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Analysis of T Cells and Major Histocompatibility Complex Class I and Class II mRNA and Protein Content and Distribution in Antiglomerular Basement Membrane Disease in the Rabbit

Charles Eldredge,* Steven Merritt,* Meera Goyal,* Henrietta Kulaga,† Thomas J. Kindt,‡ and Roger Wiggins*

From the Department of Internal Medicine, The University of Michigan, Ann Arbor, Michigan; and the Neuropsychiatry Branch,† National Institute of Mental Health, and the Laboratory of Immunogenetics,‡ National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland*

The major interacting components of the immune system, major histocompatibility complex (MHC) class I and class II proteins and T cells were analyzed in a model of anti-GBM (glomerular basement membrane) disease in the rabbit that progresses to develop cellular crescents and glomerular and interstitial fibrosis. Class I and II mRNA and protein were measured in isolated glomeruli and whole renal cortex using cDNA probes and monoclonal antibodies. The distribution of T cells and class I and II proteins was assessed by immunofluorescence. Normal glomeruli contained no T cells and were class II negative. By day 4, glomeruli contained MHC class I and II mRNA and protein and class II positive T cells. Although some animals had T cells in the periglomerular area, these cells were class II negative. By day 7 periglomerular T cells were largely class II positive (activated) and there was increased MHC class I and II mRNA and protein in whole renal cortex. Later T cells accumulated in the tubulo-interstitial compartment, which became diffusely positive for MHC classes I and II, but to a variable extent in different animals. Those with high class II mRNA expression also had detectable T cell antigen receptor mRNA by Northern analysis. The authors conclude 1) in this model there was a close association between mRNA abundance and protein expression for both MHC classes I and II in glomeruli and renal cortex as a whole; 2) in this model of glomerular injury there are three phases of activation. The first phase takes place in the glomerulus and is associated with accumulation of activated T cells and MHC class I and II protein in the glomerulus. Phase 2 is associated with the accumulation of periglomerular T cells and their becoming class II positive. There is subsequent dissemination (phase 3) of activated T cells and accumulation of class I and II mRNA and protein throughout the interstitial compartment. This spacial progression of glomerulocentric inflammation is likely associated with degree of injury and permanent loss of renal function. (Am J Pathol 1991, 139:1021–1035)

Glomerular crescent formation is commonly associated with scarring of both glomerular and interstitial compartments and permanent loss of renal function. To understand the renal fibrotic process during crescent formation, we analyzed in detail the time course of matrix protein (collagen I and IV and fibronectin) mRNA, protein synthesis, accumulation, and distribution in a model of anti-glomerular basement membrane (GBM)–induced crescentic nephritis in the rabbit.[1–3] We found that transforming growth factor-β (TGF-β) appeared to be an important cytokine driving matrix synthesis in glomeruli and renal cortex in the model.[4] This study was designed to test the hypothesis that the scarring process seen in the model is driven by T cells directly or indirectly. We have focused on analyzing T cell infiltration and activation and class I and II protein expression. Analysis of monocyte/macrophage infiltration and activation, which also is rel-

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Address reprint requests to Dr. Roger C. Wiggins, Chief, Nephrology Division, The University of Michigan Medical Center, 3914 Taubman Center, Box 0364, Ann Arbor, MI 48109.

evant to understanding the fibrotic process in this model, form the basis of a subsequent report.

The evidence that T cells play a role in some forms of glomerular disease is rapidly accumulating. In man, T cells are often found in proliferative types of glomerulonephritis, particularly in crescentic nephritis.[5–10] T cells are also found within glomeruli in experimental anti-GBM disease in rats.[11,12] The most direct data linking T cells to glomerular injury in a casual fashion, however, come from two types of experiment. First transfer of sensitized T cells to rats in which the target antigen has been planted in the recipient's glomeruli causes glomerulonephritis.[13–15] Recent studies showing crescent formation with this model are particularly striking.[15] Second in bursectomized chickens, which do not produce antibody, immunization with bovine GBM causes glomerulonephritis.[16] The involvement of T cells in interstitial nephritis is well accepted.[17] T cells are often a predominant cell in the interstitial infiltrates of human renal biopsies with primary glomerular disease of various types,[17–20] and interstitial lymphocytes are often seen in severe anti-GBM disease in humans.[21–30] In experimental interstitial nephritis, a number of elegantly dissected models have helped elucidate complex interacting elements of the T cell regulatory system.[17] Studies in other organs have implicated T cells in driving the fibrotic response to inflammatory injury,[31] and we hypothesize that the same process occurs in the kidney.[32]

T cells recognize and respond to peptides bound to major histocompatibility complex (MHC) class II proteins on the APC (antigen-presenting cell) surface through the T-cell antigen receptor (TCR) on the T cell surface. We therefore analyzed the distribution and activation state of T cells and the appearance of MHC class I and class II mRNA and protein in glomeruli and interstitium of a model of anti-GBM disease in the rabbit. In this model, rabbits preimmunized against guinea pig gamma G immunoglobulin (IgG) mount both a cellular and a humoral immune response that targets guinea pig immunoglobulin bound along the glomerular basement membrane. The result of this immune-driven process is characterized histologically by glomerular proliferation, necrosis, crescent formation, and scarring in both the glomerular and interstitial compartments.[1] The histologic sequence of events in the model, including the periglomerular accumulation of lymphocytes, is very similar to that described in anti-GBM disease in humans.[25]

Materials and Methods

Model of Anti-GBM Disease in the Rabbit

The experimental model used was as described previously.[1,33] Briefly, New Zealand White rabbits, weighing 2.0 to 2.5 kg, were injected subcutaneously with 200 μg guinea pig IgG in Complete Freund's Adjuvant (Sigma Chemical Co., St. Louis, MO) Five days later they were given an intravenous injection of guinea pig anti-rabbit basement membrane (anti-GBM) IgG. Control animals received no injections. Rabbits were kept in individual cages and were given water and regular rabbit chow *ad libitum.* In the text, all times are noted as the number of days after the intravenous anti-GBM antibody injection. Previous studies have established that proteinuria begins on approximately day 4, serum creatinine is increased by day 7, the peak of glomerular procoagulant activity and formation of the fibrin-containing proteinaceous cast in Bowman's space occurs by day 7, and cellular crescents are well established by day 14. The time course of the collagen mRNA accumulation, synthetic rate, hydroxyproline accumulation, and stainable collagens in cortex and glomeruli has been reported.[1,2] At the indicated times, renal tissue samples were obtained for analysis from these rabbits, after anesthetization with 50 mg/kg intravenous sodium pentobarbital.

Glomerular Isolation

Kidneys were perfused with cold phosphate-buffered saline (PBS) until blanched and then with 180 ml iron oxide suspension (5 mg/ml in normal saline) through the inferior aorta as previously described.[1] The kidneys then were harvested and the capsule was removed. The cortex was trimmed off with scissors. In each case, cortex was saved for histologic analysis by fixing in formalin, and for immunofluorescence and for cortical RNA extraction by snap freezing in liquid nitrogen. The remaining cortex was homogenized in chilled PBS by a Polytron (Brinkman Instruments Co., Westbury, NY) and then passed through a 200-μ nylon screen (Tetko Inc., Elmsford, NY) into a 250-ml beaker. The filtrate then was placed on a magnet, and the iron-embolized glomeruli were purified by successively washing away the nonmagnetized material. All glomerular preparations used were more than 94% glomeruli, with small tubular contamination. Glomerular yield was assessed by wet mount counts of 10-μl samples. Glomerular purity and representation was assessed by plastic embedding a formalin-fixed aliquot of each glomerular preparation, and then doing counts at the light microscopic level of 1-μ sections of the isolated glomeruli. As reported previously, the isolated glomeruli are representative of glomeruli in the histologic sections from the same animals for control and day 4, but that the day 7 and to a larger extent the day 14 glomeruli were biased toward more normal glomerular histologic appearance.[1]

Histologic Analysis

Sections fixed in formalin were stained by hematoxylin and eosin (H&E) and by the Masson-Trichrome method.

For glomerular cell counts, H&E-stained sections (6 μmol/l [micromolar]) from five rabbits per time point were assessed by an individual who was blinded to the time point (SM). He counted the number of nuclei per glomerular cross section in 10 glomeruli (per histologic section) that appeared to have been cut transversely through the tuft. The mean value for each animal was calculated, and these values were used to calculate the mean ± standard error of the mean (SEM) for each time point.

Tissue for immunofluorescence was snap frozen in liquid nitrogen, stored at −70°C, and was sectioned (3- to 5-μ sections cut on a HistoStat microtome [Scientific Instruments, Buffalo, NY]). Sections were fixed in acetone, blocked with goat serum (10%), incubated with the primary antibody (a monoclonal mouse antibody of the IgG1 subclass), and, after a washing step, incubated with a fluorescein-labeled goat anti-mouse secondary antibody (Cappel, Malvern, PA) that had been preabsorbed with both guinea pig and rabbit serum (10%). Sections were viewed and photographed on a Zeiss Photomicroscope 111 or an Olympus Photomicroscope (Scientific Supply Co., Schiller Park, IL). Sections were compared on each occasion with sections stained with a control monoclonal antibody of the same subclass, and were scored according to the following method: Semiquantitative grades were assigned to the glomerular density (proportion of glomeruli involved), number of positive cells per glomerulus, and number of interstitial cells as outlined below. No attempt was made to grade differences in the intensity of labeling. At least 25 glomeruli were evaluated in each section. The pattern of tubular labeling, if any, also was noted.

Semiquantitative grading of immunofluorescence was done according to the following scheme: Proportion of glomeruli positive: 0 = none, 1+ = 1% to 25% positive, 2+ = 26% to 50% positive, 3+ = 51% to 75% positive, 4+ = 76% to 100% positive; Glomerular cell density: 0 = no cells labeled, 1+ = less than 5 cells per glomerular section labeled, 2+ = 5 or more distinct cells per glomerular section labeled, 3+ = glomerulus packed with labeled cells; Interstitial density: 0 = none, 1+ = less than 10 cells or small aggregates (2 to 3 cells) per high-power field (HPF [250×]), 2+ = 10 or more cells or small aggregates per HPF, without large aggregates, 3+ = large aggregates of cells present, 4+ = labeled cells confluent through large areas of the section.

Monoclonal Antibodies

The L11/135 monoclonal antibody was obtained from ATCC (Rockville, MD) and is an IgG1 pan rabbit T cell antibody that recognizes a 120-kd cell surface glycoprotein.[34] The 2C4 monoclonal antibody was provided by Dr. Katherine Knight (Chicago, IL) and was also obtained from ATCC.[35] This reagent recognizes an isotypic determinant on all rabbit class II molecules, and it immunoprecipitates polypeptide chains of 28, 31, and 35 kd.[35] The class I monoclonal antibody, 61-183-3, recognizes a 42-kd class I cell surface glycoprotein that is present in only about 50% of animals.[36] Therefore for all animals studied with this antibody, the spleen also was examined as a positive control to ensure that the monoclonal antibody would recognize class I if it were present. If the spleen was negative, the kidney of that animal was not used for class I analysis. Monoclonal antibody 1.1B (IgG 1), which does not recognize rabbit antigens, was used as a negative isotype control for immunofluorescent studies.

Double-label Studies

Gamma G immunoglobulin purified from ascites produced in mice injected intraperitoneally with L11/135 hybridoma (ATCC, Rockville, MD) was biotinylated according to the method of Jackson et al.[37] Frozen sections were first blocked with goat serum and processed for class II staining with the 2C4 monoclonal and fluorescein-labeled anti-mouse immunoglobulin as described above. Sections then were reblocked with mouse serum before incubation with the biotinylated L11/135 IgG (1/50), washed, and then incubated with the avidin-rhodamine complex (Cappel, Malvern, PA). As a positive control, an IgG1 monoclonal that recognizes the brush border of proximal tubular cells and glomerular epithelial cells (1F2) and a second IgG1 monoclonal that does not recognize rabbit kidney (1.1) were biotinylated and used in parallel sections.

Glomerular and Cortical Extraction Procedure

Fifty thousand isolated glomeruli or 50 mg minced cortex separately were lysed in 1 ml freshly made PBS buffer containing detergents Triton X-100 (1%), sodium dodecyl sulfate (SDS; 0.1%), and protease inhibitors (phenylmethylsulfonyl fluoride [2 mmol/l]), N-ethylmaleimide (5 mmol/l), ethylenediaminetetra-acetic acid (EDTA, 2 mmol/l), and 8 mol/l urea by quick sonication in short burst while on ice. Iron oxide and debris was removed by centrifugation at 12,000 rpm at 4°C (Beckman Microfuge) for 5 minutes. Supernatant was stored at −70°C until ready to use.

Western and Dot-blot Procedure

The extracts from glomeruli and cortex were dotted (3 μl) onto nitrocellulose membrane (Bio-Rad Laboratories,

Richmond, CA). Membranes were air dried and soaked in 1% bovine serum albumin (BSA) in PBS–Tween solution for 30 minutes. After four washes in PBS–Tween, the membranes were incubated with the primary antibody (2C4 [class II], 61-183-3 [class I], or 9G7-3 [control IgG I]) and rocked for 60 minutes. Membranes were washed five times in PBS–Tween (10 ml) and then incubated with secondary antibody (0.125 μCi/ml) ^{125}I-labeled sheep anti-mouse IgG, F(ag')2 fragment (NEN Research Products) in 3% rabbit serum in PBS for 60 minutes. After further washes (× 5) in PBS–Tween, the membranes were air dried and exposed to x-ray film. The radioactivity in each sample was quantitated by the use of a Gamma Counter (4000 Multiwell Gamma Counter, Corning) by cutting out each dot and measuring the radioactivity.

For Western blots, aliquots of glomerular and cortical extracts were mixed with SDS sample buffer containing B-mercaptoethanol and loaded onto 7% SDS-polyacrylamide gels. Molecular weight standards (Bio Rad Laboratories, Richmond, CA) were included in each gel. Gels were run by the method of Laemmli[38] for 1+ hours at 100 volts. The proteins were transferred from the gel onto nitrocellulose by Polyblot (Model SBD-1000, American Bionectics) using a discontinuous buffer system. The blots were overlaid with primary antibody (mouse IgG as above), followed by ^{125}I-labeled sheep anti-mouse IgG, and then exposed to x-ray film as described. The class I antibody (61-183-3) recognized a band on the Western blot, whereas the class II antibody (2C4) did not, probably because of the denaturing conditions used.

Purification of RNA

RNA was purified using a modification of the method of Chirgwin et al.[39] Glomeruli were purified as described above, counted, centrifuged, and the pellet containing 100 to 250 thousand glomeruli was suspended in 3 ml guanidine isothiocyanate (GITC) solution and quickly sonicated (Heat System—Ultrasonics, Inc., Farmingdale, NJ) in short bursts while on ice. Iron oxide and debris was removed by centrifugation (Beckman Microfuge) for 5 minutes. The supernatant was loaded onto two cesium chloride gradients, each consisting of 2.5 ml 5.7 mol/l CsCl, 0.5 ml 2.3 mol/l CsCl, and 0.5 ml 1.1 mol/l CsCl in a diethylpyrocarbonate (DEPC) treated Beckman Sw55Ti polyallomer tube. The gradients were centrifuged at 130,000*g* for 16 hours at 16°C. After centrifugation, the GITC layer plus any cloudy material was removed from the upper portion of the tube. Fifty percent CsCl was used to float off any remaining visible contaminants. Then all but approximately 0.5 ml CsCl was removed with a sterile transfer pipet. The tubes were cut near the bottom seam with a clean razor blade and the remaining liquid was poured off. The gelatinous pellet was resuspended in 250 μl 0.3M DEPC-treated sodium acetate (pH 6) per tube, pooled, and precipitated with two volumes of ethanol at −70°C for 1 hour. After microcentrifugation for 10 minutes, the pellet was resuspended in 50 μl DEPC-treated water. Absorbance at 260 and 280 nm was measured to assess protein contamination and to quantitate RNA. Samples were aliquotted and stored at −70°C.

For isolation of cortical RNA, the perfused kidney cortex was snap frozen in liquid nitrogen and stored at −70°C until needed. The cortical fragments were quickly homogenized with a polytron in GITC solution (as above) at approximately 150 to 180 mg wet tissue per milliliter GITC solution. The solution was microcentrifuged for 5 minutes, and then the RNA was purified on a CsCl gradient as described above for glomerular RNA.

To assess quality of RNA, the RNA was electrophoresed on a 1% agaroseformaldehyde gel and stained with ethidium bromide to confirm clear visualization of the 18 and 28S rRNA bands. Northern transfers (gene screen, New England Nuclear, Dupont) were stained with methylene blue.[40]

cDNA Probes and Hybridization Conditions

The cDNA probes for class I, class II, the TCRβ, and the 28S rRNA probes were as follows: The class I probe was a 390 bp *Eco*R1-*Pst*1 fragment (PR9) 19-1 exon 4 cDNA.[41] The class II probe was a 2.2 Kb *Hin*dIII fragment of DQ alpha genomic DNA.[42] The T cell antigen receptor probe was 0.38 Kb fragment of the C beta region, designated CT beta 92 as previously described.[43] A plasmid containing portions of the 28S rRNA gene was used to correct for differences in loading of gels as described below.[44] Nucleic acid probes were labeled to a specific activity of 10^9 dpm/μg using a random primer method.[45]

To determine specific hybridization conditions for each probe, Northern filters (Gene Screen, New England Nuclear, Dupont) were hybridized in 0.5 mol/l $NaH2PO_4$ buffer, pH 7.0, containing 1 mmol/l EDTA, 7% SDS, 1% BSA, and 100 μg/ml denatured salmon sperm DNA at temperatures between 50°C and 65°C. After 16 to 18 hours, the filters were washed (at the same temperature) twice with 40 mmol/l $NaPO_4$, pH 7.0, containing 1 mmol/l EDTA, 5% SDS, and 0.5% BSA, and four times with 40 mmol/l $NaPO_4$, pH 7.0, containing 1 mmol/l EDTA and 1% SDS. Optimal hybridization and washing conditions were 65°C for the 28S rRNA and class I probes and 60°C for the class II probe.

Autoradiograms were prepared from each Northern or dot-blot filter by exposing X-OMAT AR film at −70°C with intensifying screens. Exposures were varied to pro-

duce signals within the linear range of the film. The radio density was quantitated with a laser densitometer and the integrated peak areas for MHC class I and II probes were corrected for loading/transfer inequities with the area for the 28S rRNA probe derived from the same sample on the same blot. Mean areas for each probe were expressed relative to the mean of control animals (designated 100%). The statistical variation about the meaned values for each time then was calculated.

For the Northern blot shown in Figure 11, 1.5% agarose gels were blotted onto nitrocellulose. Filters were dried and baked for 2 hours at 80°C and prehybridized at 42°C for 16 hours. Filters then were hybridized with 10^7 cpm of (alpha-^{32}P) nick-translated DNA probe in 7 ml hybridization mixture (containing dextran sulfate) at 42°C for 18 to 20 hours. Filters were washed twice with 0.03 mol/l NaCl, 0.003 mol/l sodium citrate (0.2 × SSC), and 0.1% SDS for 15 minutes, twice at 52°C with 0.1 × SSC 0.1% SDS for 30 minutes, and once at 60°C with 0.1 × SSC for 30 minutes. Filters were dried and exposed to XAR-2 films with an intensifying screen at −70°C. Transcript size was estimated in relation to the migration of 18S and 28S RNA subunits.

Statistics

All data were analyzed using analysis of variance except where noted. Grading of histologic section was compared with the chi-square test. A P value of 0.05 was considered to be statistically significant. All data are expressed as the mean ± the standard error of the mean (SEM).

Results

Light microscopic findings in the model of anti-GBM disease are shown in Figure 1. The major change by day 4

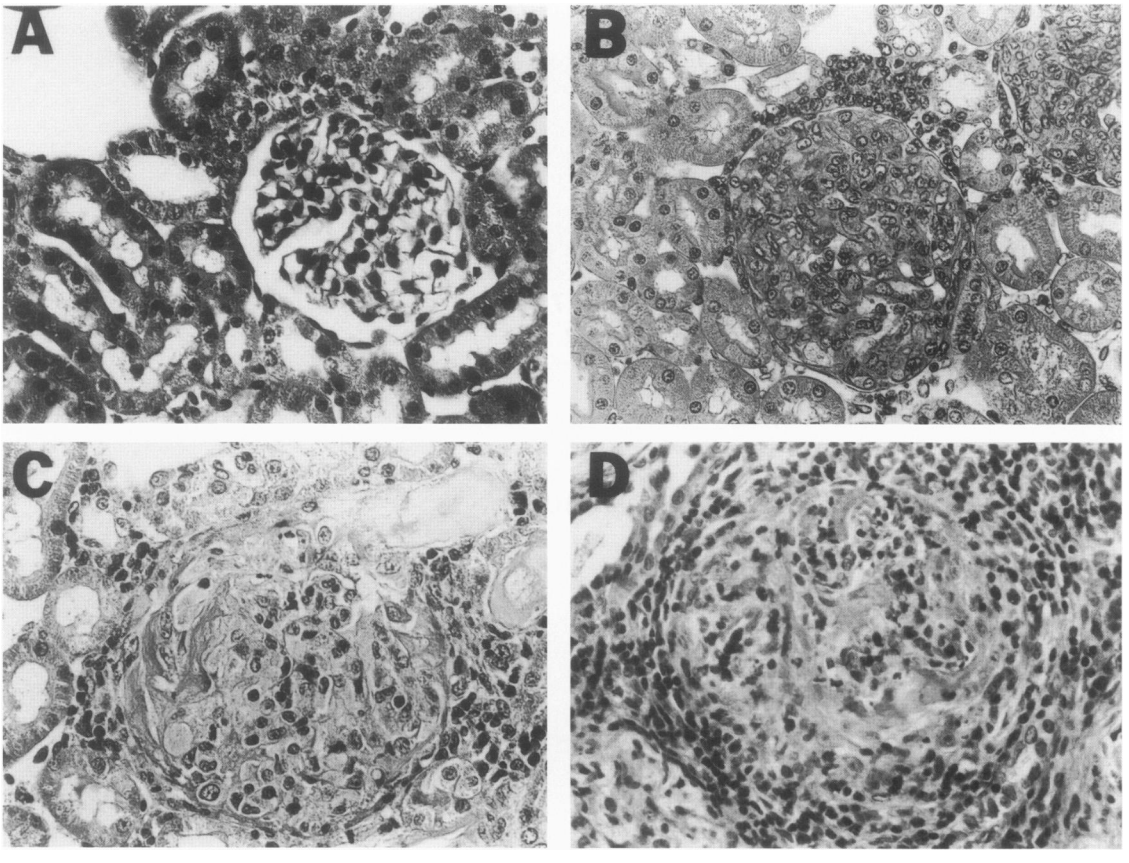

Figure 1. *Light micrographs of kidney from control* **(A)**, *day 4* **(B)**, *Day 7* **(C)**, *and day 14* **(D)**, *rabbits with anti-GBM disease. At day 4* **(B)**, *glomeruli contain increased numbers of cells and in some animals there is a periglomerular accumulation of mononuclear cells (seen particularly above the glomerulus in* **B**). *At day 7, Bowman's space and tubules contain proteinaceous material which contains fibrin and cells are present in this matrix. There is also a periglomerular and interstitial accumulation of mononuclear cells. By day 14, many glomeruli show areas of necrosis with accumulation of cells in Bowman's space, a dense periglomerular accumulation of mononuclear cells and widespread interstitial mononuclear cell accumulation; magnification, ×300. The mean nuclear counts per glomerular cross-section were control = 71 ± 9, day 4 = 140 ± 14, day 7 = 91 ± 27 and day 14 = 177 ± 39 (n = 5 per group). The day 14 count is probably an overestimate of the true glomerular cell number as the bounds of the glomerulus are lost during the development of the crescent and the periglomerular cell accumulation, making it difficult to assess true glomerular cell number; magnification, ×300.*

was a doubling of the number of cells per glomerular cross section, from 71 ± 9 to 140 ± 14. In some animals there was a periglomerular accumulation of cells at day 4, as shown above the glomerulus in Figure 1B. By day 7 (Figure 1C) the number of cells per cross section had decreased to 91 ± 27, although this was not statistically significantly different from day 4. By day 7, proteinaceous material containing fibrin had accumulated in Bowman's space and proximal tubular lumen, and there was a more widespread interstitial infiltrate. By day 14 on average, about 50% of glomeruli showed areas of necrosis and crescent formation, and there was a marked periglomerular and interstitial accumulation of mononuclear cells. The remaining 50% of glomeruli return toward normal by this time, as we have previously reported.[1]

Figure 2 shows Northern blots for MHC class I and class II mRNA using RNA preparations obtained from whole renal cortex and from isolated glomeruli of individual animals. Normal glomeruli contained no detectable class II mRNA and a barely detectable band for class I mRNA. Both glomerular and renal cortical RNA preparations showed a marked increase in specific mRNA for MHC class I and class II with injury. The transcript sizes were 1.3 Kb for class II and 1.7 (major) and 2.7 (minor) Kb for class I.

Figure 3 (lower panel) shows semiquantitation of class

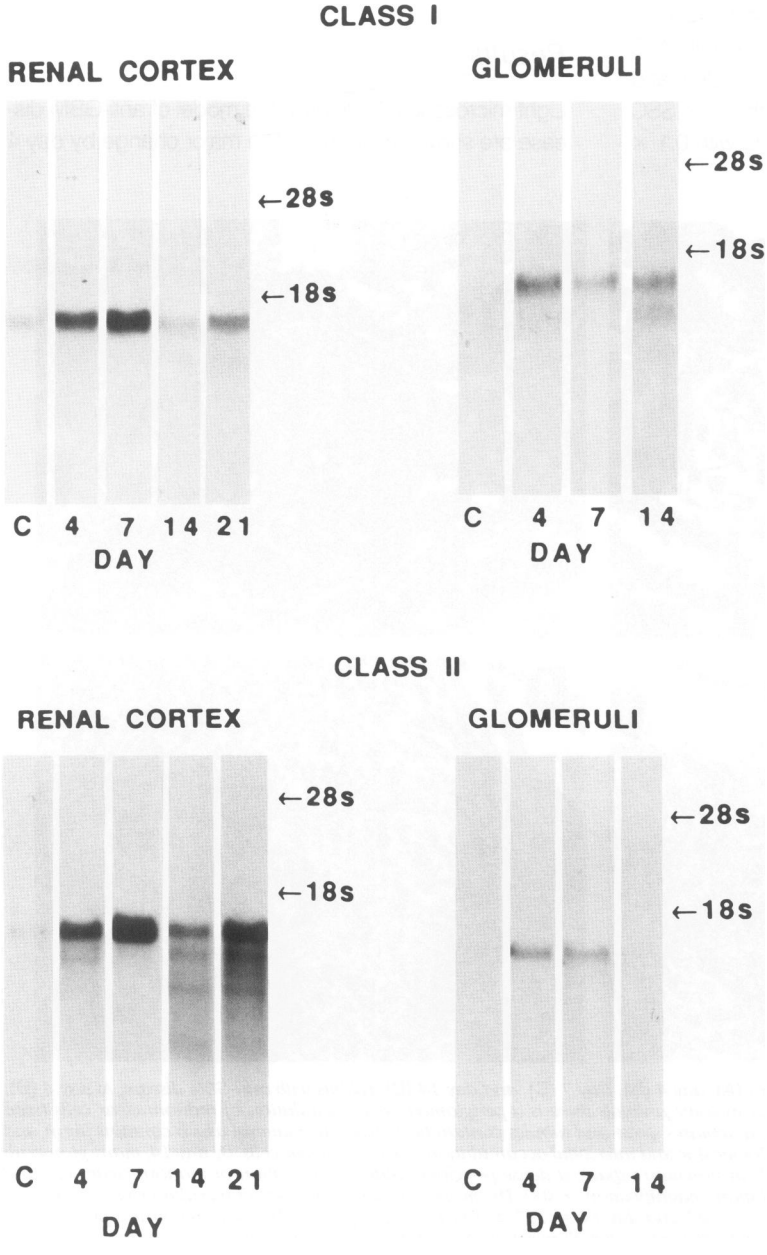

Figure 2. *Northern blots of RNA obtained from whole renal cortex (left panels) or isolated glomeruli (right panels) probed with ^{32}P-labelled cDNAs for Class I (upper panels) and Class II (lower panels). The individual RNA preparations used were identical for the two studies. The position of migration of the 28S and 18S ribosomal bands are shown. The data show specific hybridization for MHC Class I and II and intact RNA isolated from glomeruli and renal cortex. These hybridization conditions were used for subsequent studies.*

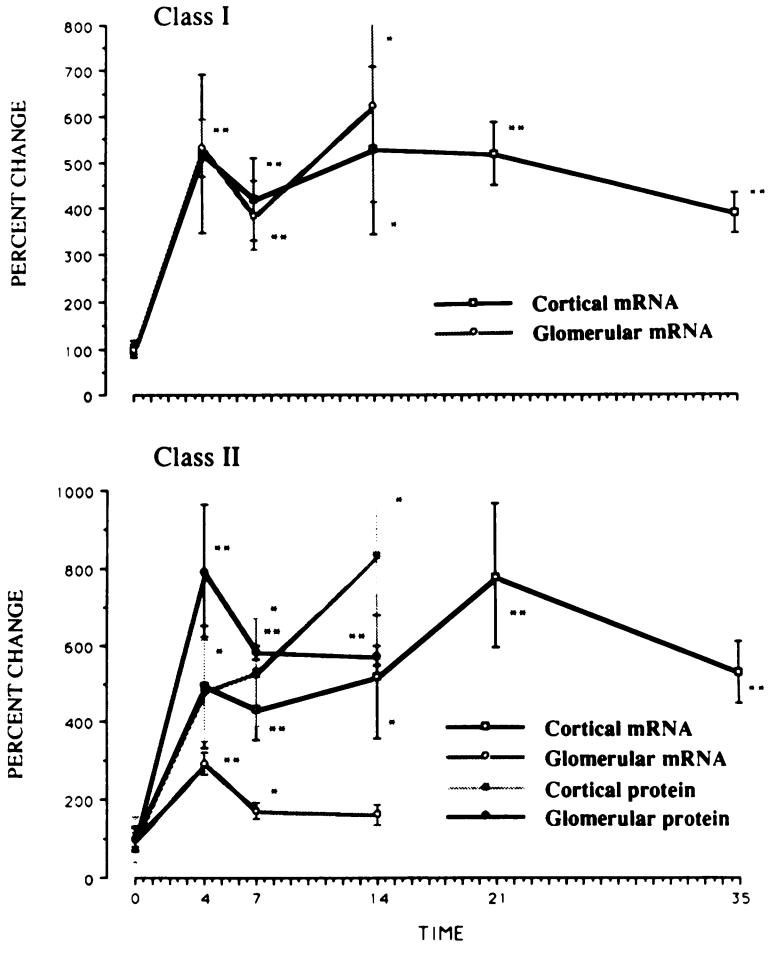

Figure 3. *(Top) Quantitation of Class I mRNA from cortex (open squares) and isolated glomeruli (open circles) corrected for total 28S rRNA loaded and expressed as percent control ± SEM. *P < 0.05, **P < 0.01 compared with control. n = 5 per group. (Bottom) Quantitation of Class II mRNA from cortex (open squares) and isolated glomeruli (open circles) corrected for total 28S rRNA loaded and expressed as percent control ± SEM. Quantitation of Class II protein from cortex (closed squares) and isolated glomeruli (closed circles) expressed as percent control ± SEM. The time is in days. *P < 0.05, **P < 0.01 compared with control. n = 5 per group.*

II mRNA and protein in glomeruli and whole renal cortex (n = 5 per group). At day 4 there was a marked and statistically significant increase in glomerular class II mRNA. By day 7, glomerular class II mRNA remained significantly elevated above baseline, but by day 14 the increase was no longer statistically significant. Whole renal cortical class II mRNA was significantly increased above baseline through day 35.

Quantitation of the total class II protein using the 2C4 monoclonal antibody in Triton/urea extracts of glomeruli and renal cortex is also shown in Figure 3. There was little class II protein detectable in normal glomeruli or cortex. Quantitation by dot blot showed that by day 4 glomeruli contained eightfold more class II protein than controls ($P < 0.01$). By days 7 and 14, glomerular class II had decreased, although the levels were still significantly above baseline. Whole cortical class II protein was increased significantly by day 7 and, in contrast to glomeruli, the cortical class II protein continued to increase through day 14.

The distribution of Class II proteins as assessed by immunofluorescence in kidney sections using the 2C4 monoclonal antibody is shown in Figure 4. Control renal cortex contained bright class II positive cells sparsely distributed in the interstitial compartment, with occasional tubules staining faintly as has been previously reported.[46] Control glomeruli in perfused animals contained only an occasional positive cell, probably a resident monocyte/macrophage, as has been described in the rat.[47] In contrast, by day 4 glomeruli were brightly positive, with most cells appearing to be class II positive. Periglomerular cells were rarely class II positive at day 4, and there was no increase in class II positive cells in the tubulo-interstitial compartment. By day 7, glomeruli were surrounded by class II positive cells, whereas the glomeruli themselves appeared to be relatively less brightly stained. At this time the interstitial compartment contained more class II positive cells than control, and some tubules were class II positive. By day 14, the class II staining was present in periglomerular and tubulo-interstitial compartments. Semiquantitation of these findings for four to nine animals per time point is shown in Table 1.

We conclude that the data for class II mRNA, total

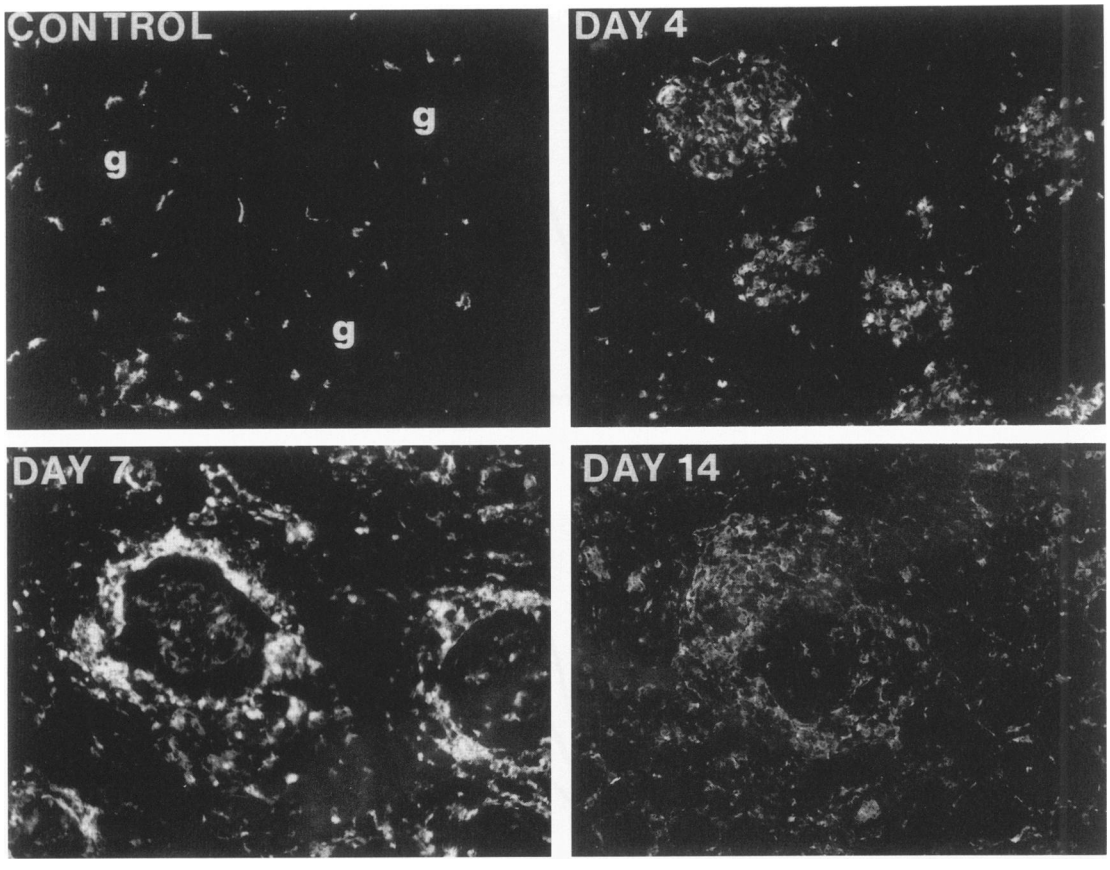

Figure 4. *Immunofluorescent analysis of Class II protein expression in renal cortex of control, day 4, day 7, and day 14 animals. In the control panel, the position of three glomeruli is indicated (g). Quantitation of immunofluorescent results for five animals per group is shown in Table 1; magnification, ×150.*

class II protein, and immunofluorescence all showed essentially the same thing. The initial expression of class II at day 4 is predominantly in glomeruli. At later time points, class II expression is markedly increased in the extraglomerular compartment. The data also confirm the close relationship between mRNA, total class II protein expression, and immunofluorescent findings in this model of immunologic renal injury.

Figure 2 (upper panel) shows the quantitation of class I mRNA in glomeruli and renal cortex. By day 4, class I mRNA expression in glomeruli was increased, and it remained high through day 14. In whole cortex at day 4, the

Table 1. *Semiquantitative Immunofluorescent Analysis of T-cell and Class II Expression During Evolution of Crescentic Nephritis*

Group	N	Glomerular fluorescence present	Interstitial† fluorescence present	Glomeruli positive (±SEM)	Cells per glomerulus (±SEM)	Interstitial† cell density (±SEM)
Pan T cell (L11/135)						
Control	4	0	2	0.0 (0)	0.0 (0)	0.8 (0.5)
Day 3–4	9	7	9	2.3 (0.8)**	1.4 (0.4)**	2.9 (0.1)*
Day 7–9	5	3	5	2.4 (1.0)*	1.2 (0.6)	3.2 (0.4)**
Day 14–16	5	2	5	1.6 (1.0)	0.6 (0.4)	3.2 (0.2)**
Day 21–23	5	3	5	2.4 (1.0)*	0.8 (0.4)	3.0 (0)**
Class II (2C4)						
Control	4	0	4	0.0 (0)	0.0 (0)	2.3 (0.3)
Day 3–4	9	9	9	4.0 (0)**	3.0 (0)**	2.5 (0.2)
Day 7–9	5	5	5	3.2 (0.5)**	2.8 (0.2)**	3.2 (0.2)**
Day 14–16	5	5	5	2.8 (0.7)**	3.0 (0)**	3.4 (0.2)**
Day 21–23	5	4	5	3.2 (0.8)**	2.4 (0.6)	3.2 (0.4)**

† The interstitial T cells at day 4 were predominantly in the immediate periglomerular area. At later time points they accumulated in the general interstitial compartment.

* $P > 0.05$, ** $P > 0.01$.

See Methods section for the scoring used for these studies. The numbers provided refer to the score rather than absolute cell numbers.

result was variable, but class I was not statistically above baseline. At later time points through day 35, class I was significantly increased above baseline in whole renal cortex.

Western blots of Triton/urea extracts from glomeruli and renal cortex were performed to confirm that the monoclonal antibody 61-183-3 was specific for class I. The control animals had only a faint band detectable in the glomerular extract. In contrast, the day 4, 7, and 14 animals each showed a single heavy band at approximately 42 kd as has been reported previously for rabbit class I protein.[36] A similar increase in a single band of the same size was seen in extracts of whole renal cortex (data not shown). We conclude that the monoclonal antibody was specific for class I under the conditions used.

Figure 5 shows the distribution of class I proteins in sections of renal cortex as detected by immunofluorescence using the monoclonal antibody (61-183-3) over the same period. In normal kidney, there was weak class I protein detectable in glomeruli and interstitium. By day 4, this was increased particularly in glomeruli, but also in extraglomerular interstitial cells. In some animals there was detectable staining in tubular cells. At days 7 and 14, there continued to be increased staining in all compartments. These data confirm a close relationship between mRNA, protein, and immunofluorescence for class I in glomeruli and cortex.

We next examined the distribution of T cells (Figure 6). Normal renal cortex contains occasional T cells, which are markedly reduced if the tissue is perfused before analysis. By days 3 to 4 in 7 of 9 animals, glomeruli contained 2 to 25 T cells per cross section (Table 1). In those animals in which light microscopy showed periglomerular accumulation of cells at day 4 (Figure 1B), glomeruli were surrounded by cells that stained brightly with the L11/135 anti–T-cell monoclonal antibody (Figure 6). By days 7 to 9, T cells were fewer in glomeruli, but were present in crescents, and periglomerular T cells increased in number. At later points, T cells were present in crescents and in the periglomerular and interstitial compartment, whereas in some animals glomeruli had returned toward normal by histologic analysis and few T cells were present. Semiquantitation of T cells for the group of animals is shown in Table 1.

Because of the similarity in the T cell and class II distribution at day 7, we performed double labeling studies

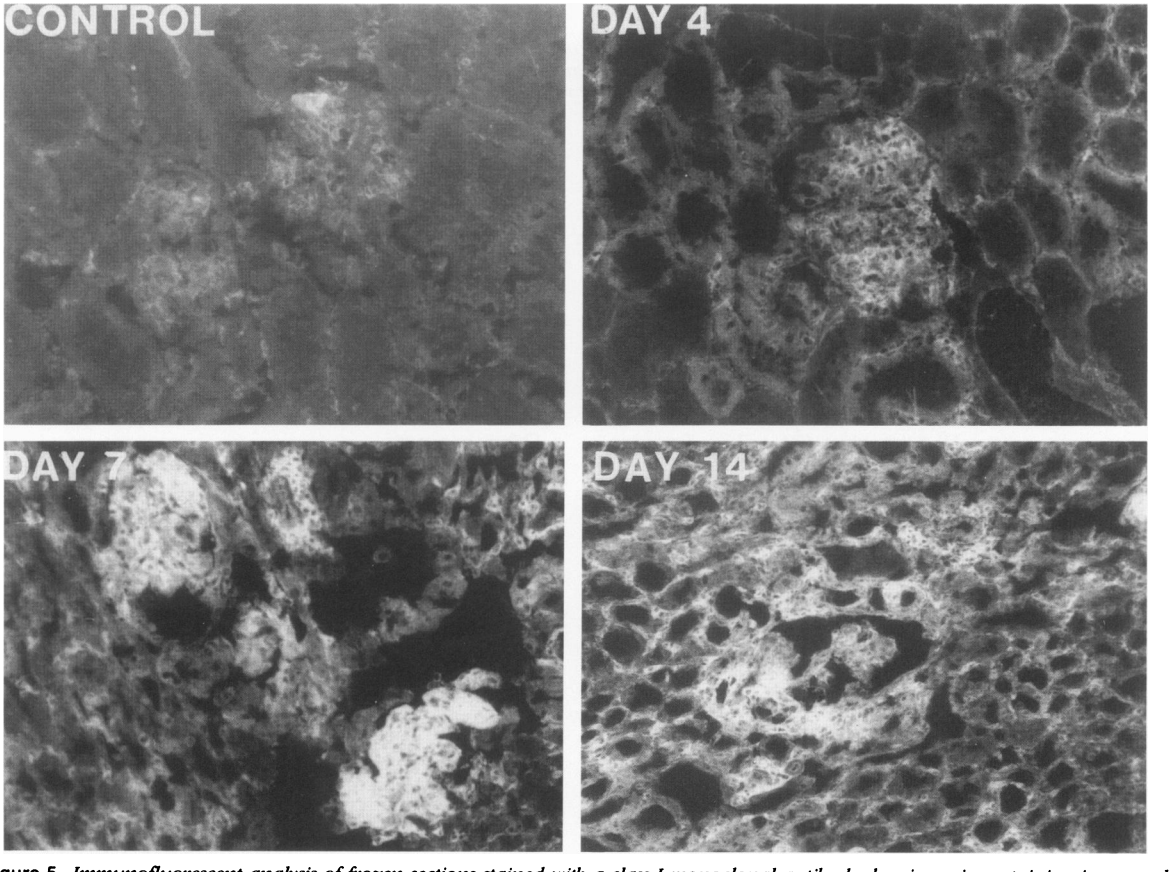

Figure 5. *Immunofluorescent analysis of frozen sections stained with a class I monoclonal antibody showing minor staining in control animals, but a marked increase in staining by 4 to 14 days in glomeruli and a continued increase in Class I staining in the tubulointerstitial compartment at days 7 to 14; magnification, ×150.*

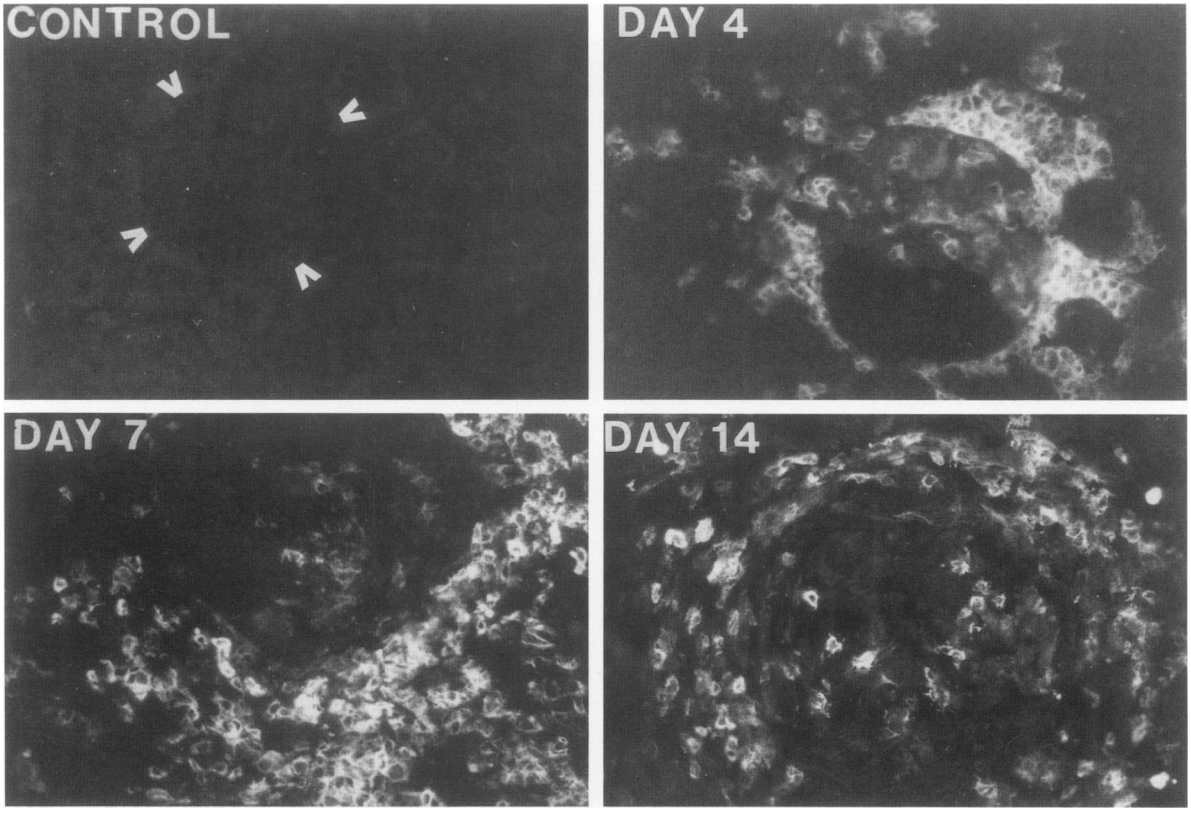

Figure 6. *Distribution of T cells in control and day 4, 7, and 14 animals. The glomerulus (position indicated by arrowheads) in normal kidney contains only an occasional T cell. By day 4, most glomeruli contain T cells. In some day-4 sections, there is an intense periglomerular infiltrate of T cells which is present through day 7 and 14 as the glomerular crescent develops. For quantitation of T cells for glomerulus and interstitium at various time points see Table 1; magnification, ×300.*

with rhodamine-conjugated L11/135 (anti-T cell) and fluorescein-conjugated 2C4 (anti-class II) to determine the distribution of class II positive (activated) and class II negative (nonactivated) T cells. As shown in Figure 7 (upper panel), at day 4 periglomerular T cells were largely class II negative (89 ± 12% in three animals). In contrast, intraglomerular T cells at day 4 were class II positive (76 ± 11%). By day 7 (Figure 7, lower panel), periglomerular T cells were mostly class II positive (64 ± 7%). T cells in the remainder of the interstitial compartment were also class II positive (76 ± 14%) at this time point. We conclude that at day 4 intraglomerular T cells were already activated, whereas extraglomerular T cells were not. By days 7 to 14, activated T cells were initially present in the periglomerular region of glomeruli, forming fibrocellular crescents, and subsequently in the interstitial compartment as a whole. At later points, T cells persisted in the interstitial compartment rather than in glomeruli.

Figure 8 shows Northern blots of cortical mRNA, comparing mRNA for class I, class II, and T cell antigen receptor B chain mRNA for five experimental animals and a control animal. As can be seen on the blot, T cell antigen receptor β chain mRNA was only detectable in the three animals with high class II mRNA expression. Of the four animals with high class I expression, two had low class II mRNA expression and undetectable T cell antigen receptor mRNA expression. These data show that there is heterogeneity between individual animals in this model and provide further support for the close association between class II expression and activated T cells.

Discussion

In this model, initial immune events targeted at guinea pig immunoglobulin bound along the glomerular basement membrane included rabbit anti-guinea pig antibody bound to the GBM as well as accumulation of class II positive T cells (and monocyte/macrophages) in glomeruli. These changes were associated with markedly increased expression of MHC class I and II mRNA and protein within glomeruli, probably as a consequence of cytokines produced by T cells and monocyte/macrophages. At this early time, there were some T cells present in the immediate periglomerular area in some animals, but they did not predominate on histologic sections, and they were not class II positive. This first phase of intraglomerular cell accumulation and activation was

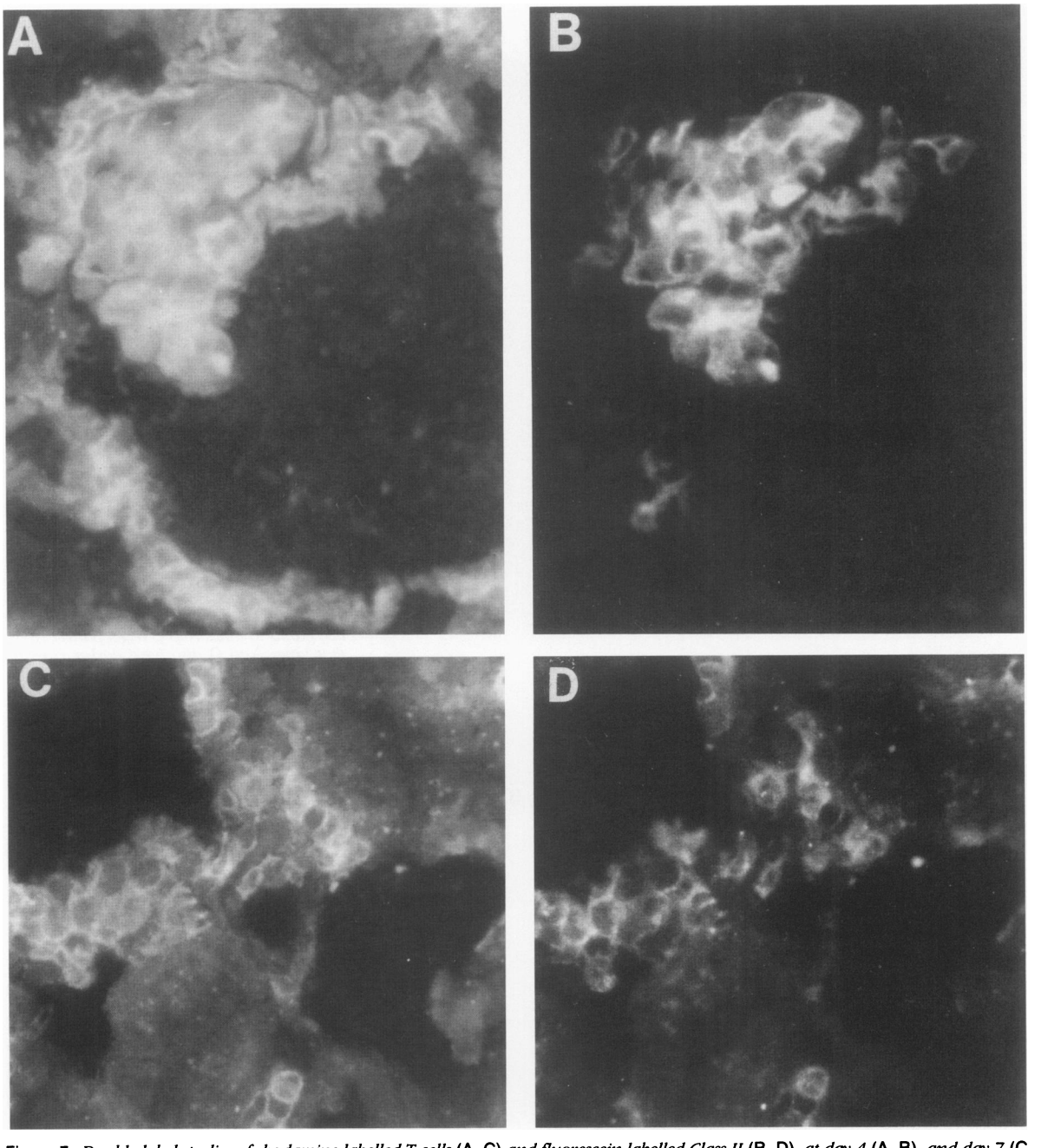

Figure 7. *Double label studies of rhodamine-labelled T cells* (A, C) *and fluorescein-labelled Class II* (B, D), *at day 4* (A, B), *and day 7* (C, D). *In the upper panel, the ring of rhodamine fluorescence seen in* (A) *is the periglomerular ring of T cells surrounding the glomerulus. Note that in* (B) *the periglomerular ring of cells does not stain with green FITC fluorescence showing that the periglomerular T cells are Class II negative at day 4. In contrast many of the cells in the glomerular tuft squashed into the upper left corner of the circular Bowman's space are Class II positive at day 4. In* (C, D), *Bowman's space is seen in the upper left corner. Note that the segment of the ring of periglomerular T cells is broader than at day 4* (C) *and that many of these cells are now Class II positive* (D); *magnification, ×530.*

followed within 3 days by a second phase associated with the accumulation of activated (class II positive) T cells in the periglomerular compartment, and subsequently over the following 7 to 14 days by a third phase associated with the acquisition of more generalized expression of MHC class I and II mRNA and protein by cells in the nonglomerular renal cortex.

One interpretation of this result is that the phase 1 intraglomerular injury triggers the phase 2 periglomerular immune and inflammatory cell accumulation and activation. This initially periglomerular inflammation subsequently may spread outward to involve the whole renal cortex. This sequence of events could occur through production of cytokines that pass from the glomerulus to the extraglomerular compartment by various pathways. These could include the peritubular capillary network that

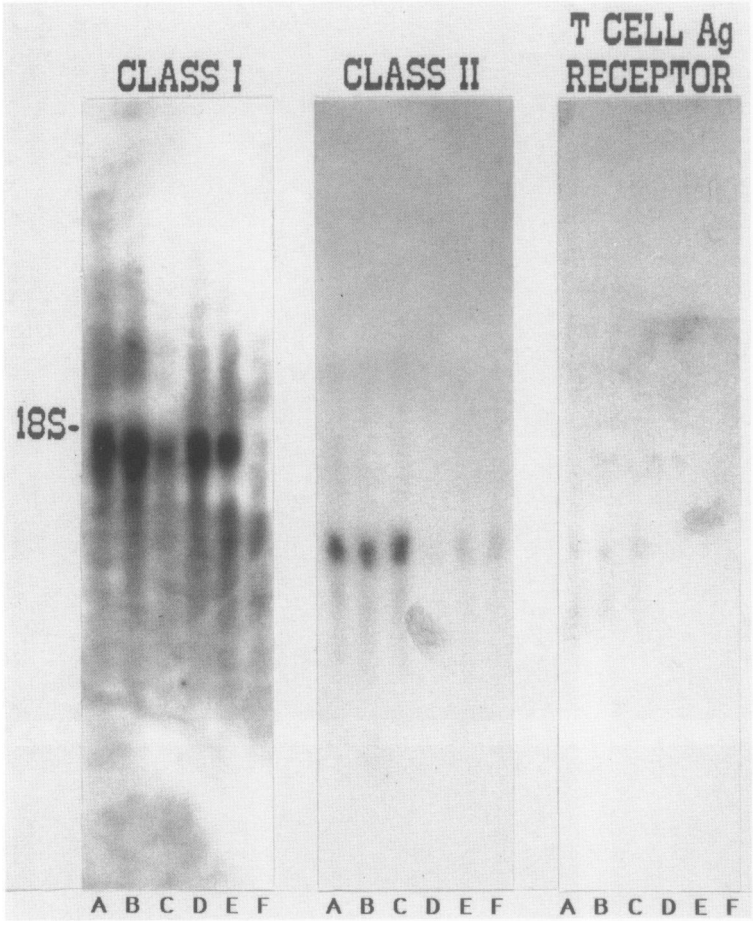

Figure 8. *Northern blot of cortical RNA from day 5 to 7 animals* (**A–E**) *and a control animal* (**F**) *successively probed for Class I, Class II, and T-cell antigen receptor mRNA. The position of the 18S rRNA band is shown. The T-cell antigen receptor mRNA is faintly seen as two bands in lanes* **A** *to* **C** *(right panel). Note that lanes* **A** *to* **C** *also have darker class II bands, but that class I is present in high amounts in lane* **A**, **B**, **D**, *and* **E**. *There is thus heterogeneity between individual animals, and the three animals with high class II also have the detectable TCR mRNA. The control animal had no detectable TCR mRNA and only faint bands for MHC class I and II seen as also noted in Figure 2.*

drains the inflamed glomerulus, the tubular lumen that drains Bowman's space, or through holes in Bowman's capsule. The distribution of these T cells immediately adjacent to the outer aspect of Bowman's capsule suggests attraction of these cells to factors coming directly from within the glomerulus.

A second mechanism to account for the observed result could be that guinea pig (GP) anti-rabbit GBM antibody bound not only to the GBM but also to the tubular basement membrane (TBM), and that this triggered an immune response in the interstitium similar to that seen in the glomerulus. This possibility is less likely for three reasons: 1) TBM staining by immunofluorescence was not noted when GBM staining was bright; 2) the time course of cell accumulation in the extraglomerular compartment was delayed 3 to 4 days behind glomerular cell accumulation, suggesting a different triggering method; and 3) as noted above, cells predominate in the immediate periglomerular region at early times.

A third possibility is that as the GBM became damaged and leaked protein into Bowman's space, this allowed GP IgG to be reabsorbed by the proximal tubule, processed, and presented to T cells in association with class II. These antigen-presenting cells could have been macrophages or B cells or they could have been renal epithelial cells.[48,40] Macrophages were present in glomeruli by day 4 and in the interstitial compartment by later times as detected by monoclonal antibody, although few B cells were detected at any stage (data not shown). Class II positive renal tubular epithelial cells were seen in some animals as noted in the results section.

The finding that class II positive (activated) T cells were present in glomeruli at day 4 when glomerular cell MHC class I and II mRNA and protein expression was significantly increased would be compatible with the hypothesis that intraglomerular activated T cells may have been a source of interferon-γ (IFN-γ) causing MHC class I and II expression in glomeruli.[51–53] However interleukin-1 or tumor necrosis factor alpha (TNF-α) produced by monocyte/macrophages or other cells also could have modulated class I/II expression.[54,55]

The histologic sequence of events in the rabbit closely parallels the sequence of events in anti-GBM disease in humans.[25] Similar structural findings have been previously described in the rabbit.[56–59] The periglomerular T cell accumulation has been described in association with severe anti-GBM disease in humans,[25] as well as in models of glomerular injury.[60] The interstitial mononuclear cell

accumulation is a common feature of severe anti-GBM disease in humans, and as in other forms of renal disease,[19,61–63] may be associated with progression to sclerosis and permanent loss of renal function. The causal relationship between glomerulonecrosis (usually associated with glomerular crescent formation) and the accumulation of activated T cells in glomeruli in association with widespread expression of MHC class I and II proteins by glomerular cells is uncertain and not proven by this study. It is possible that T cells play an active role in causing death of glomerular cells, however. This might be indirectly by activating monocyte/macrophage through cytokines or directly by T cytotoxic mechanisms.

How do these data compare with those of our prior studies of matrix synthesis using the same model? There was a clear temporal dissociation between glomerular accumulation of class I/II mRNA and activated T cells at day 4 and relative accumulation of matrix (collagen I/IV and fibronectin) mRNAs in glomeruli that did not occur until day 7. Thus there was a delay of about 3 days between immune cell accumulation and activation in the glomerulus and the onset of matrix synthesis as assessed by steady state mRNA levels and measurement of collagen synthetic rate.[1,2] What is the basis for this delay? Our previous study implicated TGF-β as an important profibrogenic molecule in this model, and in those studies we found that TGF-β mRNA was markedly increased in glomeruli by day 4, but that TGF-β protein was released from isolated glomeruli only in latent *inactive* form at that time.[3] There is therefore also a temporal dissociation or delay of several days between accumulation of activated T cells in glomeruli and the presence of active TGF-β together with ongoing glomerular matrix synthesis. We therefore cannot directly link T cell accumulation and activation in glomeruli with the presence of active TGF-β driving the scarring process, at least as we interpret the data from these studies. The interactions of TGF-β with events in the glomerulus are probably complex, however, because TGF-β probably plays a role in directly suppressing the glomerular T cell activation,[64] and IFN-γ and TNF-α synthesized in glomeruli at day 4 could be interfering with TGF-β–driven matrix protein synthesis, as has been previously reported for fibroblasts.[65]

In the extraglomerular compartment in this model, collagen and fibronectin mRNA synthesis *precedes* the accumulation of interstitial mononuclear cells (including class II positive T cells) by several days.[2,3] The mechanism for this finding may be similar to that suggested above for periglomerular T cell accumulation, in which we suggest that mediators may be leaking out of the inflamed glomerulus into the interstitial space and that these cytokines of glomerular origin may have effects on distant targets. Thus in severe glomerular inflammation the intraglomerular events could have effects on interstitial cells and structures, causing, for example, periglomerular and interstitial fibrosis.

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