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Initiation and evolution of interstitial leukocytic infiltration in experimental glomerulonephritis

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Initiation and evolution of interstitial leukocytic infiltration in experimental glomerulonephritis. Most forms of glomerulonephritis have a significant interstitial leukocytic infiltrate which is associated with disease progression. However, there is little data concerning the timing, initial location, and development of this interstitial component. Therefore, we have addressed these issues in a study of passive accelerated anti-GBM glomerulonephritis in the rat. In this model, interstitial leukocytic infiltration was an early event in the disease process with a significant infiltrate apparent at 12 hours after administration of nephrotoxic serum (NTS). This initial infiltrate was restricted to a perivascular sheath surrounding the hilar arterioles. The sheath infiltrate then spread to include the whole hilar area by day 1, the entire periglomerular area by day 3, and became widespread throughout the cortical tubulointerstitium by day 7. The early sheath infiltrate was composed of macrophages and T cells. Both cell types continued to increase as the infiltrate expanded, and a significant accumulation of activated cells (IL-2R$^+$) was evident from day 7 onwards. There was a highly significant correlation between interstitial macrophage infiltration and renal function impairment, proteinuria, and histologic damage. Interstitial T cell infiltration correlated with proteinuria and histologic damage, while the appearance of immune-activated mononuclear cells (IL-2R$^+$) exhibited a highly significant correlation with all disease parameters. This study demonstrates the importance of the glomerular hilar arteriolar region as a focus for mononuclear leucocytic migration and accumulation which not only affects the structure and function of the glomerulus but subsequently the entire tubulointerstitium.

The involvement of immunological processes in the induction of glomerular injury is widely accepted [1]. The infiltration of leukocytes, particularly macrophages, into the glomerulus has been known for some time [2, 3] and a variety of human and animal studies have implicated these cells as being involved in glomerular damage [4]. However, recent studies have revealed the importance of interstitial leukocytic infiltration in disease progression [5, 6]. An extensive study of human biopsy material by Hooke, Gee and Atkins [7] demonstrated the presence of interstitial macrophages and T cells in all types of glomerulonephritis except minimal change disease, and that the intensity of interstitial leukocytic infiltration correlated with the degree of renal function impairment. This finding has been confirmed in other human studies, with long term prognosis of disease apparently predicted by the degree of interstitial leukocytic infiltration at the time of biopsy [8–10].

In an experimental model of focal glomerular sclerosis induced by chronic administration of puromycin aminonucleoside and protamine sulphate, progressive renal impairment correlated with the development and degree of interstitial leukocytic infiltration [11]. In addition, improvement in renal function produced by prednisolone treatment (begun following initiation of glomerular injury) was associated with the suppression of interstitial leukocytic infiltration, while development of glomerular damage was unaltered in this model [11]. Hence chronic disease progression in glomerulonephritis may involve common interstitial cellular immune-mechanisms which are independent of the nature of the insult responsible for the initial glomerular injury.

Although interstitial leukocytic infiltration has been demonstrated in almost all forms of glomerulonephritis, very little is known of the development of this component. For example, the location of the initial infiltrate, when infiltration begins, how the infiltrate develops throughout the disease process, and how the infiltrate relates to events in the glomerulus are questions yet to be investigated. Therefore, in the present study we have used a well established rat model of rapidly progressive glomerulonephritis to study the initiation and evolution of interstitial leukocytic infiltration.

Methods

Animals

Male Sprague-Dawley inbred rats aged 3 to 4 months (250 to 300 g body weight) were obtained from the Monash Medical Centre Animal House.

Nephrotoxic serum

Rabbit nephrotoxic serum was raised by repeated immunisation of a rabbit with particulate rat glomerular basement membrane (GBM) emulsified in Freund's incomplete adjuvant (FIA) as previously described [12]. The nephrotoxic serum was decomplemented, and absorbed extensively against normal rat erythrocytes.

Experimental protocol

Passive accelerated anti-GBM glomerulonephritis was induced in male Sprague-Dawley inbred rats by subcutaneous immunization with 5 mg normal rabbit IgG (Silenus, Australia) in FIA followed five days later by intravenous injection of

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nephrotoxic serum (10 ml/kg body weight). Control animals had the same schedule as above, but received normal rabbit serum in place of nephrotoxic serum.

Passive accelerated anti-GBM glomerulonephritis was induced in 32 animals, with groups of four rats killed at times 12 hours, days 1, 2, 3, 7, 14, 21 and 28 after injection of nephrotoxic serum. Control animals, also in groups of four, were killed at 12 hours, days 1, 7 and 21 after injection of normal rabbit serum. One group of four normal animals was also studied. In addition, in order to investigate very early events, groups of two rats were killed at 30 minutes, 1, 3, 6 and 9 hours after injection of nephrotoxic serum.

Immunohistochemistry

Tissues for immunofluorescence staining were frozen in liquid nitrogen and stored at −80°C. Cryostat sections (6 μm) were stained with: fluorescein isothiocyanate (FITC)-conjugated goat antibodies recognizing rat IgG, C3 or fibrinogen; or FITC-conjugated sheep anti-rabbit IgG (Nordic, the Netherlands).

Tissues for immunoperoxidase staining were fixed in paraformaldehyde-lysine-periodate as previously described [13]. Serial cryostat sections (6 μm) were adhered to gelatin coated microscope slides, air-dried and preincubated with 10% fetal calf serum (FCS) and 10% normal rabbit serum for 10 minutes. Sections were then incubated with mouse anti-rat monoclonal antibodies (MoAbs) for 30 to 60 minutes, washed twice in phosphate-buffered saline (PBS), treated with 0.3% hydrogen peroxide in methanol for 20 minutes to block endogenous peroxidase, and incubated with rabbit anti-mouse IgG conjugated to horseradish peroxidase (Dako, Denmark) for 30 minutes. Sections were developed with either 3-amino-9-ethylcarbazole (AEC, Sigma) or 3'3'-diaminobenzidine (DAB, Sigma), counterstained with Harris' hematoxylin and mounted in aqueous mounting medium or Depex, respectively. All steps were carried out at room temperature.

Antibodies

Monoclonal antibodies used in this study were as follows: OX-1, leukocyte common antigen [14]; OX-19, CD5 antigen, pan T cells [15]; ED1, most macrophages and some dendritic cells [16]; ART-18, p55 chain of the rat interleukin-2 receptor (IL-2R) [17].

Quantitation of leukocytes on tissue sections

Cells labelled by MoAbs were counted in high-power fields (× 400) by means of a 0.02 mm^2 graticule fitted in the eyepiece of the microscope. To examine the early interstitial infiltrate, serial sections of glomeruli containing distinct hilar areas were used to count the number of labelled cells in the glomerular cross section, hilar region (area surrounding and between glomerular arterioles and tubules), and the periglomerular areas. Ten such glomeruli were evaluated for each animal. In addition, a point-counting method was used to assess the number of positive cells per unit area in glomerular and hilar regions. To assess tubulointerstitial leukocyte infiltration, cortical areas were selected at random. The number of labelled cells was assessed from 20 consecutive high power fields; these progressed from the outer to inner cortex, avoiding only large vessels and glomeruli. For each tissue, the same area was examined in serial sections labelled with different MoAbs. No adjustment of the cell count for tubules or the luminal space was made. The mean of 20-field counts from each group of four animals was expressed as cells per square millimeter ± standard error of mean (SEM).

Renal function and proteinuria

Rats were housed in metabolic cages for 24 hours prior to sacrifice. Protein concentrations in 24-hour urine samples were determined using the turbidity assay [18]. Blood samples, taken at the time of sacrifice, were used to determine serum creatinine and serum urea concentrations using the Jaffe rate reaction (alkaline picrate) or NED/OPA assays, respectively, in the Kone Progress Random Access Analyser.

Evaluation of tubulointerstitial and glomerular lesions

To evaluate the degree of tubulointerstitial lesions, tubular atrophy and fibrosis were semiquantitatively assessed on hematoxylin and eosin stained sections and graded on a scale of 0 to 4 as previously described [19]: (0) no apparent damage; (1) tubular atrophy involving less than 5% of the cortex; (2) scattered tubular atrophy over 5 to 25% of the cortex; (3) focal areas of tubular atrophy and mild fibrosis over 25 to 50% of the cortex; (4) severe tubular damage and widespread fibrosis involving more than 50% of the cortex.

Glomerular lesions, assessed by examination of at least 50 glomeruli per animal, were categorized into four grades [20]: (0) no apparent damage; (1) minor change; (2) moderate hypercellularity (80 to 120 cells/glomerular cross section, compared to normal 50 cells) or minor exudate into Bowman's space involving less than 25% of glomeruli; (3) severe hypercellularity, focal segmental necrosis or sclerosis, heavy exudation, or crescent formation involving 25% to 50% of glomeruli; (4) segmental and/or global sclerosis or crescent formation involving greater than 50% of glomeruli.

Statistical analysis

Data from monoclonal antibody labelling in each particular renal area for both experimental and control animals was compared using one way analysis of variance with Tukey's comparison program from SAS (Statistical Analysis Systems). The glomerular or cortical interstitial infiltrate for all 32 experimental animals for each leukocyte population was compared with measurements of renal function and proteinuria using the single correlation program from C.S.S. (Complete Statistical Systems), while comparison of leukocyte infiltration with the histological damage index used the Spearmans' rank correlation coefficient.

Results

Immunoglobulin deposition

Strong linear deposition of rabbit IgG along the glomerular basement membrane (GBM) is shown in Figure 1. This deposition of anti-GBM antibody was found at 30 minutes following administration of NTS and was clearly detectable throughout the 28 day timecourse. No deposition of rabbit IgG was found in glomerular arterioles or any other interstitial area during the experimental timecourse. In control animals, no deposition of rabbit IgG was detected.

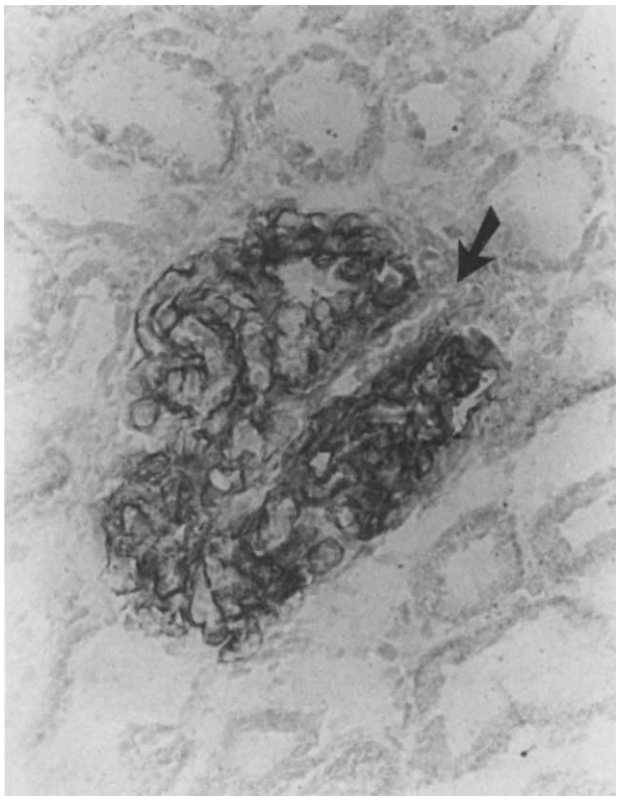

Fig. 1. *Immunoperoxidase staining showing linear deposition of rabbit IgG along the GBM at day 1.* No deposition was detected in the hilar arteriole (arrow), periglomerular, or other interstitial area. Magnification, × 400.

Mild linear deposition of rat IgG along the GBM was first seen at 12 hours in experimental animals, becoming moderate at day 2 and strong by day 7. Rat IgG was found in the Bowman's urinary space at day 14, and deposition on the capsular basement membrane was evident at later times. In control animals, some granular deposition of rat IgG on the GBM and in some mesangial areas was detected at day 7; this was reduced by day 21.

Mild linear C3 deposition on the GBM and on some tubular basement membrane was evident at day 3 in experimental animals. This became moderate at day 7 and strong by day 14. In control animals, weak granular C3 deposition followed the pattern of rat IgG deposition.

Initial location of interstitial leukocytic infiltration

The development of interstitial leukocytic infiltration was an early event in disease progression. Interstitial infiltration was evident at 12 hours and this was restricted to a perivascular sheath surrounding the hilar arterioles, as shown in Figure 2(a). The sheath infiltrate developed rapidly with the whole hilar area involved by day 1 (Fig. 2b). Partial infiltration of the periglomerular area was apparent at day 2 (Fig. 2c), with the entire periglomerular area involved at day 3. This produced a highly characteristic staining pattern as shown in Fig. 2(d).

Quantitation of the early interstitial infiltrate

In order to analyze leukocyte populations in the early interstitial infiltrate and relate this to events in the glomerulus, serial sections of glomeruli containing clear hilar areas were used to enumerate leukocyte populations in glomerular, hilar, and periglomerular areas.

In the hilar area, significant infiltration of both OX-19$^+$ T cells and ED1$^+$ macrophages was apparent at 12 hours; infiltration of these two populations was maximal at day 2, and remained significant at day 7 (Fig. 3). In the periglomerular area, infiltration of T cells and macrophages was not apparent until day 1 and this gradually increased over days 2 to 7 (Fig. 3). The results indicate a pattern of initial localisation of leukocytes in the hilum, followed by a rapid movement of cells throughout the periglomerular area.

In the glomerulus, a significant infiltrate of mononuclear leukocytes was apparent at 12 hours. The infiltrate was composed primarily of macrophages, although a small but significant influx of T cells was also present (Fig. 3). Glomerular macrophages continued to accumulate, but the T cell influx was only a transient event of the first three days.

A point-counting technique was also used to enumerate leukocytic infiltration in glomerular and hilar areas, although the periglomerular area could not be assessed in this fashion. The total leukocytic infiltrate (OX-1$^+$ cells) in the hilar area was 4- to 9-fold greater per square millimeter than that seen in the glomerulus (Fig. 4). This was most prominent for T cell accumulation, where there was 12 to 37 times more cells in the hilar area compared to the glomerulus over 12 hours to day 3 (Fig. 4). In addition, there were 2 to 3 times more macrophages in the hilar area than in the glomerulus. Results from point-counting studies were consistent with those shown in Figure 3.

In normal animals, a small number of leukocytes were seen in the glomerulus and the periglomerular area, but no leukocytes were evident in the hilum (Fig. 3). In control animals, no hilar or periglomerular infiltrate was evident, although macrophage infiltration in the glomerulus was seen at day 7 which was associated with granular deposition of rat IgG.

Development of leukocytic infiltration

Early interstitial leukocytic infiltration was confined to the hilar and periglomerular areas with development of cortical tubulointerstitial infiltration not apparent until day 7 (Fig. 5a). OX-19$^+$ T cells and ED1$^+$ macrophages were significant components of tubulointerstitial infiltration at day 7 and both cell types increased during the experimental timecourse (Table 1). Immune-activated mononuclear cells were identified on the basis of expression of the p55 chain of the IL-2R. Small but significant numbers of IL-2R$^+$ cells were apparent in the tubulointerstitial infiltrate (Table 1). An example of IL-2R$^+$ cells localized to an area of tubular damage is shown in Figure 5b.

Widespread leukocytic infiltration in the medulla was first evident at day 14. This followed an accumulation of cells at the corticomedullary junction on day 7 (not shown). There was no significant tubulointerstitial infiltration in control animals.

The development of glomerular leukocyte infiltration is shown in Table 2. Significant glomerular leukocyte infiltration was evident at 12 hours and was a feature throughout the

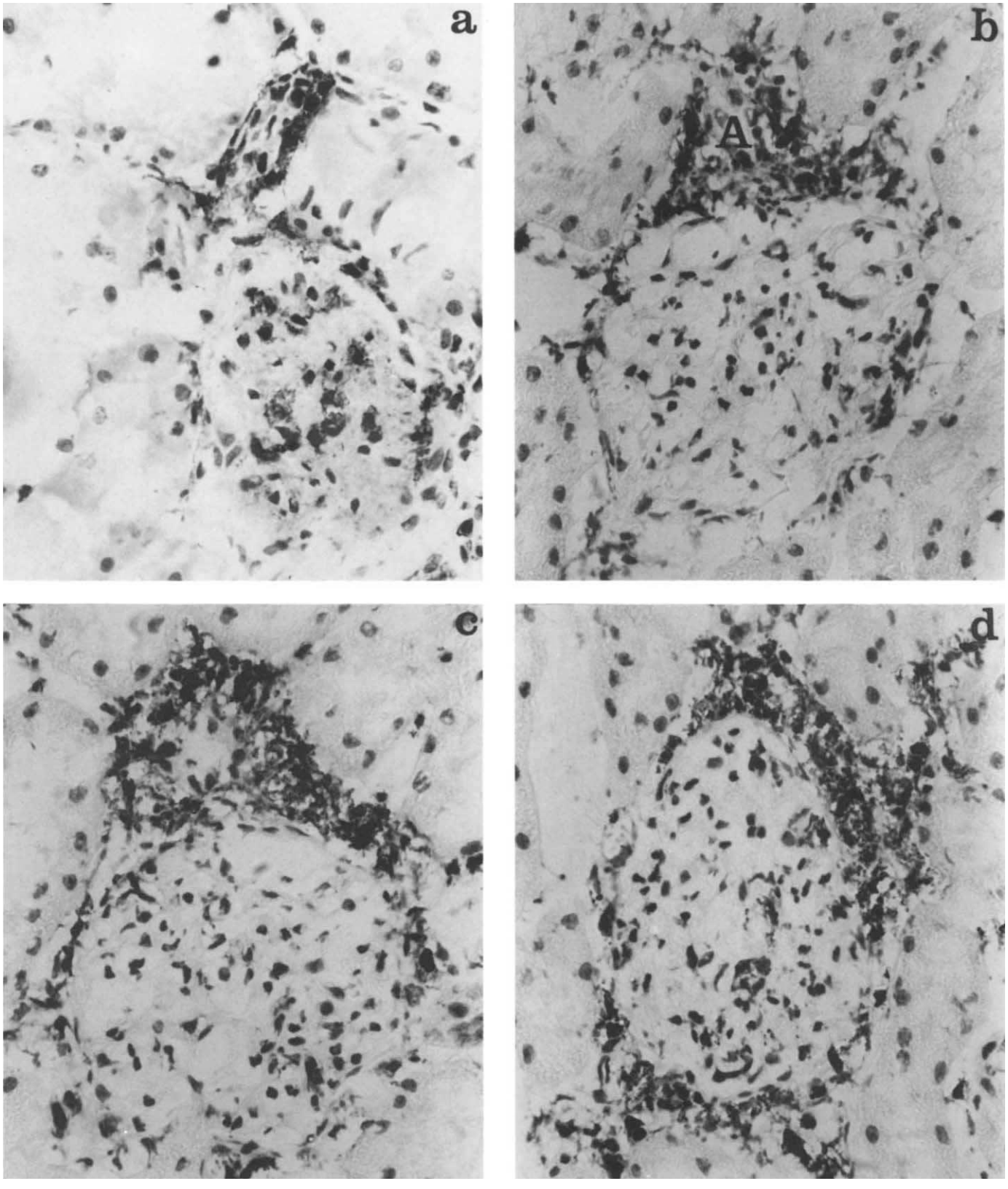

Fig. 2. *Pattern of initial interstitial leukocyte infiltration shown by immunoperoxidase staining with the OX-1 MoAb.* **(a)** At 12 hours, infiltrate in a sheath surrounding the hilar arteriole (arrows) and within the glomerulus. **(b)** Day 1, infiltration throughout the hilar area (A, glomerular arteriole). **(c)** Day 2, hilar and partial periglomerular infiltration. **(d)** Day 3, infiltration throughout the entire hilar and periglomerular areas. Magnification, × 400.

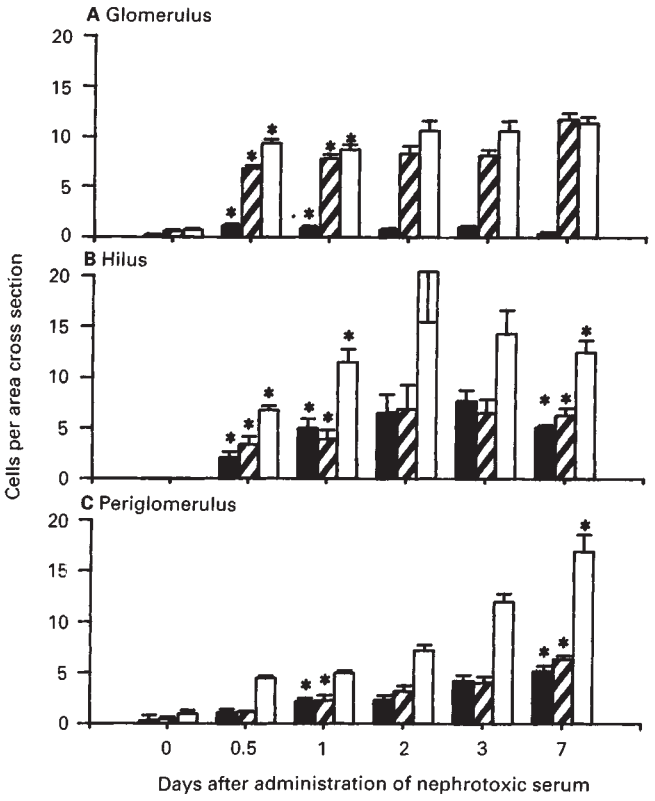

Fig. 3. *Analysis of early leukocyte infiltration.* Glomeruli with distinct hilar areas were scored for OX-19$^+$ T cells (black bars), ED1$^+$ macrophages (hatched bars), and total OX-1$^+$ leukocytes (open bars) in adjacent glomerular, hilar and periglomerular areas. Control animals at 12 hours and day 1 were not different from normal, whereas at day 7 a significant glomerular infiltrate was apparent (Table 1). At this time, 3.3 ± 1.1 and 3.8 ± 1.1 OX-1$^+$ cells were present in the hilar and periglomerular areas, respectively. *Significant difference ($P < 0.05$) compared to time-matched control groups.

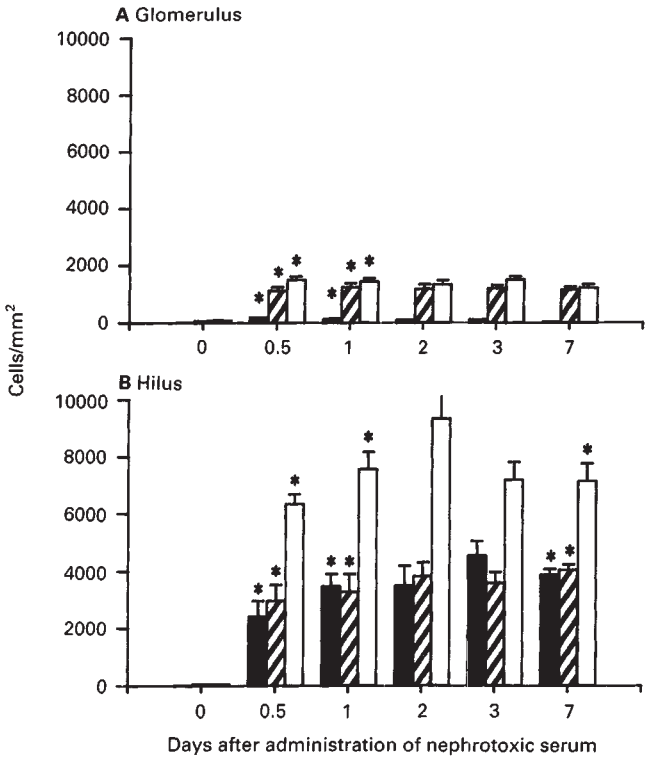

Fig. 4. *Comparison of glomerular and hilar leukocyte infiltation.* Point-counting of OX-19$^+$ T cells (black bars), ED1$^+$ macrophages (hatched bars), and total OX-1$^+$ leukocytes (open bars) in adjacent glomerular and hilar areas. Control animals at 12 hours and day 1 were no different to normal, whereas day 7 control animals exhibited a significant glomerular infiltration, and 3178 ± 905 OX-1$^+$ cells were present in the hilar area. *Significant difference ($P < 0.05$) compared to time-matched control groups.

experimental timecourse (Table 2). This infiltrate comprised mainly ED1$^+$ macrophages, with a small but significant influx of OX-19$^+$ T cells evident during the first three days. No immune-activated cells were apparent in the glomerulus early in the experimental timecourse, although small numbers of IL-2R$^+$ cells were evident from day 7 onwards. In control animals, a transient glomerular infiltration of ED1$^+$ macrophages was evident at day 7 which paralleled the granular deposition of rat IgG.

Analysis of pairs of animals from 30 minutes to 9 hours identified a transient glomerular influx of polymorphonuclear (PMN) cells. PMN cells were present at 30 minutes (average of 8 cells/glomerular cross section), the influx peaked at 3 to 6 hours (18 cells/gcs) and was virtually absent by 12 hours.

Proteinuria and renal function

Experimental animals had a rapid onset of proteinuria with particular heavy amounts evident from day 7 onwards (Table 3). Significant increases in serum levels of creatinine and urea were apparent from day 14 onwards (Table 3). Control animals showed no increase in serum creatinine or urea, although they did exhibit a mild proteinuria from day 7.

Histologic changes

Minor glomerular changes were first apparent at days 1 to 2; moderate damage was evident at day 7 with severe damage at day 14 (Table 3). In the interstitium, minor tubular atrophy in close proximity to glomeruli was apparent at day 3, with moderate widespread tubular atrophy evident at day 7, and severe damage at day 14 (Table 3). The development of moderate and severe lesions in both areas was associated with the onset of heavy proteinuria and significant increases in serum creatinine and urea (Table 3).

There was an association between infiltrating leukocytes and areas of interstitial damage. From day 7 onwards, there was widespread leukocytic infiltration throughout the tubulointerstitium, with focal areas of heavy infiltration localised to sites of tissue damage. The widespread distribution of leukocytes (OX-1$^+$ cells) at day 7 is shown in Figure 5a. ED1$^+$ macrophages in the interstitium had a similar distribution pattern to that of OX-1$^+$ cells. However, OX-19$^+$ T cells were primarily restricted to areas of focal infiltrate, with only occasional T cells seen in adjacent intact regions. Similarly, IL-2R$^+$ cells were found only in damaged areas. An example of focal accumulation of IL-2R$^+$ cells in day 21 tissue is shown in Figure 5b.

Correlation of leukocytic infiltration and disease progression

Infiltration of leukocyte populations into glomerular or tubulointerstitial areas during the entire experimental timecourse

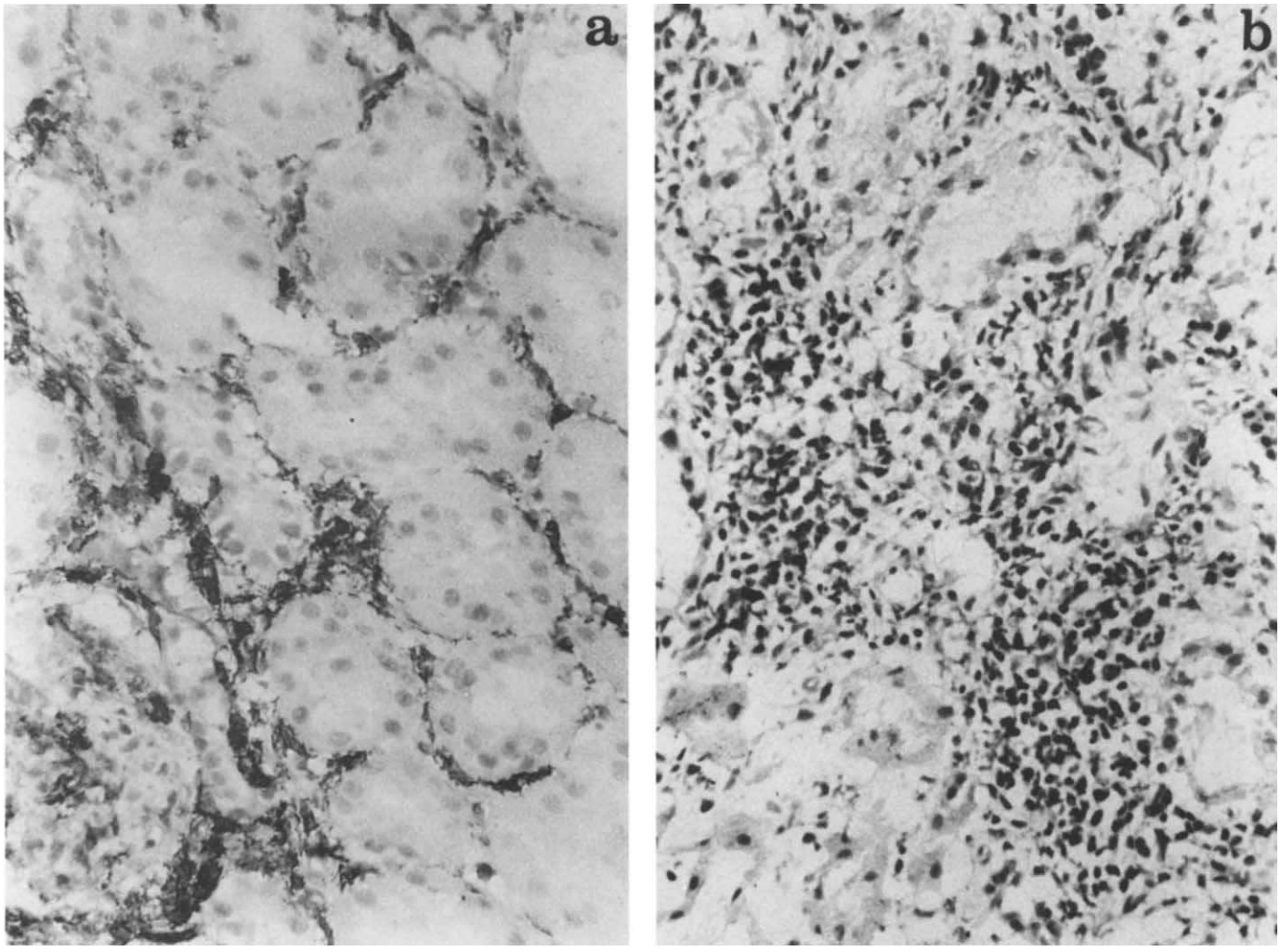

Fig. 5. *Widespread interstitial leukocyte infiltration.* (a) OX-1$^+$ total leukocytes widely distributed throughout the cortical interstitium at day 7. (b) IL-2R$^+$ (ART-18$^+$) cells exhibiting a close association with areas of tubulointerstitial damage at day 21. Magnification × 250.

was compared to parameters of renal function, proteinuria, and histological damage using single correlation analysis (Table 4). Total leukocytes (OX-1$^+$) and ED1$^+$ macrophages in both glomerular and tubulointerstitial areas showed a highly significant correlation with renal function impairment, proteinuria, and histological damage. In contrast, OX-19$^+$ T cell infiltration in the tubulointerstitium correlated with proteinuria and histological damage only, while glomerular T cell influx exhibited a negative correlation with all parameters reflecting their transient appearance prior to significant disease manifestation. Of note was the finding that the appearance of activated mononuclear cells in the interstitium produced a highly significant correlation with all parameters, and even the small numbers of IL-2R$^+$ cells seen in the glomerulus correlated with all parameters.

Discussion

The main finding of this study was that the induction of interstitial leukocytic infiltration is an early event in the devel-

Table 1. Quantitation of leukocyte subsets in the cortical tubulointerstitial area

	OX-1	ED1	OX-19	ART-18
Experimental group				
12 Hours	168 ± 7	73 ± 7	86 ± 15	0 ± 0
Day 1	251 ± 15	114 ± 12	102 ± 17	3 ± 0.5
2	293 ± 50	89 ± 23	96 ± 25	10 ± 4
3	272 ± 8	71 ± 9	75 ± 4	6 ± 1
7	618 ± 72[a]	105 ± 11[a]	137 ± 11[a]	38 ± 3[a]
14	1063 ± 72	351 ± 79	289 ± 14	104 ± 23
21	1398 ± 77[a]	388 ± 36[a]	297 ± 54[a]	112 ± 37[a]
28	1144 ± 241	325 ± 78	348 ± 90	115 ± 38
Control group				
12 Hours	98 ± 5	29 ± 1	28 ± 2	0 ± 0
Day 1	117 ± 7	40 ± 5	37 ± 5	0 ± 0
7	138 ± 4	31 ± 3	33 ± 6	0 ± 0
21	138 ± 13	46 ± 8	40 ± 8	6 ± 3
Normal rat	102 ± 8	28 ± 5	30 ± 7	0 ± 0

Data are expressed as cells per square millimeter (mean ± SEM).
[a] Significant difference ($P < 0.05$) compared to time-matched control group

Table 2. Quantitation of leukocyte subsets in glomeruli

	OX-1	ED1	OX-19	ART-18
Experimental group				
12 Hours	7.4 ± 0.5[a]	5.4 ± 0.3[a]	1.4 ± 0.2[a]	0.0 ± 0.0
Day 1	9.3 ± 0.5[a]	7.2 ± 0.5[a]	1.2 ± 0.2[a]	0.1 ± 0.1
2	9.6 ± 1.1	6.9 ± 0.5	1.2 ± 0.2	0.4 ± 0.3
3	8.1 ± 0.5	6.6 ± 0.5	0.9 ± 0.1	0.3 ± 0.4
7	11.5 ± 0.6	9.7 ± 0.8	0.8 ± 0.1	0.8 ± 0.1[a]
14	17.9 ± 0.8	13.7 ± 1.0	0.7 ± 0.1	0.8 ± 0.1
21	17.5 ± 1.3[a]	12.9 ± 1.3[a]	0.5 ± 0.1	0.9 ± 0.1[a]
28	15.2 ± 0.8	13.5 ± 0.3	0.4 ± 0.1	0.4 ± 0.1
Control group				
12 Hours	1.5 ± 0.1	1.1 ± 0.2	0.4 ± 0.1	0.0 ± 0.0
Day 1	1.9 ± 0.2	1.2 ± 0.2	0.4 ± 0.1	0.0 ± 0.0
7	10.5 ± 1.5	9.5 ± 1.6	0.8 ± 0.1	0.0 ± 0.0
21	4.6 ± 0.6	2.4 ± 0.8	0.2 ± 0.1	0.1 ± 0.1
Normal rat	1.0 ± 0.1	0.8 ± 0.1	0.3 ± 0.1	0.0 ± 0.0

Data are expressed as positive cells per glomerular cross section (mean ± SEM).

[a] Significant difference ($P < 0.05$) compared to time-matched control group

opment of experimental anti-GBM disease. Interstitial leukocytic infiltration originated in a sheath surrounding the glomerular hilar arterioles. The sheath infiltrate appeared at approximately the same time as mononuclear leukocyte infiltration in the glomerulus and before detectable interstitial damage or renal function impairment. The interstitial infiltrate developed rapidly to encompass the entire hilar and periglomerular areas, and then spread throughout the tubulointerstitium.

Accumulation of leukocytes in the hilar area could result from the ingress of cells from the hilar arterioles and adjacent capillaries, or it may derive from trafficking of glomerular leukocytes down the mesangial stalk. The latter pathway is feasible because there is no GBM in the mesangial stalk. The aspect of the glomerular capillary network which faces the mesangium is without a continuous GBM and is separated from the mesangium only by a fenestrated endothelial cell cytoplasm. The GBM, which is a continuation of the arteriolar basement membrane, merges at the hilum with the Bowman's basement membrane. Thus, movement of leukocytes and soluble molecules from the glomerular mesangium to the hilum is quite possible.

The similarity of cellular composition and kinetics of appearance of the early glomerular and interstitial mononuclear leukocytic infiltrates suggests that they arose from a common source. Direct movement of leukocytes from the Bowman's space to the periglomerular area seems unlikely since rupture of the Bowman's capsule was not evident until day 14.

There are a number of mechanisms which could be involved in the initial accumulation of leukocytes in the hilar area. Firstly, antigen deposition could attract leukocytes, but this does not appear to be the case since no anti-GBM antibody was detected in either the hilar or periglomerular areas. Deposition of small amounts of rabbit antibody or its degradation products in the hilar region, not detected by the staining method employed, could induce leukocytic infiltration. However, strong antibody deposition in the glomerulus was not sufficient to maintain T cell accumulation, which argues against antigen-directed leukocyte accumulation in the hilum.

Secondly, chemotactic molecules could be involved in the hilar leukocyte accumulation. Molecules with chemotactic properties, such as platelet activating factor, leukotrienes, tumour necrosis factor (TNFα) and interleukin 1 (IL-1) can be produced by stimulated resident glomerular cells or infiltrating glomerular leukocytes [1, 21, 22]. These molecules could diffuse down the mesangial stalk to the hilar region and thus attract leukocytes to this site.

Thirdly, expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and endothelium adhesion molecule-1 (ELAM-1) by endothelial or tubular cells in the hilar region may be involved in the accumulation of leukocytes at this site [23, 24]. In normal kidney there is variable weak to moderate ICAM-1 expression in endothelium of large vessels, peritubular capillaries, proximal tubules, and in glomeruli [25, 26]. During transplant rejection and autoimmune murine lupus nephritis there is an upregulation of ICAM-1 expression [25, 26]. In addition, fresh cultured proximal tubular cells increase ICAM-1 expression in response to cytokines interferon gamma, IL-1, or TNFα [26]. There is evidence for glomerular production of IL-1 activity in both human and experimental glomerulonephritis [22, 27–30]; thus, diffusion of these products down the mesangial stalk may result in the expression of adhesion molecules in the hilar area.

The demonstration of glomerular infiltration by macrophages and T cells at 12 hours, with continued accumulation of macrophages and the disappearance of T cell infiltration by day 3, was consistent with previous studies using the passive accelerated anti-GBM model in the rat [31, 32]. It has been proposed that the glomerular macrophage infiltration is dependent upon T cells [31], but this remains to be directly demonstrated.

We propose that the development of interstitial leukocytic infiltration is an important component of progressive renal damage, and not simply a consequence of tubular injury. This is supported by a number of findings. Firstly, initiation of leukocytic infiltration in the interstitium occurred before the onset of histologic damage. The development of widespread infiltration throughout the tubulointerstitium occurred in association with the onset of moderate histologic damage and before significant renal function impairment. There was no suggestion of a delay between onset of interstitial damage and the appearance of infiltrating leukocytes, as might have been expected had this infiltrate been simply a response to damage. Secondly, the development of macrophage and T cell interstitial infiltration showed significant correlations with the various disease parameters.

Of particular interest was the appearance of significant numbers of activated interstitial leukocytes from day 7 onwards which exhibited highly significant correlations with all disease parameters. All in vitro activated T cells and some populations of stimulated macrophages/monocytes express the IL-2R [33, 34]. IL-2R expression is a widely accepted marker of immune activation, and it can be anticipated that such cells are functionally active within the tissue. The kinetics of the appearance of IL-2R$^+$ cells and the virtually exclusive localization of IL-2R$^+$ cells to areas of focal infiltrate may reflect the action of these cells in causing and maintaining progressive injury.

This study is the first detailed investigation of the initiation and evolution of renal interstitial leukocytic infiltration. The

Table 3. Analysis of renal function, proteinuria and histologic damage

	Serum creatinine *μmol/liter*	Serum urea *mmol/liter*	Proteinuria *mg/24 hr*	Histological damage	
				Glomerular	Interstitial
Experimental group					
12 Hours	48 ± 2[a]	8 ± 1	ND	0.0[b]	0.0
Day 1	56 ± 10	11 ± 2	107 ± 38	0.75	0.25
2	48 ± 3	9 ± 0.3	71 ± 15	1.0	0.5
3	52 ± 1	8 ± 0.3	102 ± 6	1.5	1.0
7	60 ± 1	15 ± 2	414 ± 15[e]	2.5	2.0
14	74 ± 8[c]	20 ± 5[c]	379 ± 53[e]	3.0	3.25
21	81 ± 4[d]	19 ± 2[c]	306 ± 77[e]	3.5	3.5
28	92 ± 18[e]	28 ± 8[c]	275 ± 41[e]	3.75	3.75
Control group					
12 Hours	ND	ND	ND	0.0	0.0
Day 1	51 ± 2	8 ± 0.3	9 ± 3	0.0	0.0
7	48 ± 1	7 ± 0.5	20 ± 3	1.75	0.0
21	53 ± 2	9 ± 0.2	51 ± 7	0.75	0.75
Normal rat	45 ± 2	6 ± 0.2	6 ± 1	0.0	0.0

ND, not determined
[a] Data are expressed as mean ± SEM
[b] Data are expressed as average of damage index (0–4)
[c] $P < 0.05$, [d] $P < 0.01$, [e] $P < 0.001$, compared to normal

Table 4. Correlation between leukocytic infiltration and renal impairment, proteinuria and histological damage

	Site	OX-1	ED1	OX-19	ART-18	Histological damage
Serum creatinine	G	0.65[c]	0.69[c]	−0.42[a]	0.43[a]	0.71[c]
	I	0.70[c]	0.60[c]	0.33	0.57[c]	0.83[c]
Serum urea	G	0.63[c]	0.70[c]	−0.46[b]	0.43[a]	0.73[c]
	I	0.68[c]	0.58[c]	0.27	0.53[c]	0.84[c]
Proteinuria	G	0.63[c]	0.71[c]	−0.61[c]	0.70[c]	0.65[c]
	I	0.64[c]	0.48[b]	0.37[a]	0.62[c]	0.69[c]
Histological damage	G	0.83[c]	0.79[c]	−0.91[c]	0.62[c]	—
	I	0.88[c]	0.84[c]	0.73[c]	0.89[c]	—

Data are expressed as r values from all 32 rats in experimental group. Abbreviations are: G, glomerulus; I, interstitium.
[a] $P < 0.05$
[b] $P < 0.01$
[c] $P < 0.001$

results support the postulate that cell-mediated immune mechanisms are involved in progressive interstitial damage in experimental glomerulonephritis.

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References

1. KLAHR S, SCHREINER G, ICHIKAWA I: The progression of renal disease. *N Engl J Med* 318:1657–1666, 1988
2. ATKINS RC, HOLDSWORTH SR, HANCOCK WW, THOMSON NM, GLASGOW EF: Cellular immune mechanisms in human glomerulonephritis: The role of mononuclear leucocytes. *Springer Semin Immunopathol* 5:269–296, 1982
3. SCHREINER GF, COTRAN RS, UNANUE ER: Macrophages and cellular immunity in experimental glomerulonephritis. *Springer Semin Immunopathol* 5:251–267, 1982
4. ATKINS RC, HOLDSWORTH SR: Cellular mechanisms of immune glomerular injury, in *Contemporary Issues in Nephrology*, (vol 18) edited by BRENNER BM, STERN JH, 1988, pp. 111–136
5. ATKINS RC: Pathogenesis of glomerulonephritis, 1990, in *Proceedings of the 11th International Congress of Nephrology* (in press)
6. D'AMICO G: Role of interstitial infiltration of leukocytes in glomerular diseases. *Nephrol Dial Transpl* 3:596–600, 1988
7. HOOKE DH, GEE DC, ATKINS RC: Leukocyte analysis using monoclonal antibodies in human glomerulonephritis. *Kidney Int* 31:964–972, 1987
8. ALEXOPOULOS E, SERON D, HARTLEY RB, CAMERON JS: Lupus nephritis: Correlation of interstitial cells with glomerular function. *Kidney Int* 37:100–109, 1990
9. MARKOVIC-LIPKOVSKI J, MULLER CA, RISLER T, BOHLE A, MULLER GA: Association of glomerular and interstitial mononuclear leukocytes with different forms of glomerulonephritis. *Nephrol Dial Transpl* 5:10–17, 1990
10. CASTIGLIONE A, BUCCI A, FELLIN G, D'AMICO G, ATKINS RC: The relationship of infiltrating renal leukocytes to disease activity in lupus and cryoglobulinaemic glomerulonephritis. *Nephron* 50:14–23, 1988
11. SAITO T, ATKINS RC: Contribution of mononuclear leucocytes to the progression of experimental focal glomerular sclerosis. *Kidney Int* 37:1076–1083, 1990
12. HOLDSWORTH SR, THOMSON NM, GLASGOW EF, DOWLING JP, ATKINS RC: Tissue culture of isolated glomeruli in experimental crescentic glomerulonephritis. *J Exp Med* 147:98–109, 1978
13. HANCOCK WW, BECKER GJ, ATKINS RC: A comparison of fixatives and immuno-histochemical techniques for use with monoclonal antibodies to cell surface antigens. *Am J Clin Pathol* 78:825–831, 1982
14. SUNDERLAND CA, MCMASTER WR, WILLIAMS AF: Purification with monoclonal antibody of a predominant leukocyte-common antigen and glycoprotein from rat thymocytes. *Eur J Immunol* 9:155–159, 1979
15. DALLMAN MJ, THOMAS ML, GREEN JR: MRC OX-19: A monoclonal antibody that labels rat T lymphocytes and augments in vitro proliferative responses. *Eur J Immunol* 14:260–267, 1984
16. DIJKSTRA CD, DOPP EA, JOLING P, KRAAL G: The heterogeneity of mononuclear phagocytes in lymphoid organs: Distinct macrophage

subpopulations in the rat recognized by monoclonal antibodies ED1, ED2, ED3. *Immunology* 54:589–599, 1985
17. Osawa H, Diamantstein T: The characteristics of a monoclonal antibody that binds specifically to rat T lymphoblasts and inhibits IL 2 receptor functions. *J Immunol* 130:51–55, 1983
18. Kingsbury FB, Clark CP, Williams G, Post AL: The rapid determination of albumin in urine. *J Lab Clin Med* 11:981–989, 1926
19. Zakheim B, McCafferty E, Phillips SM, Clayman M, Neilson EG: 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
20. Abe S, Amagasaki Y, Iyori S, Konishi K, Kato E, Sakaguchi H: Significance of tubulointerstitial lesions in biopsy specimens of glomerulonephritis patients. *Am J Nephrol* 9:30–37, 1989
21. Mene P, Cinotti GA: Paracrine and autocrine functions of glomerular mesangial cells. *J Endocrinol Invest* 12:497–509, 1989
22. Boswell JM, Yui MA, Burt DW, Kelley VE: Increased tumor necrosis factor and IL-1 beta gene expression in the kidneys of mice with lupus nephritis. *J Immunol* 141:3050–3054, 1988
23. Springer TA: Adhesion receptors regulate antigen-specific interactions, localization, and differentiation in the immune system, in *Progress in Immunology VII*, edited by Melchers F, Berlin, Springer-Verlag, 1989, pp. 121–130
24. Haskard DO, Thornhill M, Kyan-Aung U, Kinglsey G, Pitzalis C, Panayi GS: Cytokine induced changes in the endothelial cell surface membrane: significance for lymphocyte traffic in inflammation, in *Progress in Immunology VII*, edited by Melchers F, Berlin, Springer-Verlag, 1989, pp. 780–783
25. Bishop GA, Hall BM: Expression of leucocyte and lymphocyte adhesion molecules in the human kidney. *Kidney Int* 36:1078–1085, 1989
26. Wuthrich RP, Jevnikar AM, Takei F, Glimcher LH, Kelley VE: Intercellular adhesion molecule-1 (ICAM-1) expression is upregulated in autoimmune murine lupus nephritis. *Am J Pathol* 136:441–450, 1990
27. Matsumoto K, Dowling J, Atkins RC: Production of interleukin 1 in glomerular cell cultures from patients with rapidly progressive crescentic glomerulonephritis. *Am J Nephrol* 8:463–470, 1988
28. Matsumoto K, Atkins RC: Glomerular cells and macrophages in the progression of experimental focal and segmental glomerulosclerosis. *Am J Pathol* 134:933–945, 1989
29. Matsumoto K: Production of interleukin-1 by glomerular macrophages in nephrotoxic serum nephritis. *Am J Pathol* 10:502–506, 1990
30. Werber HI, Emancipator SN, Tykocinski ML, Sedor JR: The interleukin 1 gene is expressed by rat glomerular mesangial cells and is augmented in immune complex glomerulonephritis. *J Immunol* 138:3207–3212, 1987
31. Tipping PG, Neale TJ, Holdsworth SR: T lymphocyte participation in antibody-induced experimental glomerulonephritis. *Kidney Int* 27:530–537, 1985
32. Boyce NW, Holdsworth SR, Dijkstra CD, Atkins RC: Quantitation of intraglomerular mononuclear phagocytes in experimental glomerulonephritis in the rat using specific monoclonal antibodies. *Pathology* 19:290–293, 1987
33. Paterson DJ, Jefferies WA, Green JR, Brandon MR, Corthesy P, Puklavec M, Williams AF: Antigens of activated rat T lymphocytes including a molecule of 50,000 Mr detected only on CD4 positive T blasts. *Mol Immunol* 24:1281–1290, 1987
34. Hancock WW, Kobzik L, Colby AJ, O'Hara CJ, Cooper AG, Godleski JJ: Detection of lymphokines and lymphokine receptors in pulmonary sarcoidosis. Immunohistologic evidence that inflammatory macrophages express IL-2 receptors. *Am J Pathol* 123:1–8, 1986