Kidney International, Vol. 48 (1995), pp. 1728–1737

Role of a rat membrane inhibitor of complement in anti-basement membrane antibody-induced renal injury

YUJI HATANAKA, YUKIO YUZAWA, KAZUHIRO NISHIKAWA, ATSUSHI FUKATSU, NORIKO OKADA, HIDECHIKA OKADA, MASASHI MIZUNO, and SEIICHI MATSUO

The Third Department of Internal Medicine, Nagoya University School of Medicine, and Department of Molecular Biology, Nagoya City University School of Medicine, Nagoya, Japan

Role of a rat membrane inhibitor of complement in anti-basement membrane antibody-induced renal injury. In the kidneys of anti-glomerular basement membrane (anti-GBM) antibody disease, binding of antibodies to tubular basement membrane (TBM) is often observed. The present work was performed to explore the mechanisms of binding of anti-GBM antibodies to TBM *in vivo* with special reference to 5I2Ag, a rat membrane inhibitor of complement which regulates complement activation at C3 convertase level. To suppress functions of renal 5I2Ag, F(ab′)2 fragment of 5I2 (a neutralizing mAb against 5I2Ag) was perfused in the left kidney and then blood circulation was restored. Mild proteinuria (< 10 mg/16 hr) was observed during first several days. Five days later, there were tubulointerstitial injuries defined by tubular vimentin staining and leukocyte infiltration. Significant deposition of C3 was observed in the capillaries and in TBM. In rats intravenously injected with rabbit anti-rat GBM antibodies five minutes after kidney perfusion with 5I2, strong binding of rabbit IgG to TBM was observed at one and five days after injection. Although these rats showed mild proteinuria comparable to those perfused with 5I2 and those injected with normal rabbit serum, tubulointerstitial injury was significantly enhanced at Day 5. In contrast, rats perfused with irrelevant mAb and injected with anti-GBM antibodies did not show any significant binding of antibodies to TBM nor tubulointerstitial injury. Furthermore, rats which were made proteinuric by puromycin aminonucleoside and injected with anti-GBM antibodies did not show any significant binding of rabbit IgG to TBM. These results indicate that 5I2Ag, a rat membrane inhibitor of complement at the C3 convertase level, regulates vascular permeability in the living kidney, and that dysfunction or decreased expression of this molecule leads to increased accessibility of anti-GBM antibodies to TBM.

In human anti-GBM nephritis, more than 60% of patients were reported to have antibody deposition along tubular basement membrane (TBM), and those who had antibody deposition along the TBM showed a higher degree of tubulointerstitial injury [1]. In experimental anti-GBM nephritis in rats, weak binding of anti-GBM antibodies along the TBM of occasional proximal tubules was reported, and there was mononuclear cell infiltration in these area. Eddy and coworkers proposed that the mechanism of antibody deposition along the TBM was due to absorption of anti-GBM antibodies leaked from glomeruli to tubular lumen, because the increase of glomerular permeability by puromycin aminonucleoside enhanced the antibody deposition along TBM [2]. However, the second possibility that antibodies reach the TBM directly through vasculatures cannot be excluded. It might be hypothesized that an increase of vascular permeability can make anti-GBM antibodies accessible to TBM.

A family of glycoprotein molecules, cell membrane-associated complement regulatory proteins or membrane inhibitors of complement, are present on the plasma membranes of a wide variety of host cells, and inhibit complement activation on the host cell membranes at levels of C3 amplification loop and formation of membrane attack complex (MAC) [3]. *In vivo* studies to assess the roles of these molecules have only recently begun using a rat system, and it is now considered that these molecules play crucial roles in the host cell defense against autologous complement attack both in the normal and diseased conditions. In our recent study, *in vivo* suppression of a rat complement regulatory protein 5I2Ag [4, 5], an antigen recognized by a mouse mAb (mAb) 5I2, or a rat counterpart of mouse Crry/p65 [6–8]] rendered rats susceptible to autologous complement attack and induced endotoxin shock-like symptoms in rats [9]. In these rats, vascular permeability was highly increased [9].

Suppression of renal 5I2Ag by a mAb greatly increased the accessibility of heterologous anti-GBM antibodies to TBM. Thus, this work was designed to clarify the role of a rat complement regulatory protein 5I2Ag in the experimental anti-GBM nephritis in rats. The results obtained in this study might have relevance to understanding tubulointerstitial injury in anti-GBM nephritis in humans.

Methods

Animals

Female Wistar rats weighing about 300 grams and Japanese white rabbits weighing about 2.5 kg were purchased from Chubu Kagaku Shizai Co. Ltd. (Nagoya, Japan). They were allowed free access to food and water throughout the experiments.

Antibodies

Heterologous anti-rat GBM sera were produced in rabbits according to a method described before [10]. Rabbits were immunized subcutaneously with GBM in complete Freund's adjuvant every two weeks and were bled 32 days after the first immunization. Complements were inactivated by incubating antisera at 56°C for 30 minutes. Antisera were then absorbed with normal rat liver powder and erythrocytes. These antisera (rabbit

Received for publication January 30, 1995
and in revised form June 28, 1995
Accepted for publication July 17, 1995

anti-GBM sera) showed binding to GBM, TBM and basement membranes of all vessels when tested on normal rat kidney sections by indirect immunofluorescence technique (indirect IF). As described in a previous paper [10], intravenous injection of 1.0 ml of this antisera induced a very mild cellular infiltration in the glomeruli and did not induce significant proteinuria during the heterologous phase.

Characteristics and properties of mAb 5I2 (IgG1 subclass) have been described [4]. The mAb 5I2 can inhibit the function of a cell membrane-associated rat complement regulatory protein 5I2Ag (a rat counterpart of mouse Crry/p65) which regulates C3 convertases of both classical and alternative pathways. F(ab')2 fragments of 5I2 were prepared according to a method described previously [9]. H38 is an irrelevant mAb of IgG1 subclass which does not react with rat tissues. F(ab')2 fragments were also prepared and used as a control antibody for 5I2.

Kidney perfusion

To inhibit the functions of renal 5I2Ag *in vivo* an isolated kidney perfusion technique was used. The procedure of left kidney perfusion has been described previously [11, 12]. Briefly, the left kidney of a rat was exposed under ether anesthesia. Polyethylene tubes were placed in the left renal artery and vein, and proximal portions of the vessels were temporarily ligated. The left kidney was perfused at the rate of 2 ml/min using a peristaltic pump. Modified Tyrode buffer saturated with 95% oxygen and 5% carbon dioxide was used as a vehicle. All the perfusate was discarded through a tube placed in the renal vein. After kidney perfusion, tubes were removed and the holes in the artery and vein were repaired by microsurgery. Blood circulation of the left kidney was re-established by releasing the ligature. The average time required for perfusion procedure was about 10 minutes. To see the localization of mouse IgG after kidney perfusion, rats were perfused with 0.3 mg of F(ab')2 fragments of 512 or H38 in 10 ml of buffer according to the procedure described above. Rats were sacrificed 15 minutes after recirculation of the kidney, and localization of mouse IgG and rat C3 in the kidney was examined by IF.

Experimental protocol

Rats were divided into five groups. Each group of rats were treated as described below.

Group I. The left kidney was perfused with 0.3 mg of F(ab')2 fragments of mAb 5I2. Five minutes after re-establishment of renal blood flow, 0.9 ml of rabbit anti-rat GBM antisera were injected from the tail vein.

Group II. The left kidney was perfused with F(ab')2 fragments of irrelevant mAb H38 and the same amount of anti-GBM antisera was injected.

Group III. The left kidney was perfused with 0.3 mg of F(ab')2 fragments of 5I2 and normal rabbit serum instead of anti-GBM antisera was intravenously injected. Rats of Groups I, II and III were sacrificed at 1 (Day 1), 5 (Day 5) and 14 (Day 14) days after perfusion/injection.

Group IV. Rats were intravenously injected with 25 units of cobra venom factor 12 hours before perfusion. Serum complement hemolytic activity (CH50) was undetectable for at least three days by this treatment. Rats were then treated in the same way as Group I rats. Rats were sacrificed at Days 1 and 5.

Group V. To see the effect of proteinuria on the accessibility of intravenously injected anti-GBM antibodies to TBM, five rats received intraperitoneal injection of 15 mg of puromycin aminonucleoside (PAN). Seven days later, three rats started to reveal mild proteinuria ranging from 8 to 15 mg/16 hr. These rats were then intravenously injected with 0.9 ml of anti-GBM serum. Rats were sacrificed at 24 hours after antibody injection, and left kidneys were examined by IF for the deposition of antibodies along TBM. The protocol is shown in Table 1.

Table 1. Protocol of the experiments

Group	Pretreatment	Perfusion	i.v.	Day 1	Day 5	Day 14
I	no	5I2	RbAGBM	5	5	4
II	no	H38	RbAGBM	4	5	4
III	no	5I2	NRbS	4	5	4
IV	CVF	5I2	RbAGBM	4	5	0
V	PAN	no persuion	RbAGBM	3	0	0

The number in the column indicates the number of rats examined at individual time point. Abbreviations are: CVF, cobra venom factor; PAN, puromycin of aminonucleoside; RbAGBM, rabbit antiseum against rat GBM; NRbS, normal rabbit serum.

Histology and immunohistology

At the time of sacrifice, a piece of kidney tissue was fixed in methacaln fixative and embedded in paraffin for light microscopy. Two micrometer thick sections were stained with periodic acid-Schiff reagent. For IF study, fragments of kidney tissue were snap-frozen in liquid nitrogen and kept at −70°C until use. Two micrometer thick sections were cut by a cryostat and fixed in acetone for 10 minutes at room temperature. Sections were washed in PBS and then incubated with specific antibodies conjugated with fluorescein for 15 minutes at room temperature. For the detection of mouse IgG, fluorescein-labeled rabbit anti-mouse IgG antibodies (Cappel Laboratories, Westchester, PA, USA) absorbed with normal rat serum were used. Fluoreceinated goat antibodies against rabbit IgG (Cappel) absorbed with normal rat and mouse sera were used to detect rabbit IgG in the sections. Fluorescein-labeled rabbit anti-rat C3 antibodies were used for the detection of rat C3. To assess the leukocyte infiltration in the kidney, fluorescein-labeled monoclonal antibody (OX1) against rat leukocyte common antigen (LCA) purchased from Dainippon Pharmaceutical Company (Suita, Japan) was used. For the assessment of damaged tubules [13], kidney sections were incubated first with monoclonal anti-vimentin antibody (BioMakor, Rehovot, Israel) and then with fluorescein-conjugated rabbit anti-mouse IgG. After the final wash in PBS, all the sections were mounted in media containing p-phenylenediamine [14] and were observed through an Olympus epifluorescence microscopy (Tokyo, Japan).

Quantitation of data

For quantitation of data, specimens were examined by two observers using a blinded study method. The number of nuclei in the equatorially cut, glomerular cross section was counted. Twenty glomeruli were examined and the average number was used as total glomerular cell count in each rat. The number of LCA-positive cells in the glomerular cross section was counted similarly, and the average number of 20 glomeruli was used as an index of glomerular leukocyte infiltration. Data in the cortical tubulointerstitial tissue were obtained by 20 areas randomly observed under microscope at high magnification (×400). For the

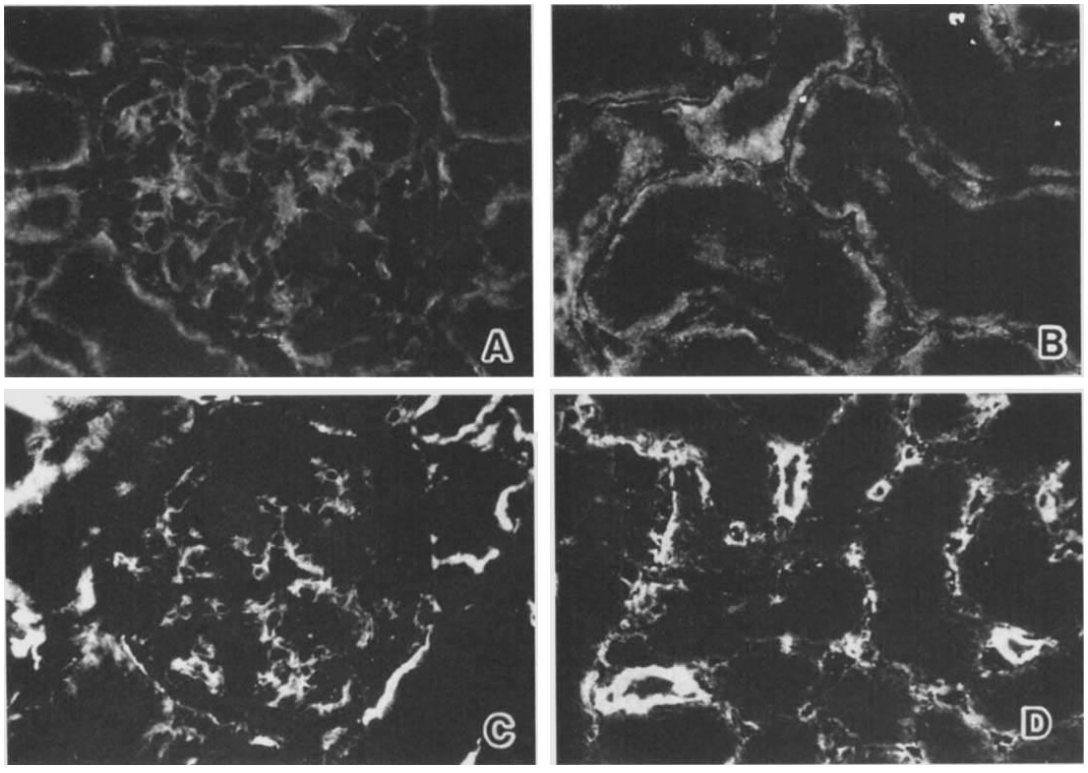

Fig. 1. *Immunofluorescence micrographs showing the binding of mAb 5I2 and rat C3 15 minutes after perfusion and recirculation in Group III rats.* 5I2 bound weakly in the mesangial area and along glomerular (**A**) and peritubular capillaries (**B**). Binding of 5I2 was also seen in the basal membrane of the proximal tubules (A, B). Deposition of rat C3 was weakly seen in the mesangial area (**C**). Strong deposition of rat C3 was seen in the peritubular capillaries (**D**). (A–D ×400)

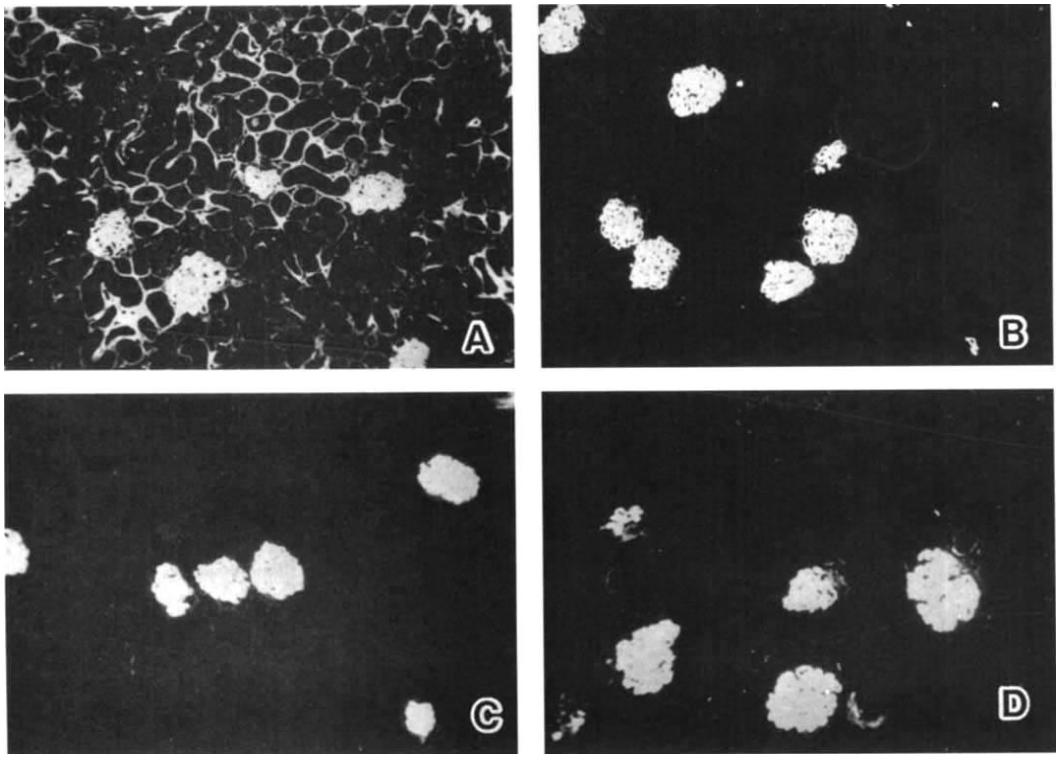

Fig. 2. *Immunofluorescence micrographs showing the binding of rabbit IgG (anti-GBM antibodies) in the kidneys one day after perfusion and recirculation.* Rabbit IgG bound to GBM strongly in Groups I (**A**), II (**B**), IV (**C**) and V (**D**). Binding of rabbit IgG to TBM was observed only in Group I rats (A). (A–D ×100)

Table 2. Summary of immunofluorescence staining

Group	Days after injection	GBM		TBM (cortex)[a]		Vessel wall		Peritubular capillaries	
		Rabbit IgG	Rat C3	Rabbit IgG	Rat C3	Rabbit IgG	Rat C3	Rabbit IgG	Rat C3
I	1	++++	++	+++	+	+/++	−/+	++	+
I	5	++++	+	+++	+	+/++	−/+	++	+
I	14	+++	+	++	−/+	−	−	−	−
II	1	++++	++	−/+	−	−	−	−	−
II	5	++++	+	−/+	−	−	−	−	−
II	14	+++	+	−	−	−	−	−	−
III	1	−	−	−	+	−	−	−	−/+
III	5	−	−	−	+	−	−	−	−/+
III	14	−	−	−	−	−	−	−	−
IV	1	++++	−	−/+	−	−	−	−	−
IV	5	++++	−/+	−/+	−	−	−	−	−
V	1	++++	++	−/+	−	−	−	−	−

Staining intensity was graded from (−) to (++++). For the evaluation of binding of rabbit IgG and rat C3 to the cortical TBM[a], following criteria were used: (−), deposition of rabbit IgG to less than 5% of TBM; (+), 5–30% of TBM; (++), 30–70% of TBM; (+++), more than 70% of TBM. In case of C3 deposition along TBM[a], circumferential deposition of C3 was defined as positive in each tubule because interrupted or discrete deposition of C3 is often observed in the TBM of normal rats.

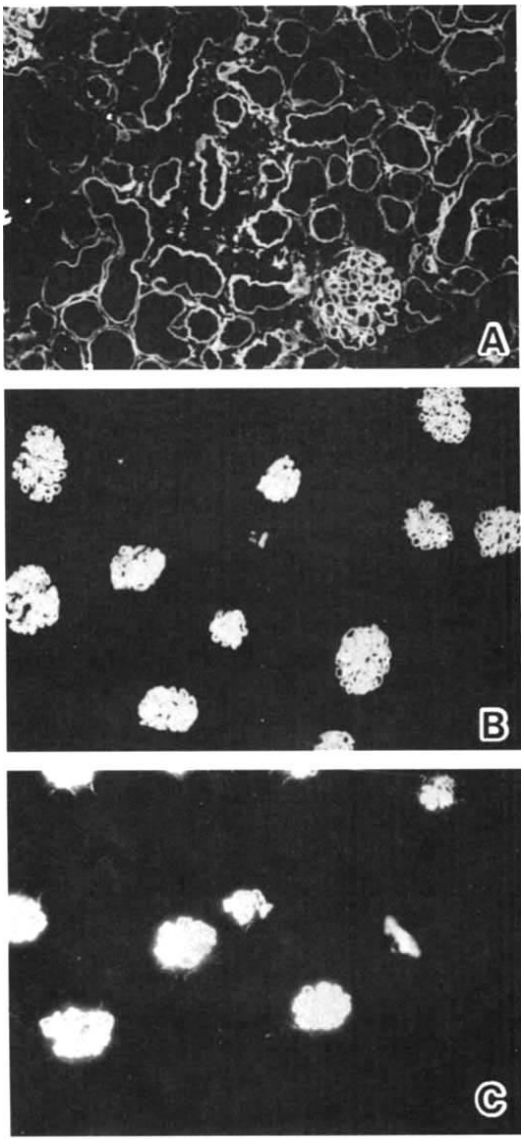

Fig. 3. *Immunofluorescence micrographs showing the binding of rabbit IgG (anti-GBM antibodies) in the kidneys five days after perfusion and recirculation.* Rabbit IgG bound to GBM strongly in rats of Group I (**A**), II (**B**), and IV (**C**). Strong binding of rabbit IgG to TBM was seen only in Group I rats (A). (A–D ×100)

evaluation of leukocyte infiltration, the average number of LCA-positive cells in a high magnification field was used in each rat. For the evaluation of proximal tubule damage, the extent of cytoplasmic staining of vimentin was graded from 0 to 3. In this case, each specimen was observed at a moderate magnification (×200). Ten areas were randomly observed and each area was graded according to the following definition: grade 0, no cytoplasmic staining; grade 1, cytoplasmic staining of proximal tubules in less than ⅓ of tubules; grade 2, positive staining between ⅓ to ⅔ of tubules; grade 3, positive staining in more than ⅔ of tubules. The average grade of 10 different areas was used as a representative grade of each rat.

Urinary protein measurements

Rats were housed in the metabolic cages overnight (about 16 hr) every other day after perfusion. Urine samples were collected and protein concentration was measured by a pyrogallol red method [15].

Statistics

The data were analyzed by one factor ANOVA. When a significant difference was indicated, statistical analysis was further performed by Scheffe's F-test to evaluate the statistical difference between any pair of groups. In case of tubular vimentin staining, only Group I and III rats showed positive staining at Day 5; no rat in the other groups (II and IV) showed positive staining. Thus, the statistical difference between Groups I and III was analyzed using the Mann-Whitney U-test. The statistical difference between two groups was determined when the P value was less than 0.05 (5%).

Results

Localization of 5I2 and C3 deposition after perfusion with 5I2

Binding of mouse IgG was observed in glomeruli, peritubular capillaries, and basal membranes of proximal tubules 15 minutes after perfusion of the kidney with 5I2 and recirculation (Fig. 1 A, B). There was weak binding of C3 in the glomerulus (Fig. 1C), and strong C3 deposition in the peritubular capillaries (Fig. 1D). In rats perfused with control mAb H38, significant binding of mouse IgG or C3 was not observed 15 minutes after perfusion.

IF staining for mouse IgG, rabbit IgG, rat IgG and rat C3

Mouse IgG. Binding of mouse IgG was observed weakly in the glomerulus and in the peritubular capillaries in Group I, III and

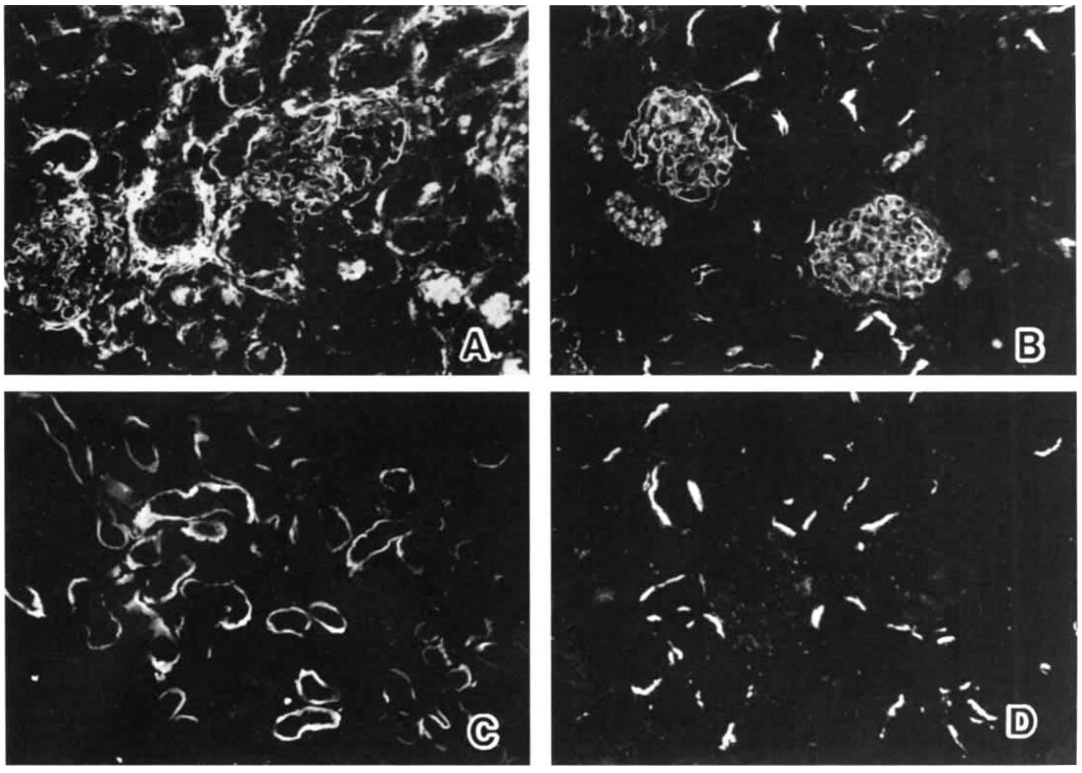

Fig. 4. *Immunofluorescence micrographs showing the deposition of rat C3 in the kidneys five days after perfusion and recirculation.* C3 deposition along GBM was seen in Groups I (**A**) and II (**B**) but not Group III (**C**) nor IV (**D**). Circumferential deposition of rat C3 along TBM was observed focally in the rat kidneys of Group I (A) and III (C). In rats of Group I, deposition of rat C3 was also observed in the perivascular tissue (A). (A–D ×200)

IV rats at Day 1. Mouse IgG was moderately seen in the proximal tubules and in the vascular bandles in these rats. At Day 5 onwards, mouse IgG was not detectable in the kidneys of these groups. Mouse IgG was not detectable in the kidneys of Group II rats throughout the experiments.

Rabbit IgG. Binding of rabbit IgG was strongly observed along GBM in Groups I, II, and IV both at Days 1 and 5 (Table 2). In Group I rats, binding of rabbit IgG was also strongly observed along TBM of proximal tubules both at Day 1 and Day 5 (Figs. 2 and 3). In addition, binding was also seen weakly to moderately in the vessel walls and peritubular capillaries. In contrast, there was practically no binding of rabbit IgG to TBM in other groups of rats throughout the experiments. In rats made slightly proteinuric by puromycin aminonucleoside, binding of rabbit IgG to TBM was seldom observed at Day 1, while there was strong binding to GBM (Fig. 2).

Rat IgG. Binding of rat IgG was not observed at Day 1 in the kidneys of all rats. At Day 5, there was weak binding of rat IgG to GBM of Groups I, II and IV. In rats of Group I, there was also weak binding of rat IgG to occasional TBM. At Day 14, linear deposition of rat IgG was clearly seen along GBM of Group I and II rats. Binding of rat IgG to TBM was not observed in rats of any group at this stage.

Rat C3. Deposition of rat C3 to GBM was weakly seen in Group I, II and V rats at Day 1, and in rats of Groups I and II at Days 5 and 14 (Table 2). There was no C3 deposition to GBM in Group III and IV rats (Fig. 4). Circumferential binding of rat C3 along TBM was seen in some tubules of Group I and III rats at Days 1 and 5 (Fig. 4). Significant C3 binding to TBM was seldom seen at Day 14 in these rats. Peritubular capillaries were positive for rat C3 in Group I rats at Days 1 and 5. In Group II, IV and V rats, abnormal binding of C3 was not seen in TBM and peritubular capillaries. These results showed that perfusion of the kidney with 512 resulted in the binding of C3 along TBM and in peritubular capillaries in concomitance with the increased binding of anti-GBM antibodies along TBM.

Glomerular pathology

Rabbit anti-rat GBM antibodies used in the present work can induce only mild glomerular pathology. Glomeruli of Group II rats (perfusion of the left kidney with an irrelevant mAb H38 followed by intravenous injection of anti-GBM antibodies) showed practically no glomerular pathology during the heterologous phase (~Day 5; Fig. 5A). At Day 14 there was a mild increase of glomerular cellularity and leukocyte infiltration in this group. Pretreatment of the left kidney with 5I2 (Group I rats) induced a significant increase of total glomerular cells (Fig. 5A) and leukocyte infiltration at Days 5 and 14 (Fig. 5B). 5I2 alone did not induce any significant change in the glomerulus (Group III; Fig. 5). Rats pretreated with CVF showed glomerular injury at Day 5 comparable to Group I rats (Fig. 5). At Day 14 when an autologous response was clearly observed, the overall glomeular pathology was mild and crescent formation was not seen in any rat. Among three groups (Groups I, II and III), rats of Group I showed most prominent changes, that is, an increase of total glomerular cells and infiltrated leukocytes. Glomeruli from rats of Group II showed significantly milder alteration than Group I.

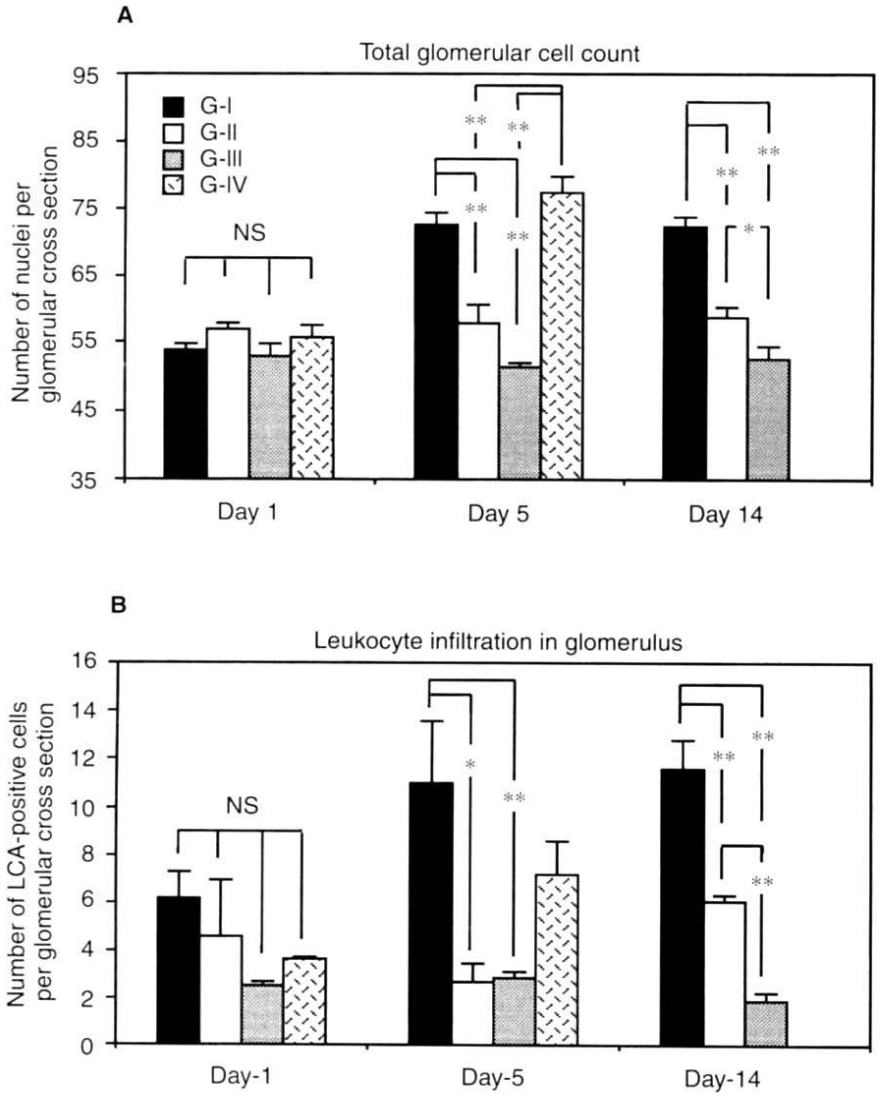

Fig. 5. *Glomerular cell number and leukocyte infiltration in glomeruli.* (**A**) Total glomerular cell number shown by the number of nuclei per equatorially cut, glomerular cross section. (**B**) Glomerular leukocyte infiltration shown by the number of LCA-positive cells per equatorially cut, glomerular cross section. (*$P < 0.05$; **$P < 0.01$; NS, not significant.)

Tubulointerstitial pathology

In Group I and III rats there was degeneration of proximal tubules and dilation of tubular lumen in the occasional area of kidney cortex at Day 1. In the other groups the tubulointerstitial tissue appeared normal. At Day 5, leukocyte infiltration and tubular damage became prominent and were most severe in Group I rats (Fig. 6A), although 5I2 itself could induce mild tubulointerstitial injury at Day 5 (Fig. 6C) as reported before [16]. Anti-GBM antibodies alone did not induce any significant tubulointerstitial pathology (Fig. 6B). Similarly, complement depletion by CVF totally abrogated the combined effects of anti-GBM antibodies and 512 (Fig. 6D). When the number of infiltrating leukocytes was examined, the greatest increase was found in Group I animals at Days 5 and 14 (Fig. 7A). In rats of Group III, there was mild to moderate increase of leukocytes in the cortex at Day 5, which became normal at Day 14. In Groups II and IV, the number of leukocytes present in the cortex was within normal range (Fig. 7A). When tubular damage was assessed by cytoplasmic expression of vimentin, proximal tubular cells in Group I rats expressed vimentin most intensely at Day 5 (Fig. 7B). Rats of Group III expressed vimentin less strongly, and rats of other groups did not show any significant vimentin expression (Fig. 7B). Thus, anti-GBM antibodies enhanced tubulointerstitial injury induced by 512. At Day 14, tubulointerstitial changes in Group I rats subsided significantly, although there was still mild cellular infiltration. Group II and III rats showed normal tubulointerstitial appearance at this stage.

Urinary protein excretion

The normal value in our laboratories for control female Wistar rats was less than 3 mg/16 hr. There was very mild proteinuria in Group I (average 4.7 mg/16 hr, range 1.3 to 6.9 mg/16 hr) and III rats (average 3.0 mg/16 hr, range 0.7 to 5.5 mg/16 hr) on Day 0/1. Proteinuria in these groups came to the upper limit of the normal value (Group I, average 2.1, range 1.4 to 3.5 mg/16 hr; Group III, average 3.0 mg/16 hr, range 1.1 to 6.8 mg/16 hr) on Day 2/3, and became normal on Day 4/5 onwards. In Group II and IV rats, urinary protein excretion was less than 3 mg/16 hr in all rats throughout the experiments. Thus, rats of Group I and III showed very mild proteinuria for the first several days of experiments.

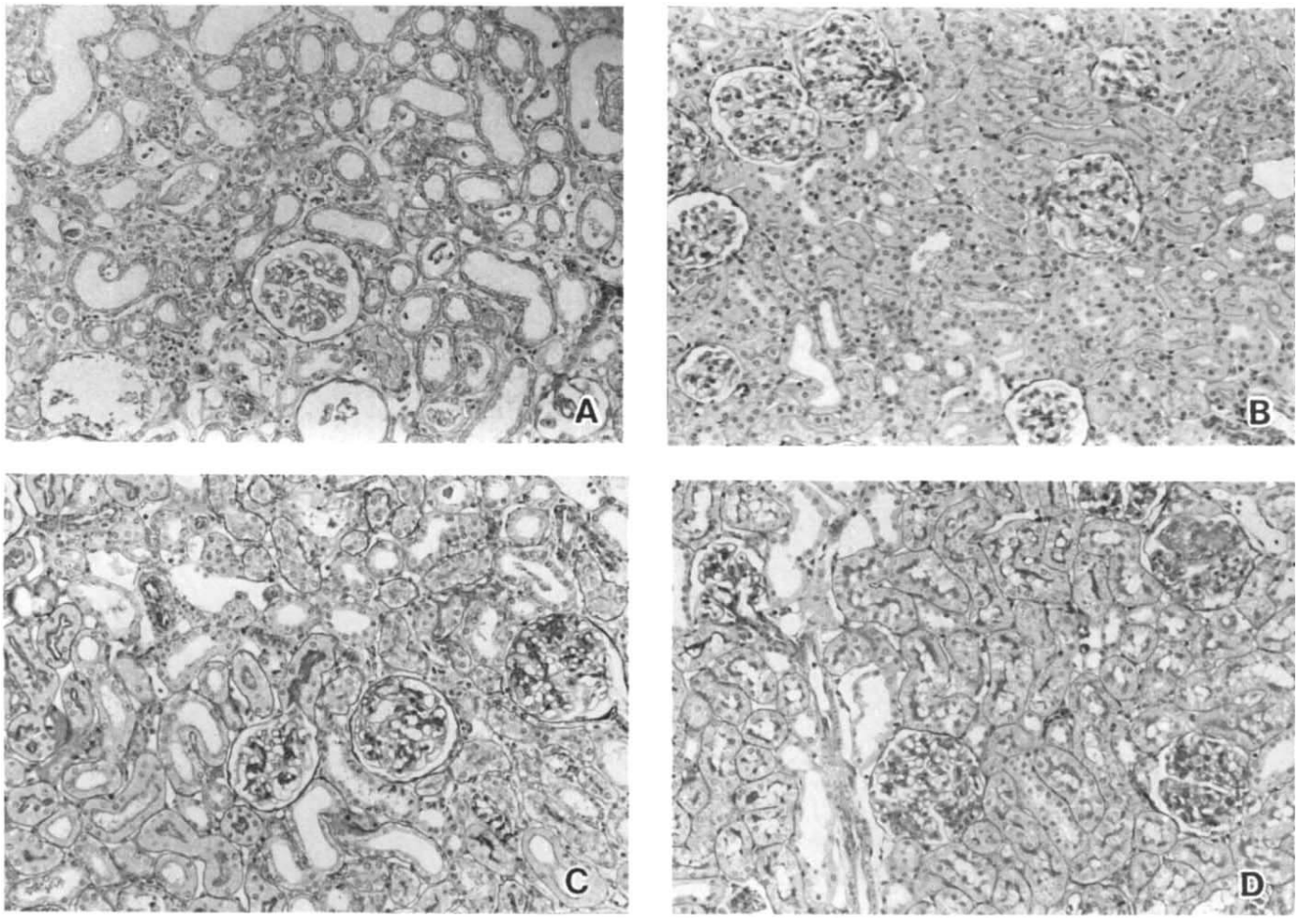

Fig. 6. *Light microscopic micrographs of the kidneys five days after perfusion and recirculation.* (**A**) Group I. (**B**) Group II. (**C**) Group III. (**D**) Group IV. (A–D ×200)

These data are shown in Figure 8. In three out of five rats intraperitoneally injected with PAN, urinary protein started to increase at the level of 8 to 15 mg/16 hr seven days after injection. These rats were used as Group V rats.

Discussion

There are several hypotheses on the mechanisms of antibody binding to TBM in human anti-GBM disease. First, anti-TBM antibodies found in patients of anti-GBM disease contained antibody (or antibodies) with different specificity against TBM [17]. Different properties among anti-TBM antibodies might determine the extent and intensity of antibody binding to TBM. Second, even when anti-TBM antibodies with the same specificity are involved, binding of antibodies to TBM was dependent on the accessibility *in vivo*. Concerning the antibody binding to alveolar basement membrane in anti-GBM disease, increased accessibility of antibodies to the cross-reactive antigens due to increased permeability of alveolar capillaries is considered to be important [18–20]. Access of antibodies to TBM is achieved via two routes. The first is direct diffusion of antibodies from the vasculature. This might be achieved by the increased vascular permeability for macromolecules. The second is the "back leak" of antibodies from the tubular lumen, which could be possible when there is significant proteinuria. Heterologous ant-rat GBM antibodies used in the present work contained antibody fraction(s) which could react with TBM because they could react with TBM when tested on the normal rat kidney section. Anti-GBM antibodies are characteristic in that they did not show any significant binding when administered intravenously. Thus, the anti-GBM antibodies used in this work were thought to be a type of tracer which could potentially react with TBM but could not bind to TBM *in vivo* in normal rats. Using these antibodies, the second hypothesis discussed above was examined in the present work.

Mouse Crry/p65 has dual functions mimicking human membrane cofactor protein (MCP) and decay accelerating factor (DAF) [21]. In rats, 5I2Ag is the only membrane inhibitor at the C3 convertase level known to date, and amino acid and cDNA sequences of 5I2Ag were highly homologous to those of mouse Crry/p65 [5]. Distribution of 5I2Ag in rats [22] was also similar to that of Crry/p65 in mice [8]. *In vivo* administration of 5I2 induced endotoxin shock-like symptoms [9] in rats, suggesting that 5I2Ag plays crucial roles in maintaining the normal physiological conditions against spontaneous and indiscriminate attack by autologous complement. When function of 5I2Ag was suppressed *in vivo* by

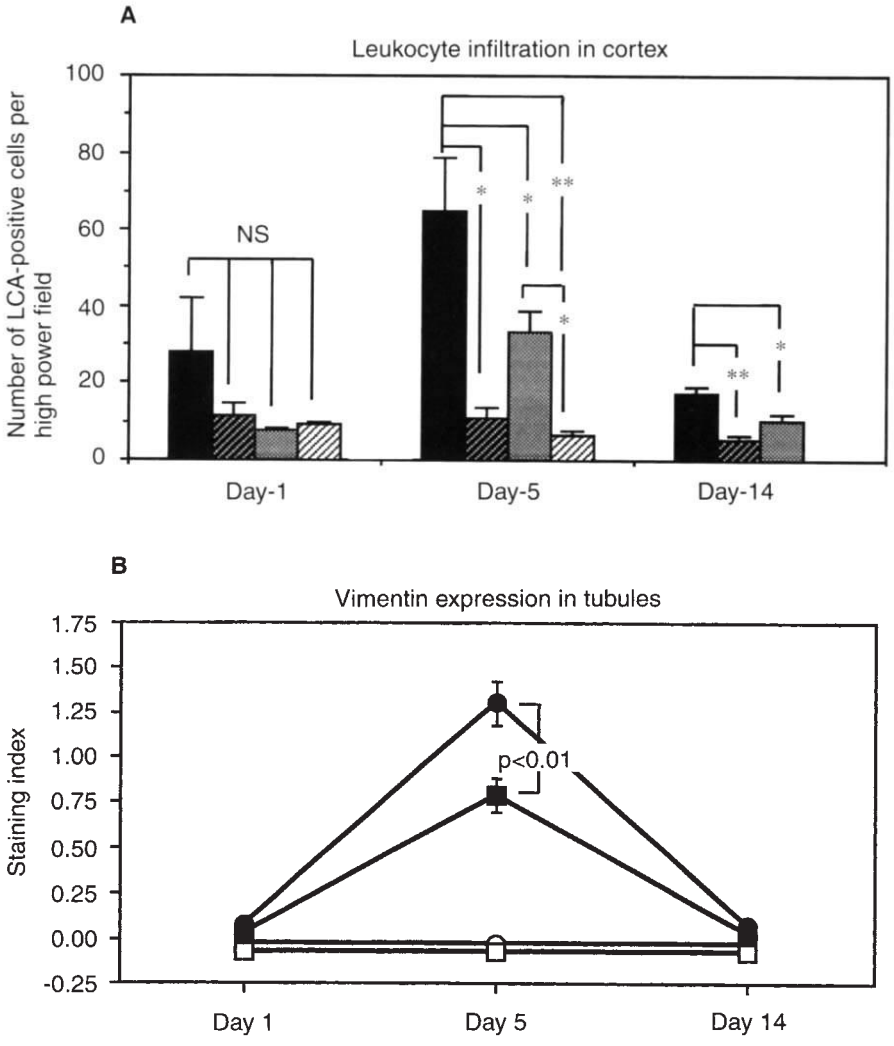

Fig. 7. *Cellular infiltration in the tubulointerstitium and cytoplasmic expression of vimentin in the proximal tubules.* (**A**) Leukocyte infiltration in the tubulointerstitial tissue. (**B**) Cytoplasmic vimentin expression of proximal tubules. (*$P < 0.05$; **$P < 0.01$; NS, not significant.)

perfusing kidneys with a neutralizing mAb 5I2, mild and transient tubulointerstitial injury pursued [16]. It is hypothesized from our recent work that the vascular permeability is increased by complement activation on the vascular endothelial cells after *in vivo* administration of 5I2 [9]. The results obtained in the present study revealed that intravenously administered anti-GBM antibodies became highly accessible to TBM when function of renal 5I2Ag was suppressed by mAb 5I2. The mechanism of binding of anti-GBM antibodies to TBM in the present work was considered as a direct diffusion of antibodies from the vasculature due to increased vascular permeability rather than reabsorption by proximal tubular cells of antibodies filtered from glomeruli. There are two reasons to support this notion. First, in the early phase (15 min after perfusion/recirculation), 5I2 bound to peritubular capillaries and basal membrane of proximal tubules. At this stage, there was strong deposition of complement C3 in the peritubular capillaries but not in the tubules or tubular lumen. This observation showed that complement activation was first induced in the peritubular capillaries. Second, there was strong binding of anti-GBM antibodies to TBM at Day 1 in Group I rats. At this stage, there was mild proteinuria in Groups I and III. To specify the route by which antibodies reached the TBM, rats were first made proteinuric by PAN, and then anti-GBM antibodies were injected (Group V). At Day 1, there was no significant binding of antibodies to TBM. This observation supported the hypothesis that the mechanism of antibody binding to TBM in rats of Group I was due to increased vascular permeability and diffusion of macromolecules (including antibodies and complement components) from the vasculatures.

mAb 5I2 itself could not induce any significant glomerular injury. In rats of Group III (perfused with 5I2 and then injected i.v. with normal rabbit serum), mild C3 deposition in the glomeruli was only transiently seen and rat C3 disappeared from glomeruli at Day 1. Anti-GBM antibodies used in this study could not induce glomerular pathology at Day 1 and Day 5 as seen in Group II rats. The fact that glomerular injury was evident in Group I suggested that pretreatment of kidney with mAb 5I2 worsened the anti-GBM antibody-mediated glomerular injury. At at Day 5, there was weak binding of rat IgG along the GBM in Groups I, II, and IV, indicating that autologous phase (antibody response to heterologous rabbit IgG) already started at this stage. The reason why the glomerular injury in Group IV (complement depleted

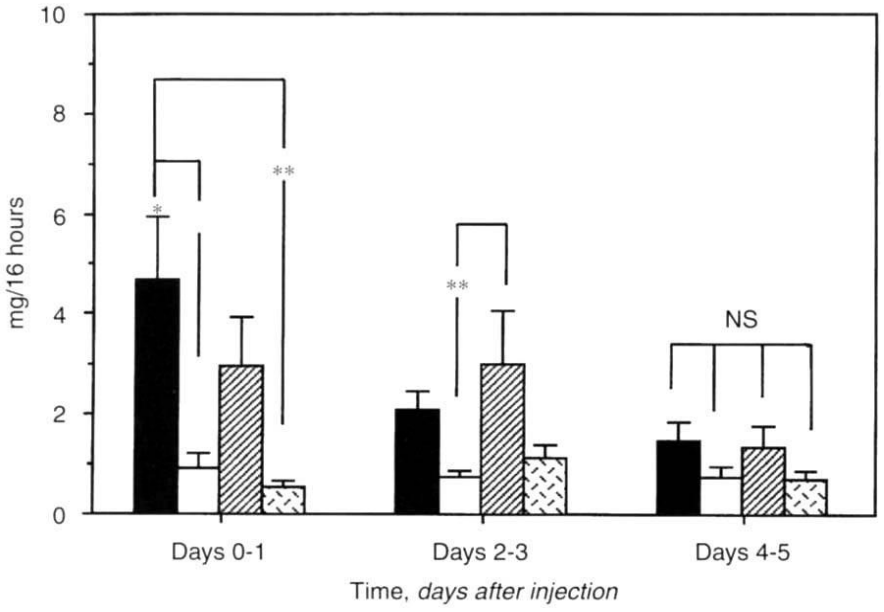

Fig. 8. *Urinary protein excretion during overnight (16 hr).* (*$P < 0.05$; **$P < 0.01$; NS, not significant.)

rats) was comparable to that of Group I at Day 5 could be explained by the previously reported findings that the autologous phase of nephrotoxic serum nephritis was independent of complement [23, 24].

Thus, intravenously administered anti-GBM antibodies (which can potentially bind to TBM) gain the increased accessibility to TBM when the function of a membrane inhibitor of complement (5I2Ag) is inhibited by F(ab')2 fragment of 5I2. The findings obtained in the present work might have relevance to the understanding of the mechanisms of antibody-mediated tubulointerstitial injuries.

Acknowledgments

Part of this work was supported by a Grant-in-Aid from the Ministry of Education and Welfare of the Japanese government (C06671136) and 1994 research grants from Nagoya Kyoritsu Hospital and Masuko Memorial Hospital. The authors thank N. Suzuki, M. Miyawaki, N. Kuno and M. Hagino for their excellent technical assistance.

Reprint requests to Seiichi Matsuo, M.D., The Third Department of Internal Medicine, Nagoya University School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466, Japan.

References

1. Andres G, Brentjens J, Kohli R, Anthone R, Anthone S, Baliah T, Montes M, Mookerjee BK, Prezyna A, Sepulveda M, Venuto R, Elwood C: Histology of human tubulo-interstitial nephritis associated with antibodies to renal basement membranes. *Kidney Int* 13:480–491, 1978
2. Eddy AA: Tubulointerstitial nephritis during the heterologous phase of nephtotoxic serum nephritis. *Nephron* 59:304–313, 1991
3. Liszewski MK, Post TW, Atkinson JP: Membrane cofactor protein (MCPor CD46): Newest family of the regulators of complement activation gene cluster. *Annu Rev Immunol* 9:431–455, 1991
4. Takizawa H, Okada N, Okada H: Complement inhibitor of rat cell membrane resembling mouse Crry/p65. *J Immunol* 152:3032–3038, 1994
5. Sakurada C, Seno H, Dohi N, Takizawa H, Nonaka M, Okada N, Okada H: Molecular Cloning of the rat complement regulatory protein, 5I2 antigen. *Biochem Biophys Res Commun* 198:819–826, 1994
6. Holers VM, Kinoshita T, Molina H: The evolution of mouse and human complement C3-binding proteins: Divergence of form but conversation of function. *Immunol Today* 13:231–236, 1992
7. Molina H, Wong W, Kinoshita T, Brenner C, Foley S, Holers VM: Distinct receptor and regulatory properties of recombinant mouse complement receptor 1 (CR1) and Crry, the two genetic homologues of human CR1. *J Exp Med* 175:121–129, 1992
8. Li B, Sallee C, Dehoff M, Foley S, Molina H, Holers VM: Mouse Crry/p65. Characterization of monoclonal antibodies and the tissue distribution of a functional homologue of human MCP and DAF. *J Immunol* 151:4295–4305, 1993
9. Matsuo S, Ichida S, Takizawa H, Okada N, Baranyi L, Iguchi A, Morgan BP, Okada H: In vivo effects of monoclonal antibodies which functionally inhibit complement regulatory proteins in rats. *J Exp Med* 180:1619–1627, 1994
10. Hara S, Fukatsu A, Suzuki N, Sakamoto N, Matsuo S: The effects of a new immunosuppressive agent, FK506, on the glomerular injury in rats with acclerated nephrotoxic serum glomerulonephritis. *Clin Immunol Immunopathol* 57:351–362, 1990
11. Matsuo S, Yoshida F, Yuzawa Y, Hara S, Fukatsu A, Watanabe Y, Sakamoto N: Experimental glomerulonephritis induced in rats by a lectin and its antibodies. *Kidney Int* 36:1011–1021, 1989
12. Matsuo S, Nishikage H, Nomura A, Yoshida F, Piddlesden SJ, Morgan BP: The role of CD59 in the complement-mediated glomerular injury in rats. *Kidney Int* 46:191–200, 1994
13. Gröne HJ, Weber K, Gröne E, Helmchen U, Osborn M: Coexpression of keratin and vimentin in damaged and regenerating tubular epithelia of the kidney. *Am J Pathol* 129:1–8, 1987
14. Platt J, Michael AF: Retardation of fading and enhancement of intensity of immunofluorescence by p-phenylene-diamine. *J Histochem Cytochem* 31:840–842, 1983
15. Watanabe N, Kamei S, Ohkubo A, Yamanaka M, Ohsawa S, Makino K, Tokuda K: Urinary protein as measured with a pyrogallol red-molybdate complex, manually and in a Hitachi 726 automated analyzer. *Clin Chem* 32:1551–1554, 1986
16. Nomura A, Yuzawa Y, Okada N, Okada H, Nishikawa K, Matsuo S: Tubulointerstitial injury by functional inhibition of a complement regulatory molecule. (abstract) *J Am Soc Nephrol* 5:760, 1994
17. Wilson CB: The renal response to immunologic injury, in *The Kidney*, edited by Brenner BM, Rector FC Jr, Philadelphia, W.B. Saunders Co., 1991, pp 1062–1181
18. Jennings L, Roholt OA, Pressmann D, Blau M, Andres GA, Brentjens JR: Experimental anti-alveolar basement membrane antibody-mediated pneumonitis. I. The role of increased permeability of

the alveolar capillary wall induced by oxygen. *J Immunol* 127:129–134, 1981

19. DOWNIE GH, ROHOLT OA, JENNINGS L, BLAU M, BRENTJENS JR, ANDRES GA: Experimental anti-alveolar basement membrane antibody-mediated pneumonitis. II. Role of endothelial damage and repair, induction of autologous phase, and kinetics of antibody deposition in Lewis rats. *J Immunol* 129:2647–1652, 1982
20. YAMAMOTO T, WILSON CB: Binding of anti-basement membrane antibody to alveolar basement membrane after intratracheal gasoline instillation in rabbits. *Am J Pathol* 126:497–505, 1987
21. KIM Y, KINOSHITA T, MOLINA H, HOURCADE D, SEYA T, WAGNER LM, HOLERS VM: Mouse complement regulatory protein Crry/p65 utilizes the specific mechanisms of both human decay-accelerating factor (DAF) and membrane cofactor protein (MCP). *J Exp Med* 181:151–159, 1995
22. FUNABASHI K, OKADA N, MATSUO S, YAMAMOTO T, MORGAN BP, OKADA H: Tissue distribution of complement regulatory membrane proteins in rats. *Immunology* 81:444–451, 1994
23. THOMSON NM, NAISH PF, SIMPSON IJ, PETERS DK: The role of C3 in the autologous phase of nephrotoxic nephritis. *Clin Exp Immunol* 24:464–473, 1976
24. HOLDSWORTH SR, NEALE TJ, WILSON CB: Abrogation of macrophage-dependent injury in experimental glomerulonephritis in the rabbit. Use of an antimacrophage serum. *J Clin Invest* 68:686–698, 1981