protein-1 (MCP-1) in proximal tubular cells in vitro [49], thus providing one of several possible links between proteinuria and interstitial infiltration of inflammatory cells. The results of the present study suggest an additional mechanism of damage involving $CD8^+$ cells in ADR nephropathy.

In conclusion, in vivo depletion of $CD8^+$ T cells significantly protected mice from renal functional impairment and reduced structural injury, including FSGS, tubular atrophy, and interstitial expansion in mice with established ADR nephropathy. The results suggest that $CD8^+$ cells are at least partially responsible for progression of functional and structural injury in this murine model of chronic progressive renal disease.

ACKNOWLEDGMENTS

This study was supported by a grant from the National Health and Medical Research Council of Australia (No. 970721). The authors thank Dr. Teresa Young, Institute of Clinical Pathology and Medical Research at Westmead Hospital, for reviewing the pathology. A part of these results was presented at the 36th Annual Scientific Meeting of the Australian and New Zealand Society of Nephrology, Melbourne, Australia, 2000.

Reprint requests to Dr. Yang Wang, Department of Renal Medicine, Westmead Hospital, Westmead, NSW 2145, Australia.
E-mail: yangw@westgate.wh.usyd.edu.au

REFERENCES

1. Rennke HG, Klein PS, Sandstrom DJ, *et al*: Cell-mediated immune injury in the kidney: Acute nephritis induced in the rat by azobenzenearsonate. *Kidney Int* 45:1044–1056, 1994
2. Sato T, Oite T, Nagase M, *et al*: Nephrotoxic serum nephritis in nude rats: The roles of host immune reactions. *Clin Exp Immunol* 84:139–144, 1991
3. Alexopoulos E, Seron D, Hartley RB, *et al*: Lupus nephritis: Correlation of interstitial cells with glomerular function. *Kidney Int* 37:100–109, 1990
4. Mann R, Zakheim B, Clayman M, *et al*: Murine interstitial nephritis. IV. Long-term cultured L3T4+ T cell lines transfer delayed expression of disease as I-A-restricted inducers of the effector T cell repertoire. *J Immunol* 135:286–293, 1985
5. Kuroda T, Kawasaki K, Oite T, *et al*: Nephrotoxic serum nephritis in nude rats: The role of cell-mediated immunity. *Nephron* 68:360–365, 1994
6. Meyers CM, Tomaszewski JE, Glass JD, *et al*: The nephritogenic T cell response in murine chronic graft-versus-host disease. *J Immunol* 161:5321–5330, 1998
7. Meyers CM, Kelly CJ: Effector mechanisms in organ-specific autoimmunity. I. Characterization of a CD8+ T cell line that mediates murine interstitial nephritis. *J Clin Invest* 88:408–416, 1991
8. Penny MJ, Boyd RA, Hall BM: Permanent CD8+ T cell depletion prevents proteinuria in active Heymann nephritis. *J Exp Med* 188: 1775–1784, 1998
9. Kawasaki K, Yaoita E, Yamamoto T, *et al*: Depletion of CD8 positive cells in nephrotoxic serum nephritis of WKY rats. *Kidney Int* 41:1517–1526, 1992
10. Wang Y, Wang YP, Tay YC, *et al*: Progressive adriamycin nephropathy in mice: Sequence of histological and immunohistochemical events. *Kidney Int* 58:1797–1804, 2000
11. Kruisbeek AM: In vivo depletion of CD4- and CD8-specific T cells, in *Current Protocols in Immunology*, edited by Coligan JE, Kruisbeek AM, Margulies DH, *et al*, New York, John Wiley and Sons, 1991, pp 4.1.1–4.1.5
12. Hathcock KS: T cell depletion by cytotoxic elimination, in *Current Protocols in Immunology*, edited by Coligan JE, Kruisbeek AM, Margulies DH, *et al*, New York, John Wiley and Sons, 1991, pp 3.4.1–3.4.3
13. Nakayama K, Nakayama K, Negishi I, *et al*: Requirement for CD8 beta chain in positive selection of CD8-lineage T cells. *Science* 263:1131–1133, 1994
14. Vleming LJ, Baelde JJ, Westendorp RG, *et al*: The glomerular deposition of PAS positive material correlates with renal function in human kidney diseases. *Clin Nephrol* 47:158–167, 1997
15. Rangan GK, Wang Y, Tay YC, *et al*: Inhibition of nuclear factor-kappaB activation reduces cortical tubulointerstitial injury in proteinuric rats. *Kidney Int* 56:118–134, 1999
16. Kim WJ, Yun SJ, Lee TS, *et al*: Collagen-to-smooth muscle ratio helps prediction of prognosis after pyeloplasty. *J Urol* 163:1271–1275, 2000
17. Ma J, Nishimura H, Fogo A, *et al*: Accelerated fibrosis and collagen deposition develop in the renal interstitium of angiotensin type 2 receptor null mutant mice during ureteral obstruction. *Kidney Int* 53:937–944, 1998
18. Zakheim B, McCafferty E, Phillips SM, *et al*: Murine interstitial nephritis. II. The adoptive transfer of disease with immune T lymphocytes produces a phenotypically complex interstitial lesion. *J Immunol* 133:234–239, 1984
19. Mizuno S, Kurosawa T, Matsumoto K, *et al*: Hepatocyte growth factor prevents renal fibrosis and dysfunction in a mouse model of chronic renal disease. *J Clin Invest* 101:1827–1834, 1998
20. Stefanini M, de Martino C, Zamboni L: Fixation of ejaculated spermatozoa for electron microscopy. *Nature* 216:173–174, 1967
21. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976
22. Ghobrial RR, Boublik M, Winn HJ, *et al*: In vivo use of monoclonal antibodies against murine T cell antigens. *Clin Immunol Immunopathol* 52:486–506, 1989
23. Grond J, Schilthuis MS, Koudstaal J, *et al*: Mesangial function and glomerular sclerosis in rats after unilateral nephrectomy. *Kidney Int* 22:338–343, 1982
24. O'Donnell MP, Michels L, Kasiske B, *et al*: Adriamycin-induced chronic proteinuria: A structural and functional study. *J Lab Clin Med* 106:62–67, 1985

25. Okuda S, Oh Y, Tsuruda H, *et al*: Adriamycin-induced nephropathy as a model of chronic progressive glomerular disease. *Kidney Int* 29:502–510, 1986
26. Eddy AA: Interstitial nephritis induced by protein-overload proteinuria. *Am J Pathol* 135:719–733, 1989
27. Diamond JR, Anderson S: Irreversible tubulointerstitial damage associated with chronic aminonucleoside nephrosis: Amelioration by angiotensin I converting enzyme inhibition. *Am J Pathol* 137: 1323–1332, 1990
28. Bricio T, Molina A, Egido J, *et al*: IL-1-like production in adriamycin-induced nephrotic syndrome in the rat. *Clin Exp Immunol* 87: 117–124, 1992
29. Ginevri F, Trivelli A, Mutti A, *et al*: Progression of chronic adriamycin nephropathy in leukopenic rats. *Nephron* 63:79–88, 1993
30. Tamaki K, Okuda S, Miyazono K, *et al*: Matrix-associated latent TGF-beta with latent TGF-beta binding protein in the progressive process in adriamycin-induced nephropathy. *Lab Invest* 73:81–89, 1995
31. Chen A, Sheu LF, Ho YS, *et al*: Experimental focal segmental glomerulosclerosis in mice. *Nephron* 78:440–452, 1998
32. Kelly CJ, Roth DA, Meyers CM: Immune recognition and response to the renal interstitium. *Kidney Int* 39:518–530, 1991
33. Amore A, Mazzucco G, Cavallo F, *et al*: Adriamycin-induced proteinuria in nude mice: An immune-system-mediated toxic effect. *Nephrol Dial Transplant* 11:1012–1018, 1996
34. Trambley J, Bingaman AW, Lin A, *et al*: Asialo GM1(+) CD8(+) T cells play a critical role in costimulation blockade-resistant allograft rejection. *J Clin Invest* 104:1715–1722, 1999
35. Slifka MK, Pagarigan RR, Whitton JL: NK markers are expressed on a high percentage of virus-specific CD8+ and CD4+ T cells. *J Immunol* 164:2009–2015, 2000
36. Bakker AB, Wu J, Phillips JH, *et al*: cell activation: Distinct stimulatory pathways counterbalancing inhibitory signals. *Hum Immunol* 61:18–27, 2000
37. Huang S, Hendriks W, Althage A, *et al*: Immune response in mice that lack the interferon-gamma receptor. *Science* 259:1742–1745, 1993
38. Kitching AR, Holdsworth SR, Tipping PG: IFN-gamma mediates crescent formation and cell-mediated immune injury in murine glomerulonephritis. *J Am Soc Nephrol* 10:752–759, 1999
39. Meyers CM, Kelly CJ: Immunoregulation and TGF-beta 1: Suppression of a nephritogenic murine T cell clone. *Kidney Int* 46:1295–1301, 1994
40. Roberts AB, McCune BK, Sporn MB: TGF-beta: Regulation of extracellular matrix. *Kidney Int* 41:557–559, 1992
41. Border WA, Okuda S, Languino LR, *et al*: Suppression of experimental glomerulonephritis by antiserum transforming growth factor beta 1. *Nature* 346:371–374, 1990
42. Border WA, Noble NA, Yamamoto T, *et al*: Natural inhibitor of transforming growth factor-beta protects scarring in experimental kidney disease. *Nature* 360:361–364, 1992
43. Schreiner GF, Kohan DE: Regulation of renal transport processes and hemodynamics by macrophages and lymphocytes. *Am J Physiol* 258:F761–F767, 1990
44. Main IW, Nikolic-Paterson DJ, Atkins RC: T cells and macrophages and their role in renal injury. *Semin Nephrol* 12:395–407, 1992
45. Bertani T, Cutillo F, Zoja C, *et al*: Tubulo-interstitial lesions mediate renal damage in adriamycin glomerulopathy. *Kidney Int* 30:488–496, 1986
46. Wehrmann M, Bohle A, Held H, *et al*: Long-term prognosis of focal sclerosing glomerulonephritis: An analysis of 250 cases with particular regard to tubulointerstitial changes. *Clin Nephrol* 33: 115–122, 1990
47. D'Amico G: Influence of clinical and histological features on actuarial renal survival in adult patients with idiopathic IgA nephropathy, membranous nephropathy, and membranoproliferative glomerulonephritis: Survey of the recent literature. *Am J Kidney Dis* 20:315–323, 1992
48. D'Amico G, Ferrario F, Rastaldi MP: Tubulointerstitial damage in glomerular diseases: Its role in the progression of renal damage. *Am J Kidney Dis* 26:124–132, 1995
49. Wang Y, Chen J, Chen L, *et al*: Induction of monocyte chemoattractant protein-1 in proximal tubule cells by urinary protein. *J Am Soc Nephrol* 8:1537–1545, 1997